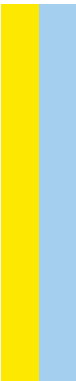


DOUTORAMENTO
CIÊNCIAS BIOMÉDICAS

Metabolic Modulation of Mitochondrial Uncoupling Proteins and Insulin on Human Spermatozoa and Sertoli Cells

David Fadista Carrageta

D
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Metabolic Modulation of Mitochondrial Uncoupling Proteins and Insulin on Human Spermatozoa and Sertoli Cells

Tese de candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Doutor Marco G. Alves
Categoria – Investigador Principal
Afiliação – Departamento de Ciências Médicas, Universidade de Aveiro

Coorientador – Professora Doutora Mariana P. Monteiro
Categoria – Professora Catedrática
Afiliação – Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto

Coorientador – Professor Doutor Pedro F. Oliveira
Categoria – Professor Auxiliar
Afiliação – Departamento de Química, Universidade de Aveiro

Any civilized society is characterized by the existence of two elements — stability and progress. If it does not offer stability, it is anarchic and progress is impossible; if it does not progress, it cannot be called a civilized society (...) Progress is a rebellion against the species (...) Creating is being not satisfied. All creation involves a rejection of what does not need to be created. Every gesture made today is a rebellion against the gesture made yesterday. Creating is to abstain. Creating is to renounce coexistence. I think I tell the truth in these absurd words:

The urge to create is an imaginative phenomenon, the atrocity of angels who thought they could improve heaven.



Qualquer sociedade civilizada caracteriza-se por a existência nela de dois elementos — a estabilidade e o progresso. Se não oferece estabilidade, resulta anárquica, e impossível o progresso; se não progride, não pode dizer-se uma sociedade civilizada (...) O progresso é uma revolta contra a espécie (...) Criar é não estar satisfeito. Toda a criação envolve uma rejeição do que não precisa ser criado. Todo o gesto feito hoje é uma revolta contra o gesto feito ontem. Criar é abster-se. Criar é dispensar-se de coexistir. Creio dizer a verdade sumária nestas palavras absurdas:

A ânsia da criação é um fenómeno imaginativo, o crime dos anjos, que julgaram poder ter um melhor céu.

In: *“Cinco Diálogos sobre a Tirania - Ultimatum e Páginas de Sociologia Política”*

Fernando Pessoa

Declaração de Honra

Declaro que a presente tese é de minha autoria e não foi utilizada previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras de atribuição e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio constitui um ilícito académico.

A handwritten signature in dark ink, reading "David Carrageta". The script is cursive and fluid, with the first letter 'D' being particularly large and stylized.

(David José Fadista Carrageta)

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A execução e término de um Doutorado exige do candidato, qual maratona, um elevado grau de esforço físico e mental. Após embarcar nesta jornada, humildade à parte, nunca tive qualquer dúvida que teria “pernas” para chegar e atravessar a meta. Posso até ser guiado pela ambição e confiar nas minhas capacidades, mas acima de tudo sei perfeitamente quais as minhas limitações e quem as poderia colmatar. O sucesso é nutrido não só pelo trabalho árduo, mas também por se estar no local certo à hora certa, e, principalmente, por se estar rodeado das pessoas certas. Se me permitem a gracinha, e se me perdoam a sinopse precoce, acredito que a maratona associada a um projeto de Doutorado, tal como a jornada do espermatozoide, é, na verdade, um trabalho em equipa. A quem me é próximo, saberá que um dos traços fundamentais da minha personalidade é a serenidade, talvez um traço demasiado forte que não me permite expressar como, por vezes, a ocasião merece. Por isso, ressalvo que os Agradecimentos que antecedem a presente tese de Doutorado são um produto inglório, dado que a minha gratidão por todos os elementos doravante mencionados transcende a minha capacidade de expressão por palavras. Talvez seja esta a principal razão pela qual estas foram as últimas palavras a ser redigidas na presente tese de Doutorado. Tentarei, no melhor que me for possível, demonstrar a gratidão que sinto pelas palavras que dedicarei a todos aqueles que, direta ou indiretamente, contribuíram não só para o desenvolvimento deste projeto, como também para moldarem a pedra basilar da pessoa e investigador que sou e ambiciono ser.

Não poderia começar os Agradecimentos sem destacar o papel fundamental da minha equipa de orientação, constituída pelo meu Orientador Doutor Marco G. Alves, e Coordenadores Doutor Pedro F. Oliveira e Doutora Mariana P. Monteiro. O papel do meu Orientador foi, provavelmente, o mais preponderante durante esta jornada, e será uma das principais vítimas da redutora e inglória secção de Agradecimentos. Começarei por referir que estou absolutamente grato ao Prof. Marco por me abrir as portas à investigação. Se dei, e darei, os passos certos para me tornar num bom investigador, será por ter ouvido atentamente os seus inúmeros e valiosos conselhos, indicações e comentários que recebi ao longo dos últimos 7 anos. Conheci o prof. Marco em 2016, ainda enquanto aluno de Mestrado em Ciências Biomédicas pela UBI. Numa aula por si lecionada, o Prof. Marco frisou veemente a importância da escolha de projeto de mestrado e da equipa de orientação.

Confesso que, ainda antes de ingressar no Mestrado, já sabia que o meu futuro passaria pela investigação, mas desconfiava que a porta de entrada estaria numa outra instituição. Estive com um pé na Universidade de Coimbra, mas em última instância optei por continuar na UBI. Foi uma escolha que durante algum tempo me assombrou, por achar que teria cedido ao comodismo, mas da qual, atualmente, em nada me arrependo. Aprendi que não temos de agarrar todas as oportunidades, mas sim as oportunidades certas. Além disso, as escolhas certas em muito dependem da nossa ambição, postura e trabalho, em detrimento, por vezes, da dita sorte. Após essa aula, a 8 de Maio de 2016, enviei um e-mail ao Prof. Marco onde descrevi a minha ambição de me tornar investigador e fazer um Doutorado, e pedi conselhos sobre as minhas áreas de interesse – Reprodução e Medicina Regenerativa. O Prof. Marco prontamente me respondeu com um longo texto, onde objetivamente descrevia ambas as áreas e enunciando alguns dos nomes nacionais de destaque em cada uma delas. Além disso, descreveu quais as características que deveria procurar no Orientador para um arranque de carreira científica de sucesso. O que não esperava foi receber igualmente uma proposta de tema de Mestrado, mas numa área diferente das quais procurei conselhos – Obesidade e Metabolismo. Na verdade, o estudo de Metabolismo, como sempre disse o Prof. Marco, é transversal a todas as áreas. E assim parti, muito muito verdinho, em direção ao ICBAS, Universidade do Porto, para estudar adipócitos. Foi neste contexto que conheci o Prof. Pedro e a Prof.^a Mariana, os quais me acompanharam igualmente ao longo dos últimos 7 anos.

Acredito que durante o Mestrado terão visto potencial em mim, resultando no convite que recebi para continuar como técnico de investigação no grupo. Serviria este período, onde comecei efetivamente a trabalhar em Reprodução, como preparação para um futuro Doutorado. E foi neste período, nos primeiros meses de 2018, que senti o “clique” e me apercebi que se queria chegar onde ambicionava, teria de traçar um plano e trabalhar arduamente em prol do mesmo. Por isso decidi, por iniciativa própria, esboçar um possível projeto de Doutorado, o qual orgulhosamente apresentei numa reunião com o Prof. Marco e o Prof. Pedro. Neste projeto, ambicionava estudar o eixo da angiotensina como um potencial regulador da homeostasia de fluídos do sistema reprodutor masculino. Este projeto acabou descartado por algumas falhas científicas, resultantes da minha inexperiência. Curiosamente, anos mais tarde, alguns dos intervenientes deste eixo estariam envolvidos no mecanismo de infeção viral do protagonista da grande pandemia global, o SARS-CoV-2, conferindo uma nova relevância a este projeto. Não obstante, a “semente” do prazer conferido ao pensar e idealizar projetos havia plantada e haveria de resultar em frutos, num futuro não muito distante. Em alternativa, o Prof. Marco e o Prof. Pedro apresentaram-me dois possíveis projetos de Doutorado: estudar os efeitos de elevados ní-

veis de glucose no metabolismo do espermatozoide humano; ou continuar a estudar a relação entre as aquaporinas e o CFTR na homeostasia de fluídos no sistema reprodutor masculino. Optei pelo primeiro, tendo concorrido com este projeto às bolsas de Doutorado da FCT em 2018. Um excelente projeto, aliado ao meu muito bom percurso académico e a uma excelente equipa de Orientação, composta pelo Prof. Marco como Orientador, e a Prof.^a Mariana e o Prof. Mário Sousa como Coorientadores, resultou na avaliação da minha candidatura como #1 a nível nacional. Aproveito a oportunidade para dedicar algumas palavras ao Prof. Mário Sousa, a quem quero agradecer a disponibilidade em iniciar este projeto e todas as correções e comentários que efetuou enquanto se manteve como Coorientador. O meu mais sincero obrigado.

Foi ao longo destes últimos 5 anos, moldado à luz da filosofia de trabalho dos Professores, que me foram dadas todas as ferramentas e oportunidades para crescer e desenvolver o meu currículo científico, do qual imensamente me orgulho. Redigi e publiquei diversos artigos científicos, ingressei em cursos especializados e apresentei o meu trabalho em congressos nacionais e internacionais. Certamente os Professores poderão confirmar que muito raramente me terão ouvido dizer “não” a um convite, tendo agarrado todas as oportunidades que me fizessem aprender e crescer. E foi assim que, no seio de um ambiente proativo, tive a oportunidade de ingressar em dois cursos especializados em Ressonância Magnética Nuclear e Metabolómica na Alemanha, um curso avançado de Experimentação Animal, e apresentar o meu trabalho em congressos espalhados por diversos países Europeus. Não posso deixar de salientar que os Professores sempre nos estimularam para a escrita e submissão de projetos, e eu raramente ignorei estas oportunidades. E foi graças ao incentivo e sugestões do Prof. Marco que consegui ganhar um Projeto de Investigação financiado pela Sociedade Portuguesa de Diabetologia em 2021, sendo este um dos momentos que mais me marcou durante a jornada deste Doutorado. Um outro momento marcante, e pelo qual não poderia deixar de agradecer, foi a confiança que o Prof. Marco depositou em mim quando me encarregou de o ajudar a gerir o laboratório. Durante cerca de um ano, foi-me incumbida a responsabilidade de gerir os *stocks* e as compras de reagentes necessários ao funcionamento do laboratório, funções que acumulei enquanto desenvolvi o meu próprio projeto e co-supervisionei projetos de outros alunos. Penso que poderei dizer, que me perdoem novamente o abandono temporário da modestia, que nenhum problema terá ficado por resolver e nenhum reagente terá faltado no nosso laboratório durante esse período. Se ficou, os meus colegas que me perdoem, pois dei o melhor de mim. Coincidência ou não, foi um dos anos mais produtivos para o nosso grupo em termos de artigos científicos. Este período, embora extremamente exigente em termos de carga laboral, foi um dos mais gratificantes que tenho em memória. O gosto que adquiri pela gestão de laboratório, aliado ao prazer derivado de materializar ideias para projetos

em papel, são as principais razões pelas quais ambiciono poder voar tão alto quanto quem voa mais alto na carreira científica. Se ambiciono tornar-me Investigador Principal, e se comecei a traçar o percurso nesse sentido, certamente terá sido mérito da minha Equipa de Orientação. Ao meu Orientador, o Prof. Marco, gostaria de reforçar o meu sincero agradecimento pela disponibilidade em aceitar liderar este Doutoramento, pela confiança, incentivos, conhecimentos, oportunidades que me facultou e por exigir nunca menos do que o melhor que tenho para dar. Agradeço todas as sugestões e correções que fez tanto na execução dos trabalhos experimentais, como na redação dos artigos científicos e na presente tese de Doutoramento. Agradeço todos os bons conselhos e por, no fundo, me educar em como ser investigador. Aos meus Coorientadores, Prof. Pedro e Prof.^a Mariana, o meu sincero obrigado pela disponibilidade em aceitar embarcar neste projeto de Doutoramento. Ao Prof. Pedro, o meu sincero obrigado pela confiança e por todas as sugestões e correções que melhoraram a qualidade dos desenhos experimentais e produção científica. Agradeço todos os bons conselhos que me deu ao longo de todos estes anos, alguns deles que transcendem o domínio científico, e por me proporcionar, juntamente com o Prof. Marco, todas as oportunidades e todos os estímulos necessários para evoluir. À Prof.^a Mariana, o meu sentido obrigado por todo o apoio, conhecimentos clínicos, sugestões e correções que forneceu ao logo deste Doutoramento. A Prof.^a Mariana é uma verdadeira inspiração, incansável e exímia, qual força da Natureza, em todas as suas funções enquanto Coorientadora e Diretora-Geral da Unidade Multidisciplinar de Investigação Biomédica (UMIB). Não houve qualquer dificuldade ou problema que a Prof.^a Mariana não conseguisse prontamente resolver e, apesar da sua agenda preenchida, sempre houve disponibilidade para me receber, ouvir, e auxiliar com todos os meios que tem ao seu alcance. A todos, coletivamente, o meu sincero obrigado por me acompanharem ao longo destes anos. Espero que a memória da minha pessoa, enquanto vosso aluno, seja marcada com orgulho, e que o percurso que almejo se materialize para que o sentimento possa ser dobrado.

Além da Equipa de Orientação, também os elementos do grupo de investigação *Sertoli cell and Gamete Biology Lab* merecem a minha gratidão. Todos os atuais e antigos membros que passaram pelo grupo contribuíram, direta ou indiretamente, para o desenvolvimento deste projeto e elaboração da presente tese de Doutoramento. Uma menção especial deverá ser dada, embora ingloria e insuficiente, ao “núcleo duro” que coexistiu durante os últimos 5 anos e transcendeu do plano profissional para o de amizade. À Bárbara, João, Luís, Mafalda, Patrícia, Raquel e Sara, o meu mais sincero agradecimento por tudo o que me ensinaram e por todo o apoio, entreaajuda, companheirismo e união. Diz-se que, numa equipa, o sucesso pessoal é o sucesso do coletivo, e prontamente direi que o término deste

Doutoramento será também mérito de todos vós. Agradeço a boa disposição e bom ambiente que proporcionaram ao longo destes anos, assim como pelas diversas boas memórias que levo para a vida. A viagem à Croácia, a gelatinização da minha chávena de café, ou a decoração dos meus frascos de cultura com o *script* da série *The Office* estarão, garantidamente, entre as minhas memórias favoritas. Os “agora” Doutora Raquel e Doutor Luís lideraram o caminho, eu almejo ser o próximo e aguardarei, fervorosamente, o momento de brilhar dos que me seguirão as pegadas. Mais do que estar grato por ter partilhado o laboratório com este fantástico grupo, desejo a todos o maior sucesso profissional e pessoal que, sem qualquer dúvida, sei que virão a ter. Fora do denominado “núcleo duro”, terei ainda de salientar o papel do Doutor David Martin-Hidalgo, atual investigador da Universidad de Extremadura, Espanha. O David é um excelente investigador e um fantástico ser humano, com um conhecimento profundo sobre o processo de capacitação e metabolismo do gâmeta masculino. O meu mais sincero obrigado ao David por todo o conhecimento que me transmitiu durante o, a meu ver, curto período em que foi um membro integrante do nosso grupo. Foi um prazer partilhar a bancada com o David e espero poder reencontrá-lo em palcos futuros. Não posso também deixar por enfatizar o papel que os meus alunos tiveram na execução deste projeto, assim como de agradecer aos Professores pela oportunidade de coorientar os mesmos. Ao Bruno Monteiro e à Laís Brito, o meu mais sincero obrigado pela vossa ajuda na execução de várias tarefas do presente projeto de Doutoramento. Contudo, não seria um Agradecimento honesto sem destacar o papel absolutamente vital que a Laís desempenhou em algumas tarefas no decorrer deste projeto de Doutoramento. A Laís foi a minha primeira aluna, tendo surgido no nosso grupo no âmbito de um estágio de licenciatura. O estágio da Laís foi toda uma situação inacreditável – estava pronto a ter as primeiras aulas de condução e fora-me atribuído um *Ferrari*. Uma excelente aluna, incrivelmente responsável e profissional, rapidamente ganhou a minha confiança e se tornou no meu braço direito. Todas as tarefas que lhe atribui sempre foram prontamente executadas e com o máximo rigor e perfeição. O Prof. Marco brincava com o nosso método de trabalho, apelidando-o de “Carragetaquistão”, dadas as longas horas de trabalho de bancada a que eu submetia a Laís. E foi graças a todo esse empenho que vários protocolos foram afinados e muitos resultados obtidos. A Laís acabou o seu estágio com 20 valores, não me podendo deixar mais orgulhoso. O meu mais sincero obrigado à Laís pela amizade, disponibilidade, empenho, e por todo o apoio na execução de uma parte significativa deste projeto de Doutoramento. A Laís terá, certamente, um futuro brilhante, que irei acompanhar e apoiar dentro do que me for possível.

Aos restantes elementos do ICBAS que me apoiaram nas mais diversas tarefas e dificuldades, o meu sincero obrigado. Aos membros do Departamento de Microscopia, dos quais não poderei deixar de mencionar a Cláudia, Elsa, Ângela, Paula, Fernanda e Célia,

o meu sincero obrigado pela amizade, apoio e boa disposição. Terei de atribuir uma menção especial à Cláudia, pela amizade, boa disposição e gargalhadas que partilhámos, juntamente com a Raquel, enquanto trabalhámos nas bancadas do laboratório de Citogenética. Com a Cláudia aprendi não só Microscopia, através das suas severas lições dadas a mão pesada, como também aprendi Genética, Astrologia, Espiritualidade e um pouco de tudo no âmbito da aleatoriedade que a caracteriza. Aos membros atuais e antigos do Departamento de Anatomia, Tiago, Sofia, Sara e Madalena, o meu sincero obrigado pela amizade, apoio e boa disposição. Seria igualmente injusto se não atribuísse uma menção especial à Madalena por toda a ajuda com as mais variadas técnicas de histologia, imunofluorescência, partilha de reagentes e pela sua boa disposição e alegria contagiante, que nunca falhou em me deixar com um sorriso. À Ana Maria, antigo membro do nosso grupo e atualmente membro do Departamento de Bioquímica, apelidada de “anjo” por todos os seus conhecidos, o meu sentido obrigado pela amizade, preocupação e ajuda que me forneceu ao longo destes anos. A Ana Maria, tal como o seu Romeu, ao qual alargo o meu sincero agradecimento, são um poço de conhecimento e sabedoria de valor incalculável para qualquer laboratório ou grupo de investigação.

Aos elementos do Centro de Genética da Reprodução Prof. Alberto Barros, representados pelo Prof. Alberto Barros, pela Dr.^a Ana Gonçalves, pela Dr.^a Margarida Gerales e pela Dr.^a Soraia Pinto, agradeço a disponibilidade e colaboração no âmbito da parceria estabelecida com o nosso grupo de investigação. Agradeço a prontidão com que sempre resolveram todos os nossos problemas e dúvidas, assim como a disponibilidade para articularem o seu trabalho com a nossa necessidade de obter amostras. Ao Serviço de Química Clínica do Centro Hospitalar Universitário de Santo António, representados pelo Dr. João Pessanha e pela Dr.^a Marta Rego, o meu agradecimento pela disponibilidade e colaboração. À Dr.^a Alexandra do CoreLab do Centro Hospitalar Universitário de Santo António, o meu agradecimento pela colaboração nas quantificações de insulina.

O agradecimento aos amigos que a ciência e a vida me proporcionaram será, igualmente, injusto devido à incapacidade de mencionar e agradecer merecidamente a todos os elementos. No entanto, terei obrigatoriamente de dedicar algumas palavras àqueles que mais contribuíram durante esta jornada. Ao João Monteiro, doravante João M., colega do Departamento de Bioquímica e amigo, o meu sincero obrigado pela amizade, disponibilidade, paciência e conselhos. Não sei se o João M. terá noção da dimensão do papel que desempenhou durante a, provavelmente, fase mais negra da minha vida pessoal. O meu mais sincero obrigado pela amizade, companhia e conselhos durante as longas horas de conversa que aconteceram na sua casa. Pelo mesmo motivo, não poderei deixar de mencionar também o João Ribeiro, doravante João R., por me ter permitido baixar os muros e demonstrar vulnerabilidade. Não terá sido coincidência, certamente, de que as casas de

ambos terão testemunhado o extremo oposto, vendo florescer uma das mais belas histórias que terei para partilhar. Terei ainda de agradecer ao João M. e João R., juntamente com o Luís, por todas as boas memórias, boa disposição e gargalhadas durante a nossa aventura pela Islândia. Podemos não ter encontrado Auroras Boreais ou a Alma, mas encontrámos uma aventura inesquecível que tarda a se repetir. Aos meus antigos colegas de casa, Nuno e Kadu, o meu obrigado por todos os momentos de boa disposição, companhia e amizade. Ao Miguel e ao Ricky, que decididamente não me coagiram a dedicar uma linha nos Agradecimentos, o meu obrigado pela amizade que surgiu durante o período final da escrita da presente tese de Doutoramento. A ambos e à Mafalda, o meu sincero obrigado por toda a companhia, boa disposição, por servirem de cobaias para as minhas experiências culinárias e por não verbalizarem qualquer desagrado pela constante presença de um parasita que a vossa colega de casa vos impingiu. A todos os meus restantes amigos, que direta ou indiretamente me auxiliaram durante o desenvolvimento do Doutoramento, e que injustamente não terei capacidade de agradecer individualmente, o meu sincero obrigado por terem feito parte desta jornada.

De todos os Agradecimentos contidos nestas páginas, talvez os mais inglórios sejam aqueles que são endereçados à família. Não há palavras que sejam suficientes para expressar o quão grato estou a quem me educou, trabalhou e lutou para me dar todo o amor, apoio e condições que levam ao término deste Doutoramento. Fernando Pessoa escrevia que “*sem estabilidade (...) impossível o progresso*”. Indubitavelmente, são estes os grandes responsáveis pela estabilidade que sustentou um progresso de 29 anos. E estes grandes pilares da minha essência serão, certamente, quem mais sofre pela minha ineficácia de expressão de sentimentos. Aos meus pais, Luísa e Fernando, aos meus avós, Adília e António, à minha irmã, Daniela, e aos meus irmãos honorários, Carlos e Pedro, garanto que, se em termos formais o Doutoramento é pessoal, a vós sempre direi que este Doutoramento é nosso. Aos meus pais, Luísa e Fernando, o maior e mais sincero obrigado não será suficiente para agradecer por todo o amor, apoio e sabedoria que me proporcionaram ao longo da vida. À minha mãe, agradeço todo o carinho e preocupação. A “intuição de mãe”, qual bola de cristal, é uma poderosa janela que permite transparecer os muros que, por vezes, teimo em erguer. Ao meu pai, que também sofre da incapacidade de expressão de sentimentos por palavras, qual doença genética que ambos partilhamos, o meu obrigado pelo carinho e valiosos conselhos ditos em meias-palavras. A ambos, espero que vos possa retribuir em dobro. Aos meus avós paternos, Adília e António, o maior e mais sincero obrigado por todo o amor, sabedoria e paciência. O meu avô, que pouco mais sabia escrever que o próprio nome, sempre frisou o quão importante era a educação. Este documento é o produto final dessa jornada que, embora não esteja presente para o ver, certa-

mente o deixaria orgulhoso. A minha avó certamente ficará, mesmo não percebendo totalmente o significado desta conquista. Ficaré mais orgulhosa, presumo, quando uma outra promessa se cumprir. À minha irmã, Daniela, o maior e mais sincero obrigado por todo o amor, paciência, companheirismo e conselhos. Não há palavras que descrevam o sentimento de quando um irmão é simultaneamente um dos nossos melhores amigos, ou que sejam suficientes para expressar o orgulho que sinto pela pessoa em que a minha irmã se tornou. Ao Carlos e ao Pedro, amigos que considero como família, o meu mais sincero obrigado por acreditarem tanto em mim e por todo o apoio, paciência, conselhos e amizade. Mais do que laços de sangue, família é quem está presente e a presença de ambos é uma constante de quase 25 anos. O meu sucesso profissional e pessoal contará sempre com a marca de ambos, tal como espero poder acompanhar e marcar positivamente a vossa jornada pela Vida.

O desenrolar deste Doutoramento, e a ciência em geral, trouxe-me imensas coisas fantásticas. Enquanto aluno de Doutoramento, tive a oportunidade de viajar imenso e conhecer novas culturas e realidades; tive a oportunidade de aprender e de me desafiar intelectualmente no dia-a-dia; tive o prazer de ter o sentimento de vitória ao receber prémios e bolsas. No entanto, nenhum destes feitos, experiências, ou vitórias me deu uma felicidade comparável ao que este Doutoramento de melhor me trouxe, que foi conhecer a Bárbara. Poderão amigos em comum dizer que esta história teve origem no São João de 2021, assumindo orgulhosamente a responsabilidade pela organização desse convívio. Poderão outros argumentar que foi graças aos longos dias, e por vezes madrugadas, onde juntos corremos centenas de amostras no gelado NMR da Universidade de Aveiro. Na verdade, poderei argumentar que a origem remonta a alguns anos antes desses acontecimentos. Foi em meados de 2019, durante o primeiro ano deste Doutoramento, que conheci a Bárbara enquanto aluna do laboratório do Prof. Rui Carvalho, na Universidade de Coimbra. A nossa primeira interação foi curta, dado o número de amostras e o curto espaço de tempo. Mais tarde, no final do mesmo ano, dirigi-me novamente à Universidade de Coimbra para entregar as primeiras amostras para Metabolómica referentes a este Doutoramento. E foi nestes moldes que reencontrei a Bárbara, a quem entreguei as amostras e que acabou por receber a responsabilidade de as processar. Agora com mais tempo, falámos durante algumas horas sobre os nossos percursos, dificuldades, e os mais variados temas. A quem me conhece, sabe que desvalorizo a espiritualidade e os chamados “destinos”, mas a verdade é que estas curtas interações foram suficientes para a intuição sinalizar a Bárbara como uma pessoa especial. Quiseram as intempéries da vida científica que a Bárbara acabasse por se mudar para o Porto, mais precisamente para vir trabalhar para o nosso laboratório. A Bárbara continuaria a ter a função de correr as amostras de Metabolómica do

grupo e, para tal, os Professores acabaram por lhe atribuir um apêndice que coincidentemente havia acabado de concluir um curso avançado de Metabolómica por NMR. Este apêndice terá de confessar que se é capaz de correr ou analisar um espectro, e até realizar *troubleshooting*, será maioritariamente pela excelência e perfeccionismo que adquiriu com os ensinamentos da Bárbara. Em troca, transmitiu os conhecimentos de macros e automação que aprendera no curso, levando a uma melhor rentabilização do tempo e ao processamento de centenas de amostras em poucos dias. Estava formada a equipa perfeita para o desempenho das nossas funções laborais. Com o passar do tempo, a Bárbara acabaria por conquistar o seu merecido espaço na minha vida, qual Cruzada ou Invasão Napoleónica, e esta equipa acabaria por desempenhar as mais variadas funções fora do horário laboral, como, por exemplo, encontrar o melhor croissant da cidade do Porto. A certo dia quiseram, novamente, as intempéries da ciência que fechássemos o laboratório mais tarde que o previsto, e o croissant desse dia ficaria por classificar. No entanto, foi graças a essa sucessão de eventos que a mais bela história se terá cumprido, nessa madrugada de verão, entre dois alentejanos deslocados no extremo oposto do país. Se devo agradecer a este Doutoramento por me levar até junto da Bárbara, também este Doutoramento muito lhe deve. Quando menciono a nossa capacidade de trabalho em equipa, não poderei eufemizar o quão bem colmatamos as limitações um do outro, tendo levado a um aumento significativo da nossa produção científica. Em termos profissionais, a Bárbara é uma brilhante Bioquímica, uma das mais inteligentes que tive o prazer de conhecer. Sempre me considereei inteligente, mas não terei qualquer problema em admitir que a Bárbara estará acima de mim e que terá um potencial superior ao meu. Penso que não será uma inverdade ao mencionar que não terá havido uma linha da presente tese de Doutoramento que não tivesse sido lida pela Bárbara, assim como muito lhe devo pelos valiosos contributos na discussão de resultados e na materialização e melhoramento estético de várias ilustrações gráficas contidas neste documento. É de uma enorme felicidade formar tão excelente equipa em termos laborais, mas maior é a felicidade pela excelente equipa que formámos na Vida. À Bárbara, devo o maior e mais sincero Agradecimento por todo o amor, carinho e energia que a caracteriza e me alegra todos os dias. À Bárbara, devo o maior e mais sincero Agradecimento pelos bons conselhos, por todo o apoio e pela infinita paciência para com os meus devaneios e ambiciosos planos irrealistas. À Bárbara, devo o maior e mais sincero Agradecimento pela sua capacidade de comunicação e por qualquer dificuldade ou problema, seja qual for a sua dimensão, ter resolução. À Bárbara, devo o maior e mais sincero Agradecimento por formarmos sonhos de Vida que almejamos partilhar. Nunca haverá linhas suficientes que farão justiça ao quão grato estou por ter a Bárbara a meu lado, e, certamente, me incumbirei de lhe lembrar dia após dia, ao longo das nossas vidas.

Atravessar uma corda-bamba torna-se mais fácil quando sabemos que a rede que nos pode aparar existe por perto. É esta rede, composta por um coletivo de seres humanos especiais, que me relembra que as derrotas e obstáculos são temporários, apenas a desistência é de carácter permanente.

A todos, coletivamente, espero que vos possa deixar tão orgulhoso como eu sou de vos ter na minha vida.

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For the following 6 months at the Gomes Lab, I absorbed the full intensity of the fast-paced and intense work environment, always at the highest standard of scientific quality, that characterizes the American Scientific Community. I had the privilege of being surrounded by the vanguard of the cancer research, which gave me the opportunity to discover new techniques, signaling and metabolic pathways, and learn from some of the best scientists in the field. When I mention that I learned a lot, I mean A LOT! And I finally had a glimpse of what it takes to work on projects that will be published at the highest level of the scientific standards. It was a fulfilling experience, and I am very grateful to all the people that took part of it.

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List of Publications

Publications included in this thesis:

1. **Carrageta DF**, Guerra-Carvalho B, Sousa M, Barros A, Oliveira PF, Monteiro MP, Alves MG. (2020). Mitochondrial activation and reactive oxygen species overproduction during sperm capacitation are independent of glucose stimuli. *Antioxidants*, 2020; 9(8):750. <https://doi.org/10.3390/antiox9080750>
2. Monteiro BS, Freire-Brito L, **Carrageta DF**, Oliveira PF, Alves MG (2021). Mitochondrial uncoupling proteins (UCPs) as key modulators of ROS homeostasis: a crosstalk between diabetes and male infertility? *Antioxidants*, 10(11): 1746. <https://doi.org/10.3390/antiox10111746>
3. Guerra-Carvalho B, **Carrageta DF**, Crisóstomo L, Carvalho RA, Alves MG, Oliveira PF (2022). Molecular mechanisms regulating spermatogenesis in vertebrates: environmental, metabolic, and epigenetic factor effects. *Animal Reproductive Sciences*, 246: 106896. <https://doi.org/10.1016/j.anireprosci.2021.106896>
4. **Carrageta DF**, Freire-Brito L, Oliveira PF, Alves MG. (2022). Evaluation of human spermatozoa mitochondrial membrane potential using the JC-1 dye. *Current Protocols*, 2, e531. <https://doi.org/10.1002/cpz1.531>
5. **Carrageta DF**, Freire-Brito L, Guerra-Carvalho B, Ribeiro JC, Monteiro BS, Barros A, Oliveira PF, Monteiro MP, Alves MG. (2023). Inhibition of mitochondrial uncoupling proteins arrests human spermatozoa motility without compromising viability. *Antioxidants*, 2023; 12(2):409. <https://doi.org/10.3390/antiox12020409>
6. **Carrageta DF**, Freire-Brito L, Guerra-Carvalho B, Bernardino RL, Monteiro BS, Barros A, Oliveira PF, Monteiro MP, Alves MG. (2023). Mitochondrial uncoupling proteins regulate the metabolic function of human Sertoli cells. *Reproduction*, REP-23-0229. <https://doi.org/10.1530/REP-23-0229>

7. **Carrageta DF**, Pereira SC, Ferreira R, Monteiro MP, Oliveira PF, Alves MG. (2023). Signatures of metabolic diseases on spermatogenesis and testicular metabolism. Accepted at: *Nature Reviews Urology* (ISSN: 1759-4820), 20th November 2023.
8. **Carrageta DF**, Guerra-Carvalho B, Pereira SC, Barros A, Oliveira PF, Monteiro MP, Alves MG. (2023). Insulin promotes capacitation through autocrine/paracrine mechanisms in human spermatozoa (unsubmitted journal article).

Abstract

Infertility is one of the most silently growing global health and societal issues, with male fertility facing a concerning decline marked by the diminishing quality of human spermatozoa. Idiopathic male infertility, *i.e.* of elusive cause, is alarmingly prevalent, highlighting the lack of knowledge in the field. Spermatozoa embarks on an extensive journey before being able to fertilize the oocyte. Spermatogenesis occurs within the testis, assisted by Sertoli cells. Then, spermatozoa progress through maturation in the epididymis and undergo the last modifications to achieve fertilizing ability in the female reproductive tract. From spermatogenesis to the final steps of maturation, capacitation and acrosome reaction, the development and function of the germ cell line are highly regulated by its environment. Depending on the stage of development or maturation, the ionic, metabolic, and hormonal requirements of the germ cell line are variable. Simultaneously, the spatial location of these cells has its own influence due to the different fluid compositions. Therefore, there is a complex interaction between the stage of maturation of the male gamete and the characteristics of the fluid where these cells are localized at a given moment. Most of the molecular mechanisms responsible for human spermatozoa development and capacitation are, in fact, currently unknown.

The main aim of this dissertation was to unravel the intricate metabolic and hormonal orchestration governing human spermatozoa and the function of Sertoli cells, with a pronounced emphasis towards scrutinizing the regulation of mitochondrial activity and the production of reactive oxygen species (ROS). This overarching goal was composed by several objectives, with the initial focus on unraveling the intricate role of glucose in shaping the metabolic profile and mitochondrial activity of human spermatozoa during capacitation. Glucose metabolism stands as a vital pathway in this process but the precise molecular mechanisms remain elusive. Within this primary objective, we conducted a thorough investigation into the effects of diverse glucose concentrations, including its absence, on the metabolic dynamics, mitochondrial function, and ROS generation during the *in vitro* capacitation of human spermatozoa. While ROS serve as natural byproducts of mitochondrial activity and contribute to essential signaling pathways, unchecked ROS production and resultant oxidative stress emerge as distinctive features of testicular dysfunction and male infertility. Mitochondrial uncoupling proteins (UCPs) stand as crucial regulators in the orchestration of key

events within cellular metabolism, influencing factors like mitochondrial membrane potential, oxidative phosphorylation efficiency, and energy dissipation as heat (thermogenesis). Moreover, UCPs shape the production of ROS contributing to the overall cellular redox state. Thus, the second objective of this dissertation comprised the study of metabolism and redox status regulation by UCPs in the “nurse” cells of the spermatogenesis, the human Sertoli cells, and also on spermatozoa. Insulin is one of the most important anabolic hormones, stimulating the uptake of glucose and being also involved in the regulation of redox balance. However, data concerning the role of insulin on human spermatozoa is limited. The third objective aimed to study the potential release of insulin by human spermatozoa and its role during capacitation. Within this objective, we aimed to clarify the presence of insulin in human spermatozoa, focusing on the potential autocrine/paracrine regulation of human spermatozoa capacitation and metabolic function. This dissertation also introduces a comprehensive protocol for the quantitative and qualitative assessment of mitochondrial health in human spermatozoa using the fluorescent dye JC-1, which is an easy and inexpensive tool to investigate the impact of various compounds, diseases, or conditions on spermatozoa health and mitochondrial function.

To achieve these objectives, we collected spermatozoa from human seminal samples of healthy men and established primary cultures of Sertoli cells (hSCs) obtained from testicular biopsies. To study glucose metabolism, we isolated the highly motile spermatozoa through density gradient centrifugation ($n = 55$) and incubated those cells in capacitating conditions for 6-hours in the absence or presence of increasing glucose concentrations (5.5, 11 and 22 mM). Then, viability and total motility were evaluated. Mitochondrial activity and intracellular ROS production were assessed using fluorogenic dyes JC-1 and CM-H₂DCFDA, respectively. Lipid peroxidation, protein tyrosine residues nitration and carbonyl groups content were evaluated as oxidative stress-induced damage biomarkers. Capacitation was evaluated through the quantification of total tyrosine residues phosphorylation. Finally, the metabolic profile was analyzed through metabolite quantification in culture media by ¹H-NMR. For the identification of UCPs, we screened for mRNA expression of all UCP homologs and assessed for its protein expression and location in human spermatozoa and hSCs. In our study of UCPs' role in human spermatozoa ($n = 16$), we used genipin — a broad-spectrum UCP inhibitor (0, 0.5, 5, and 50 μ M). After a 3-hour incubation, we evaluated parameters including viability, total motility, mitochondrial function, ROS production, and conducted a comprehensive analysis of the metabolic profile. Additionally, for hSCs ($n = 6$), we subjected those to genipin treatment (0, 0.5, 5, 50, and 100 μ M) for a 24-hour duration. Then, we evaluated cellular proliferation using the SRB assay, and assessed for cytotoxicity using the LDH release and MTT assays. Intracellular ROS production was assessed using CM-H₂DCFDA and the mitochondrial function was analyzed using JC-1 and

Seahorse Mito Stress assays. The metabolic profile was also assessed through the analysis of culture media by ^1H -NMR. To disclose if insulin could play a role in capacitation and fertilization, we collected high and low motility spermatozoa ($n = 15$) through a density gradient centrifugation and quantified the mRNA expression of *INS*, *PCSK1* and *PCSK2*, genes encoding the proteins responsible for proinsulin cleavage into insulin, by RT-qPCR. We also quantified the *INS* mRNA content in high motility spermatozoa from men undergoing fertility treatment ($n = 97$) to explore whether there was any association with assisted reproduction techniques (ART) outcomes. The protein expression of insulin, PC1, PC2, and insulin and IGF-1 receptors were evaluated by Western Blot and immunofluorescence. Insulin content in lysates was determined by electrochemiluminescence. Finally, the effects of insulin on viability, total motility, and mitochondrial and metabolic function were also assessed, and the insulin signaling pathway was analyzed by Western Blot.

We were able to demonstrate that glucose, even at high concentrations, is essential for human spermatozoa capacitation and motility. Remarkably, heightened mitochondrial activity manifested even in the absence of glucose. This surge in mitochondrial activity was succeeded by an overproduction of ROS, with no observable damage resulting from oxidative stress. These findings suggest that mitochondrial activation occurs during capacitation independently from a glucose stimulus, although glucose is necessary for a complete capacitation. Interestingly, our observations also detected the production of lactate and malate even in the absence of any external energetic substrates in the media, hinting at the presence of endogenous energy sources within human spermatozoa. This intriguing data challenges the prevailing paradigm on capacitation and propels us to explore the intricate roles of mitochondria and the regulation of ROS production in human spermatozoa.

We then targeted UCPs due to its regulation of mitochondrial function and ROS production. The mRNA expression of all UCP homologs (UCP1-6) by human spermatozoa is reported here for the first time. Using immunofluorescence, we observed that UCP1-3 are predominantly expressed outside the mitochondria, more precisely at the head equatorial segment, even though UCP1 and UCP2 are also located in the mitochondria. Despite not compromising spermatozoa viability, the inhibition of UCPs by 50 μM genipin had a striking effect on motility, leading to the irreversible total motility loss that persisted despite washing or incubation with theophylline, a cAMP activator. The inhibition of UCPs also had a negative impact on spermatozoa glycolytic metabolism, as these effects were associated with decreased lactate production, and impaired mitochondrial activity and ROS production. Overall, we demonstrated that UCPs are major regulators of human spermatozoa motility and metabolism, highlighting its potential as targets for the development of male contraceptives. Given the high impact of UCPs in human spermatozoa physiology and metabolism,

and the pronounced role of metabolic dysfunction and oxidative stress in testicular dysfunction, we investigated its function in hSCs. Notably, we also identified the mRNA expression of all UCP homologs (UCP1-6) and protein expression of UCP1, UCP2, and UCP3 in hSCs. The 24-hour inhibition of UCPs by genipin resulted in a decrease in hSCs proliferation, with no observed cytotoxicity. Furthermore, the inhibition of UCPs exhibited a dose-dependent reduction in mitochondrial membrane potential, oxygen consumption rate, and endogenous ROS production. The metabolism of hSCs was also affected as UCPs inhibition shifted its metabolism towards an increased pyruvate consumption. Taken together, these findings demonstrate that UCPs play a role as regulators of the mitochondrial function in hSCs, emphasizing its potential as targets in the study of male (in)fertility.

We were also able to demonstrate that insulin and the enzymes responsible for the cleavage of proinsulin into insulin, PC1 and PC2, are expressed by human spermatozoa. Insulin mRNA content was found to be higher in highly motile spermatozoa, however, we found no significant association between insulin mRNA expression and ART outcomes. Since insulin was found in spermatozoa (1.25 ± 0.71 ng/ 10^6 cells), we hypothesized that insulin could regulate motility and capacitation in an autocrine/paracrine manner. We successfully demonstrated the expression of insulin and IGF-1 receptors in human spermatozoa. Notably, the insulin signaling pathway remained active during capacitation, even in the absence of exogenous insulin. Supplementation with insulin (1 nM) had no additional effects on spermatozoa viability, motility, and metabolic function, suggesting that the stored insulin is sufficient to stimulate capacitation. Overall, our findings reveal that human spermatozoa possess the molecular machinery for insulin production and secretion. The active insulin signaling pathway during capacitation suggests the hormone's involvement in its autocrine/paracrine regulation.

In summary, this study delves deeper into the intricate metabolic and hormonal regulation governing male fertility. Our findings underscore the indispensable roles of glucose and mitochondrial metabolism in the functionality of human spermatozoa and Sertoli cells, with a notable emphasis on UCPs as key contributors to the orchestration of these processes. Additionally, we demonstrate an extrapancreatic production of insulin by human spermatozoa, unveiling its potential importance for capacitation. More importantly, our data suggests that human spermatozoa are insulin-producing cells and that a metabolic and hormonal cooperation may occur between spermatozoa. Overall, this study challenges the prevailing dogma of fertilization as a competitive and solitary swimming marathon. In truth, it reveals a nuanced perspective where fertilization emerges as a sophisticated and finely orchestrated team endeavor.

Resumo

A infertilidade é um dos problemas de saúde mais silenciosos a nível global, com a fertilidade masculina a sofrer um declínio preocupante, marcado pelo decréscimo de qualidade dos parâmetros espermáticos. Dentro dos casos de infertilidade masculina, a infertilidade idiopática, ou seja, com causas desconhecidas, é altamente prevalente, destacando a lacuna presente no conhecimento nesta área clínica. Antes de terem a capacidade de fertilizar o oócito, os espermatozoides embarcam numa extensa jornada com início no testículo. É neste órgão que ocorre a espermatogénese, altamente protegida e regulada pelas células de Sertoli. Após a espermiogénese, os espermatozoides progridem para o epidídimo, onde sofrem um novo processo de maturação. No entanto, as derradeiras alterações que levam à aquisição da capacidade de fertilização ocorrem no trato reprodutor feminino. Da espermatogénese às etapas finais de maturação, processos denominados de capacitação e reação acrossómica, o desenvolvimento e a função das células germinativas masculinas são altamente regulados pela composição do meio envolvente. Dependendo do estágio de desenvolvimento ou maturação, as necessidades iónicas, metabólicas e hormonais do gameta masculino são variáveis. Simultaneamente, também a localização espacial do gameta masculino é um fator a ser considerado, devido à composição característica de cada fluido envolvente. Desta forma, uma interação complexa entre o estágio de maturação do gameta masculino e as características do fluido envolvente, que depende da localização espacial, regulam a função do gameta e a capacidade de fertilização. No entanto, a maioria dos mecanismos moleculares envolvidos nesta interação, assim como as necessidades metabólicas e hormonais do espermatozoide, são ainda desconhecidos.

O principal objetivo desta dissertação passou por estudar a complexa regulação metabólica e hormonal que rege a função do espermatozoide humano e das células de Sertoli, com ênfase na regulação da atividade mitocondrial e na produção de espécies reativas de oxigénio (ROS). Este objetivo foi estratificado em vários objetivos secundários, onde o primeiro passou por estudar o papel da glucose no perfil metabólico e na atividade mitocondrial do espermatozoide humano durante o processo de capacitação. O metabolismo da glucose é uma via metabólica considerada como essencial para a capacitação do espermatozoide humano, no entanto, os mecanismos moleculares ainda não são totalmente conhecidos. Neste âmbito, procedeu-se ao estudo do impacto de concentrações crescentes

de glucose, assim como da sua ausência, no metabolismo, função mitocondrial e produção de ROS durante a capacitação *in vitro* de espermatozoides humanos. Enquanto a produção de ROS é uma consequência natural da atividade mitocondrial, assim como a sua contribuição como moléculas intervenientes em vias de sinalização é essencial, uma produção exacerbada resulta em stress oxidativo, que constitui um dos principais fatores associados à disfunção testicular e infertilidade masculina. Neste âmbito, as proteínas desacopladoras mitocondriais (UCPs) são intervenientes-chave no metabolismo celular, regulando vários processos como o potencial de membrana mitocondrial, a eficiência da fosforilação oxidativa e a dissipação de energia sob a forma de calor (termogénese). Adicionalmente, as UCPs regulam a produção mitocondrial de ROS, contribuindo para a homeostasia *redox* celular. Desta forma, o segundo objetivo passou pelo estudo da função das UCPs na regulação do metabolismo e homeostasia *redox* em espermatozoides e células de Sertoli humanas. A insulina é uma das mais importantes hormonas anabólicas, amplamente associada à captação e metabolismo da glucose, estando também envolvida na homeostasia *redox* celular. No entanto, o papel da insulina na função do espermatozoide humano não é totalmente conhecido. O terceiro objetivo incidiu no estudo da libertação de insulina pelos espermatozoides humanos, com ênfase no seu potencial papel autócrino/parácrino na regulação do metabolismo e processo de capacitação. Por último, esta dissertação também descreve um protocolo para a avaliação quantitativa e qualitativa da atividade mitocondrial em espermatozoides humanos usando a sonda fluorescente JC-1. Esta sonda é caracterizada pela sua fácil utilização e baixo custo, permitindo investigar o impacto de compostos ou doenças, entre outras condições experimentais, na função mitocondrial de espermatozoides.

De forma a cumprir os objetivos estipulados, procedeu-se à recolha de amostras seminais humanas de homens saudáveis e estabeleceram-se culturas primárias de células de Sertoli humanas (hSCs) obtidas de biópsias testiculares. Para o estudo do metabolismo da glucose, isolámos espermatozoides de elevada motilidade através da técnica de centrifugação por gradiente de densidade ($n = 55$), tendo estas células sido posteriormente incubadas em meio de capacitação na ausência ou presença de concentrações crescentes de glucose (0, 5.5, 11 e 22 mM). Após 6 horas de incubação, avaliámos a viabilidade e motilidade total; a atividade mitocondrial e produção intracelular total de ROS usando as sondas JC-1 e CM-H₂DCFDA, respetivamente; biomarcadores de stress oxidativo, nomeadamente a peroxidação lipídica, a nitração de resíduos de tirosina e o conteúdo de grupos carbonilo em proteínas; a capacitação pela quantificação da fosforilação total dos resíduos de tirosina; e o perfil metabólico, através da quantificação de metabolitos no meio de cultura por ¹H-NMR. Para a análise da expressão de UCPs, procedemos à avaliação da expressão de

mRNA de todos os homólogos, assim como avaliámos a expressão da proteína e imunolocalização por Western Blot e Imunofluorescência, respetivamente, em espermatozoides humanos e hSCs. Para o estudo da função das UCPs no espermatozoide humano ($n = 16$), foi utilizada a genipina, um inibidor geral de UCPs (0, 0.5, 5 e 50 μM). Após 3 horas de incubação, procedemos à avaliação da viabilidade e motilidade total, função mitocondrial, produção intracelular total de ROS, e perfil metabólico. No estudo em hSCs ($n = 6$), o tratamento com genipina (0, 0.5, 5, 50 e 100 μM) teve uma duração de 24 horas. Após incubação, avaliámos a proliferação celular, utilizando o ensaio de SRB; a citotoxicidade, utilizando os ensaios de libertação de LDH e MTT; a produção intracelular total de ROS foi analisada com recurso à sonda fluorescente CM-H₂DCFDA; a função mitocondrial, utilizando a sonda JC-1 e o ensaio de Seahorse Mito Stress; e o perfil metabólico, através da análise do meio de cultura por ¹H-NMR. Para o estudo do potencial papel da insulina no processo de capacitação e fertilização do espermatozoide humano, procedemos ao isolamento de espermatozoides humanos de elevada e baixa motilidade ($n = 15$) a partir de amostras seminais de indivíduos saudáveis. Após a extração do RNA total, quantificámos o conteúdo em mRNA da insulina e das enzimas necessárias à clivagem de pro-insulina em insulina, PC1 e PC2, por RT-qPCR. Para explorar uma eventual associação com os resultados de técnicas de procriação medicamente assistida (ART), procedemos à quantificação do conteúdo em mRNA de insulina em espermatozoides de elevada motilidade derivados de homens pertencentes a casais submetidos a tratamento na clínica de fertilidade ($n = 97$). Posteriormente, a presença da proteína da insulina, PC1, PC2, e recetores da insulina e IGF-1 foi avaliada por Western Blot e Imunofluorescência e o conteúdo em insulina em espermatozoides foi avaliado por eletroquimioluminescência. Por último, os efeitos da suplementação do meio de capacitação com insulina foram avaliados através da avaliação da viabilidade, motilidade total, análise da função mitocondrial e metabólica por JC-1 e MTT, respetivamente. Foi também avaliada a ativação da via de sinalização da insulina por Western Blot.

Os resultados obtidos demonstram que a glucose, mesmo em elevadas concentrações, é essencial à capacitação e motilidade dos espermatozoides humanos. Surpreendentemente, observámos um aumento na atividade mitocondrial na ausência de glucose ou outros substratos energéticos exógenos. Este aumento de atividade mitocondrial foi acompanhado por um aumento na produção de ROS, embora nenhuma alteração nos biomarcadores de stress oxidativo tenha sido registada. Estes resultados sugerem que o aumento da atividade mitocondrial decorrente do processo de capacitação ocorre independentemente do estímulo da glucose, embora este substrato energético seja necessário para completar o processo de capacitação. Curiosamente, observámos que os espermatozoides

humanos produzem lactato e malato na ausência de substratos energéticos exógenos, sugerindo a existência de substratos energéticos endógenos.

Devido ao seu papel na regulação da produção de ROS e metabolismo mitocondrial, o próximo objetivo incidiu sobre o estudo das UCPs. Pela primeira vez, descrevemos a presença de mRNA de todos os homólogos (UCP1-6) em espermatozoides humanos. Curiosamente, observámos por imunofluorescência que as UCP1-3 são predominantemente presentes fora das mitocôndrias, mais precisamente no segmento equatorial da cabeça, embora a UCP1 e UCP2 também se encontrem presentes na mitocôndria dos espermatozoides humanos. Após a inibição das UCPs com genipina, observámos um efeito drástico na motilidade, caracterizado pela perda total de motilidade de forma irreversível, dado que os efeitos persistiram após a lavagem das células ou tratamento com teofilina, um conhecido ativador da motilidade de espermatozoides pela via do cAMP. Surpreendentemente, a viabilidade dos espermatozoides não se encontrava comprometida. A inibição das UCPs apresentou ainda um impacto negativo no metabolismo glicolítico dos espermatozoides, caracterizado pela diminuição da produção de lactato e redução na atividade mitocondrial e produção de ROS. De uma forma geral, demonstrámos que as UCPs são importantes reguladores da motilidade e metabolismo do espermatozoide humano, assim como evidenciamos o seu potencial papel no desenvolvimento de novos métodos contraceptivos masculinos. Dado o elevado impacto das UCPs no metabolismo do espermatozoide humano, procedemos à análise do seu potencial papel na disfunção metabólica e stress oxidativo no testículo, através do estudo da sua função em culturas primárias de hSCs. Surpreendentemente, também as hSCs expressam o mRNA de todos os homólogos (UCP1-6), assim como a expressão da proteína da UCP1, UCP2 e UCP3. Após 24 h de inibição das UCPs pela genipina, observámos que a proliferação celular havia diminuído, embora sem qualquer indício de citotoxicidade. Adicionalmente, a inibição prolongada das UCPs inibiu de forma dose-dependente o potencial da membrana mitocondrial e a taxa de consumo de oxigénio, sugerindo um impacto negativo na função mitocondrial. Observou-se ainda que a produção endógena de ROS estava igualmente diminuída. A inibição das UCPs também afetou o metabolismo das hSCs, levando a uma alteração do fluxo metabólico para um maior consumo de piruvato. Conjugando estes resultados, as UCPs desempenham um papel importante na regulação da função mitocondrial e no metabolismo das hSCs, evidenciando a sua relevância no estudo da disfunção testicular e (in)fertilidade masculina.

Por último, demonstrámos que a insulina, assim como as enzimas responsáveis pela clivagem de pro-insulina em insulina, PC1 e PC2, estão presentes em espermatozoides humanos. Observámos que o conteúdo em mRNA de insulina é maior em espermatozoides de elevada motilidade, comparativamente com os de baixa motilidade; no entanto, não encontramos qualquer correlação com as taxas de sucesso de técnicas de ART. O conteúdo

em insulina em espermatozoides humanos foi determinado em $1.25 \pm 0.71 \text{ ng}/10^6$ espermatozoides, colocando a hipótese de que esta hormona poderia regular a motilidade e o processo de capacitação de uma forma autócrina/parácrina. Neste âmbito, identificámos a presença do recetor de insulina e IGF-1 na cauda dos espermatozoides humanos. Em suporte desta hipótese, observou-se que a via de sinalização da insulina se encontrava ativa, mesmo na ausência de insulina no meio de cultura. Suplementação com insulina (1 nM) não teve qualquer efeito adicional na viabilidade, motilidade e função metabólica e mitocondrial, sugerindo que a insulina armazenada pelo espermatozoide humano será suficiente para estimular a capacitação. De uma forma geral, os nossos resultados demonstram que o espermatozoide humano possui as enzimas necessárias para a produção de insulina, que estimamos ser libertada durante a capacitação para a sua própria regulação de forma autócrina/parácrina.

Em suma, o trabalho da presente Dissertação descreve a complexa regulação metabólica e hormonal que governa a fertilidade masculina. Os nossos resultados demonstram os papéis indispensáveis da glucose e do metabolismo mitocondrial no funcionamento do espermatozoide e células de Sertoli humanas, com particular ênfase no papel central que as UCPs desempenham nestes processos. Adicionalmente, demonstrámos a existência de produção extra-pancreática de insulina, exibida pelo espermatozoide humano, assim como o seu potencial papel regulatório no processo de capacitação. Estes resultados sugerem uma potencial cooperação metabólica e hormonal entre espermatozoides, destacando o espermatozoide como uma potencial célula produtora de insulina. Em conjunto, este trabalho desafia o dogma vigente de que a fertilização é o resultado final de uma corrida extremamente competitiva entre espermatozoides quando, na realidade, poderá ser um complexo e finamente regulado trabalho de equipa.

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List of Abbreviations

¹H-NMR	Proton Nuclear Magnetic Resonance	BMI	Body Mass Index
4-HNE	4-Hydroxy-2-nonenal	BPA	Bisphenol A
ABHD2	Abhydrolase Domain-Containing 2 Acylglycerol Lipase	BSA	Bovine Serum Albumin
ACADL	Long-Chain Acyl-CoA Dehydrogenase	BTB	Blood-Testis Barrier
ACADVL	Very Long-Chain Acyl-CoA Dehydrogenase	BWW	Biggers, Whitter and Whittingham Medium
ACOX1	Acyl-CoA Oxidase 1	cAMP	Cyclic Adenosine Monophosphate
ACSL1	Acyl-CoA Synthetase Long-Chain Family Member 1	CASA	Computer Assisted Sperm Analyzer
Akt	Protein Kinase B	CatSper	Sperm Cation Channel
AMPA	Aminomethylphosphonic Acid	CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
AMPK	AMP Kinase	CPT1	Carnitine Palmitoyltransferase 1
ANT	Adenine Nucleotide Translocase	DHT	5 α -Dihydrotestosterone
ART	Assisted Reproductive Technology	DMSO	Dimethyl Sulfoxide
BAT	Brown Adipose Tissue	DNP	2,4-Dinitrophenyl
BFA	Branched Fatty Acids	DNPH	2,4-Dinitrophenylhydrazine
		ECAR	Extracellular Acidification Rate

EDC	Endocrine-disrupting Chemical	HBSS	Hanks' Balanced Salt Solution
EDSP	Endocrine Disruptor Screening Program	hCG	Human Chorionic Gonadotropin
FCCP	Carbonyl Cyanide-p-trifluoromethoxyphenylhydrazone	HM	High Motility Human Spermatozoa
FRAP	Ferric Reducing Antioxidant Power Assay	HPG	Hypothalamic-Pituitary-Gonadal Axis
FSH	Follicle-stimulating Hormone	hSC	Human Sertoli Cell
FTO	Fat Mass and Obesity Gene	Hv1	Voltage-gated Proton Channel
G6PDH	Glucose-6-phosphate Dehydrogenase	IGF-1	Insulin-like Growth Factor 1
GHS-R	Growth Hormone Secretagogue Receptor/Ghrelin Receptor	IGF1R	Insulin-like Growth Factor 1 Receptor
GLP-1	Glucagon-like Protein 1	IKBA	NF-κB Inhibitor Alpha
GLP-1R	Glucagon-like Protein 1 Receptor	InsR	Insulin Receptor
GLUT	Glucose Transporter	IP₃	Inositol Triphosphate
GNPDA2	Glucosamine-6-phosphate Isomerase 2	IP₃R	Inositol Triphosphate Receptor
GnRH	Gonadotropin-releasing Hormone	IQR	Interquartile Range
GSIS	Glucose-stimulated Insulin Secretion	IRS	Insulin Receptor Substrate
GSK3β	Glycogen Synthase Kinase 3 Beta	IVF	In Vitro Fertilization
HADHA	Hydratase Subunit A	JAK2	Kanus Kinase 2
		JC-1	5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
		LDH	Lactate Dehydrogenase
		LepR	Leptin Receptor

LH	Luteinizing Hormone	PI3K	Phosphoinositide 3-Kinase
LM	Low Motility Human Spermatozoa	PKA	Protein Kinase A
MAPK	Mitogen-activated Protein Kinase	PKB	Protein Kinase B
MC4R	Melanocortin 4 Receptor	PLC	Phospholipase C
MCT	Monocarboxylate Transporter	PVDF	Polyvinylidene Fluoride
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	qPCR	Quantitative Polymerase Chain Reaction
NBC	Sodium Bicarbonate Cotransporter	RIPA	Radioimmunoprecipitation Assay
NEC1	Neuroendocrine Convertase 1	RMR	Rest Metabolic Rate
NEC2	Neuroendocrine Convertase 2	ROS	Reactive Oxygen Species
NF-κB	Nuclear Factor-κB	RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
OCR	Oxygen Consumption Rate	sAC	Soluble Adenylyl Cyclase
ORG	Obesity-Related Genes	SC	Sertoli Cells
OS	Oxidative Stress	SCNT	Somatic-Cell Nuclear Transfer
PBL	Peripheral Blood Leukocytes	SNARE	Soluble N-ethylmaleimide-Sensitive-Factor attachment Protein Receptor
PBS	Phosphate Buffer Saline	SRB	Sulforhodamine B
PC1	Neuroendocrine Convertase 1	STAT	Signal Transducer and Activator of Transcription
PC2	Neuroendocrine Convertase 2	SYT	Synaptotagmin-1
PGCs	Primordial Germ Cells	TCA cycle	Tricarboxylic Acid Cycle
		TMEM18	Transmembrane Protein 18

UCP Mitochondrial Uncoupling Proteins

WAT White Adipose Tissue

WHO World Health Organization

Chapter 1

Introduction



This chapter was adapted from the following published works:

Monteiro BS, Freire-Brito L, **Carrageta DF**, Oliveira PF, Alves MG (2021). Mitochondrial uncoupling proteins (UCPs) as key modulators of ROS homeostasis: a crosstalk between diabetes and male infertility? *Antioxidants*, 10(11): 1746. <https://doi.org/10.3390/antiox10111746>

Guerra-Carvalho B, **Carrageta DF**, Crisóstomo L, Carvalho RA, Alves MG, Oliveira PF (2022). Molecular mechanisms regulating spermatogenesis in vertebrates: environmental, metabolic, and epigenetic factor effects. *Animal Reproductive Sciences*, 246: 106896. <https://doi.org/10.1016/j.anireprosci.2021.106896>

Carrageta DF, Pereira SC, Ferreira R, Monteiro MP, Oliveira PF, Alves MG. (2023). Signatures of metabolic diseases on spermatogenesis and testicular metabolism. Accepted at: *Nature Reviews Urology* (ISSN: 1759-4820), 20th November 2023.

The integral texts are available in the Annex A of this Dissertation.

1.1. Male infertility

Infertility is one of the most challenging health and socioeconomic issues worldwide. Once relegated, fertility care has now ascended to a pivotal position within the realm of advancing the United Nations' Sustainable Development Goals for 2030, particularly in the domains of "Good Health and Well-Being" (Goal 3) and "Gender Equality" (Goal 5). According to the latest World Health Organization (WHO) report "Infertility prevalence estimates 1990-2021", infertility affects one in every six people (16.7% of the global population) (WHO, 2023). In this report, the highest prevalence of lifetime infertility was found to be the Western Pacific Region (23.2%), followed by the Region of the Americas (composed by the North, Central, and South America; 20.0%), and European Region (16.5%). However, these findings may be conflicting due to the social constrictions and lack of studies from the Eastern Mediterranean Region and South-East Asia Region. Besides this limitation, another major limitation highlighted in the report is the lack of studies with male participants (WHO, 2023). Comparing to the female counterpart, conducting studies on male infertility can be a difficult task due to social and religious motives in certain regions of the globe. Nonetheless, there is a consensus among the scientific community that studying the male factor is as important as the female factor. In fact, it is estimated that in 50% of infertile couples, infertility is attributed either to male factors or a combination of male and female factors (Agarwal et al., 2021). In 2015, Agarwal et al. conducted a systematic review and reported that up to 12% of men are infertile (Agarwal et al., 2015). More perilously, a recent meta-regression analysis by Levine et al. containing data from 288 studies and 57168 men, reported that the concentration and total count of spermatozoa in the ejaculate steadily decreased, between 1973 and 2018, by 51.6% and 62.3%, respectively (Levine et al., 2023). Curiously, the decline of sperm quality was found to be steeper post-2000, doubling the percent decline per year. This trend was observed in every region of the globe, suggesting that the etiology may be more complex than the simplistic explanation of different lifestyles. In fact, the WHO reported that the estimated lifetime prevalence of infertility is similar in high- (17.8%) and low- and middle-income countries (16.5%), further suggesting multifactorial causes (WHO, 2023). In fact, up to 75% of infertility attributed to male factor is idiopathic, *i.e.* of unknown causes (Kothandaraman et al., 2016). Notwithstanding, a few etiologic factors for male infertility have been already identified. Among those, genetic factors, hormonal imbalances, testicular diseases, inflammation, oxidative stress, exposure to pollutants or certain drugs, and metabolic disorders were highlighted as likely drivers (Bernardino et al., 2019; Carrageta et al., 2019; Krausz & Riera-Escamilla, 2018; Martin-Hidalgo et al., 2019; Shahrokhi et al., 2020).

To gain deeper insights into the underlying factors influencing male fertility and the potential avenues for therapeutic intervention in cases of impairment, it becomes imperative to delve into the intricate molecular mechanisms governing spermatogenesis. This further encompasses comprehending the intricacies of spermatozoa function, along with the complex biochemical transformations these cells must undergo to acquire fertilization competence. In the ensuing sections, we will discuss the present-day understanding of the full spectrum of molecular processes spanning from spermatogenesis to spermatozoa capacitation, centering our focus on the nuanced interplay of metabolic and hormonal regulation.

1.2. Sertoli cells and the metabolic and hormonal regulation of spermatogenesis

Spermatogenesis orchestrates the intricate production of male gametes known as spermatozoa, taking place within the testes. This remarkable process unfolds within the seminiferous tubules, the fundamental working unit of the testes. The seminiferous tubules exhibit distinctive attributes, characterized by its elongated and intricate tubular architecture, ultimately converging into the *rete testis*. These tubules, constituting the microcosm of sperm development, are enveloped by a lining of Sertoli cells (SCs) and germ cells, establishing intimate physical connections with the underlying basement membrane. Notably, SCs represent approximately 17-20% of the seminiferous tubules (Russell et al., 1990) and form a highly polarized epithelium, due to its prominent basement membrane, presenting tight junctions between adjacent cells that are the basis of the blood-testis barrier (BTB) (Mruk & Cheng, 2015). The BTB serves as a functional barrier, controlling the exchange of molecules that enter or exit the testis microenvironment due to its complex and finely regulated network of transporters and channels in the basal membrane. In addition, the BTB acts as an immunological barrier that minimizes the presence of immune cells and inflammatory responses in the seminiferous tubules (Su et al., 2011; Wong & Cheng, 2005). Hence, SCs are responsible for maintaining the delicate microenvironment balance within the seminiferous tubules. SCs offer not just safeguarding but also extend mechanical and nutritional sustenance to the germ cells, thus playing a pivotal role in its development and well-being. If germ cells do not receive the appropriate quantity of energetic substrates and the adequate ionic environment for its development, degenerate through apoptosis (Boussouar & Benahmed, 2004; Jutte et al., 1981). Each SC supports up to 30-50 germ cells at all stages of development due to its numerous cytoplasmic prolongations (Cheng et al., 2010; Weber et al., 1983). At each stage, SCs deeply associate with the germ cells and provide all the

nutrients and metabolic intermediates needed, including amino acids, carbohydrates, lipids, vitamins, and ions (Mruk & Cheng, 2004; Robinson & Fritz, 1981).

Spermatogenesis occurs from the basement membrane to the seminiferous tubules' lumen (Fig. 1.1). In brief, spermatogonia, which are located near the basement membrane, undergo mitosis to replicate and maintain the germ cell line (spermatogonia A), while generating a new population (spermatogonia B) that differentiate into spermatozoa. Then, spermatogonia B differentiate into primary spermatocytes, which cross the BTB and enter the first meiotic division to generate secondary spermatocytes. Upon the second meiotic division, round spermatids are generated, and elongated spermatids are then formed through spermiogenesis. Finally, spermiation occurs and elongated spermatids are released into the lumen as immature spermatozoa. Non-motile immature spermatozoa are transported in the testicular fluid into the epididymis through peristaltic contractions. In the epididymis, spermatozoa become mature and gain motility (Hess & de Franca, 2008).

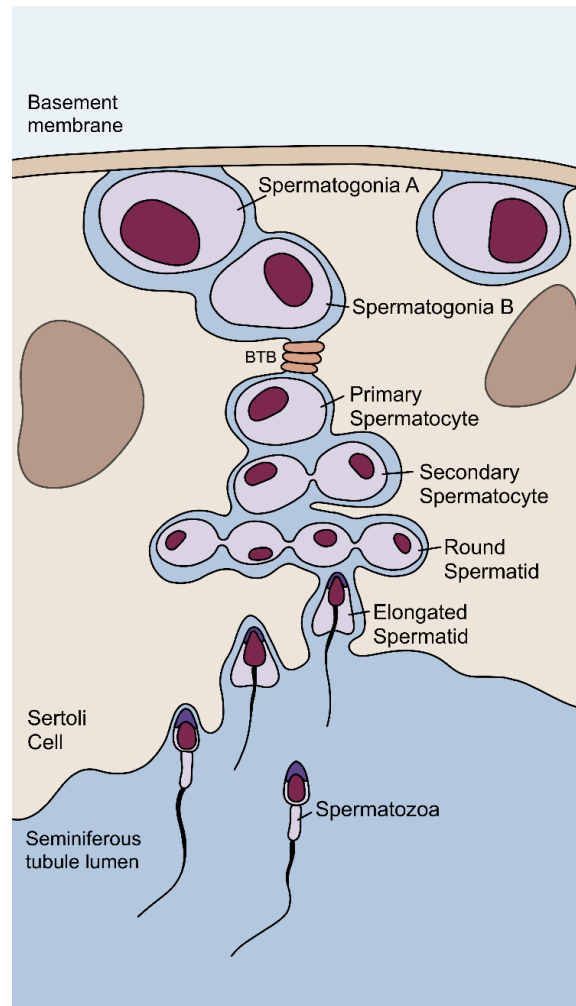


Fig. 1.1 – Schematic illustration of mammalian spermatogenesis. Spermatogonia A are located near the basement membrane and undergo mitosis to generate spermatogonia B, which will differentiate into spermatozoa. Spermatogonia B differentiate into primary spermatocytes, cross the blood-

testis barrier (BTB), and enter the first meiotic division to generate secondary spermatocytes. Round spermatids are formed through the second meiotic division and then elongated spermatids are generated through spermiogenesis, which are released into the lumen as immature spermatozoa in a process known as spermiogenesis. Immature spermatozoa are transported into the epididymis, in order to complete maturation (adapted from Guerra-Carvalho et al. 2022).

Spermatogenesis is a very complex and finely regulated process that is still poorly understood at the molecular level. Currently, the testis is regarded as a highly active metabolic and endocrine organ, sensitive to several pathophysiological conditions and a myriad of environmental or nutritional cues that are on the genesis of a poor testicular function and impaired spermatogenesis that could compromise reproduction (Oliveira et al., 2017).

1.2.1. Sertoli cells and the metabolic regulation of spermatogenesis

The testes are very specialized organs where the production of spermatozoa depends on the metabolic state of the individual. As aforementioned described, SCs integrate many of these metabolic cues, which are later reflected in spermatogenesis due to its functions: formation of BTB and nutritional support (Mruk & Cheng, 2004, 2015). The human germ cell line exhibits a unique metabolism, where lactate is used as the preferential energy source even though these cells possess glucose transporters (GLUTs) and the molecular machinery for glycolysis (Kokk et al., 2004; Oliveira et al., 2012). In order to guarantee the provision of optimal lactate levels essential for the proper development of germ cells, SCs have evolved a range of metabolic adaptations aimed at prioritizing lactate production over any other competing metabolic pathways (Fig. 1.2). SCs present a “Warburg-like” metabolism, as detailed by Oliveira et al. (Oliveira et al., 2015). Glucose is the primary source for lactate production by the SCs (Jutte et al., 1982). Lactate production by SCs starts with the uptake of glucose from the interstitial space by high-affinity GLUTs, mainly GLUT1 and GLUT3 (Oliveira et al., 2015). Glucose is subsequently metabolized into pyruvate in the cytoplasm through glycolysis. The resulting pyruvate can be further metabolized in three pathways: conversion to lactate (1) or alanine in the cytoplasm (2) or converted to acetyl-CoA after been transported into the mitochondria (3) (Rato et al., 2012). However, in SCs glucose is preferentially metabolized into lactate. Interestingly, the unique metabolism of SCs can be compared to cancer cells, although the high glycolytic rate is not associated with rapid energy production or cellular proliferation. As a matter of fact, the human SCs population remains roughly the same throughout an individual’s life (Cortes et al., 1987). The majority of lactate produced by SCs is necessary to fuel spermatogenesis, and thus, exported to the intratubular fluid of seminiferous tubules by the specific proton/monocarboxylate transporter 4 (MCT4). There, developing germ cells obtain lactate mainly through MCT2 and MCT1,

which is once again converted to pyruvate, fueling its metabolic needs (Boussouar & Benahmed, 2004). SCs also export high amounts of acetate into the intratubular fluid. Although the exact role of acetate in spermatogenesis remains to be elucidated, it is proposed that this metabolite is used to *ново* lipogenesis by germ cells, given these cells high demand for lipids (Oliveira et al., 2015).

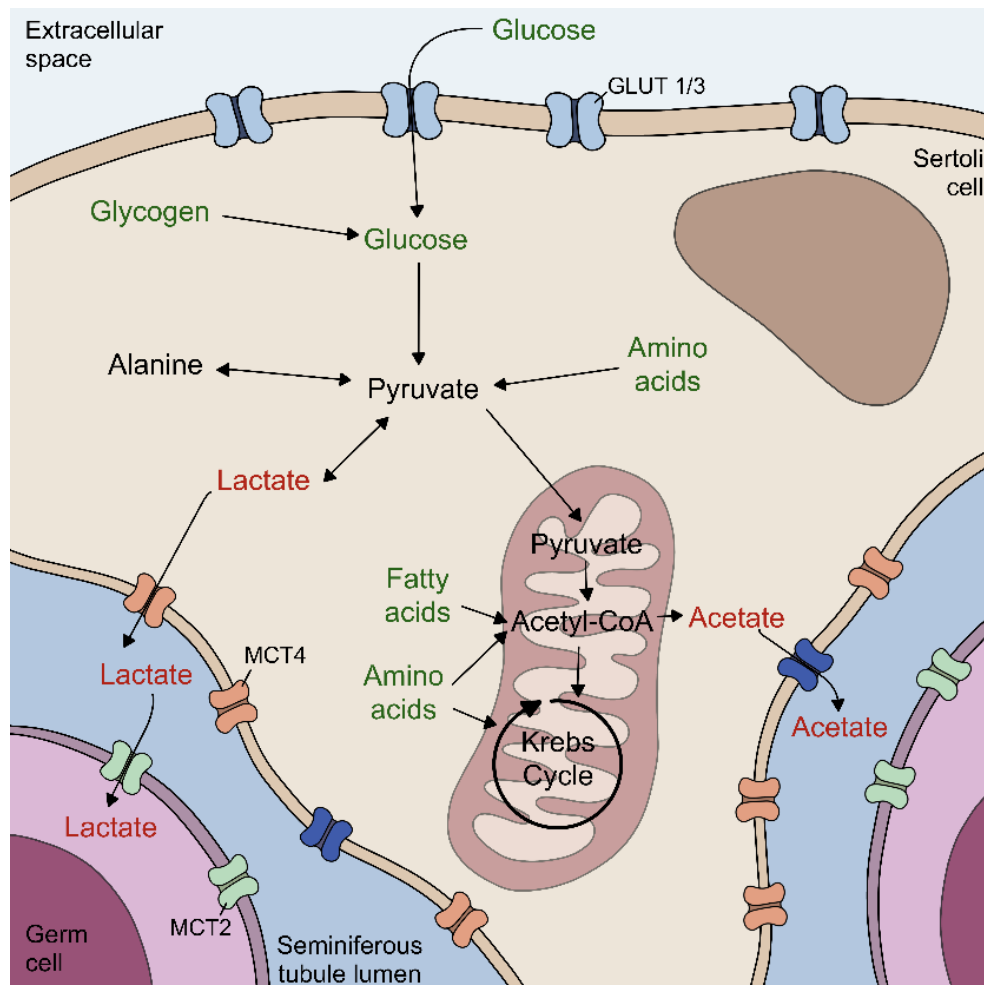


Fig. 1.2 – Metabolic pathways of Sertoli cells. Glucose enters the Sertoli cell through the high-affinity glucose transporters 1 (GLUT1) and 3 (GLUT3). Inside the cytoplasm, glucose is converted to pyruvate, as a result of aerobic glycolytic processes. Pyruvate can then be metabolized into (1) lactate or (2) alanine in the cytoplasm or (3) oxidized into acetyl-CoA after being transported into the mitochondria. In Sertoli cells, glucose is mostly converted into lactate, which is released into the seminiferous tubule lumen, by the monocarboxylate transporter 4 (MCT4). Lactate is taken up by the developing germ cells, via the MCT2, to meet energetic requirements. Sertoli cells also secrete acetate into the seminiferous tubule fluid, which might be used by germ cells to support *de novo* lipogenesis. Additionally, to sustain metabolism and meet energetic requirements, Sertoli cells can also metabolize several amino acids, glycogen, and fatty acids (adapted from Guerra-Carvalho et al. 2022).

Under unfavorable conditions, SCs adapt metabolically to ensure adequate lactate production for the support of spermatogenesis. SCs viability and lactate production are maintained even when glucose concentrations are low or completely absent. In these circumstances, GLUT1 and MCT4 expression increases to maximize glucose uptake and lactate secretion, respectively (Galardo et al., 2007; Riera et al., 2009). Interestingly, and even though there is a large amount of glycolytic activity, glucose is hypothesized not to be the main substrate used by SCs for ATP synthesis to accomplish its own metabolic needs. Most of the energy transformed by SCs into ATP comes from lipids derived from apoptotic germ cells and residual bodies, through the oxidation of fatty acids. Briefly, upon entering the mitochondria, fatty acids are metabolized into acetyl-CoA (via β -oxidation), and acetyl-CoA is subsequently processed in the Krebs cycle and oxidized to CO_2 . The electrons produced during these metabolic processes (β -oxidation and Krebs cycle) are transferred to the electron transport chain, resulting in the synthesis of ATP (Xiong et al., 2009). Additionally, SCs can use several amino acids, including glutamine, glycine, and branched-chain amino acids, and also glycogen to ensure adequate nutritional support for germ cell development and sustain metabolic processes (Kaiser et al., 2005; Rato et al., 2015; Slaughter & Means, 1983).

1.2.2. Sertoli cells and the hormonal regulation of spermatogenesis

Testes are hormonally regulated through the hypothalamic-pituitary-gonadal (HPG) axis (Fig. 1.3). In brief, the gonadotropin-releasing hormone (GnRH) is synthesized and released in pulsatile fashion by the hypothalamus, which stimulates the anterior pituitary to produce and release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both these hormones regulate the testicular function, although through different targets – LH stimulates steroidogenesis and the release of testosterone in Leydig cells; FSH modulates the function of SCs and spermatogenesis. In turn, testosterone and inhibin, produced by Leydig and SCs, respectively, regulate the activity of the hypothalamus-pituitary gland axis in a negative feedback mechanism (Clavijo & Hsiao, 2018).

Besides the hormonal regulation through the HPG axis, testes are also hormonally regulated in response to the individual metabolic status. Gut hormones and adiposity signal hormones, which regulate food intake and energy balance, play a crucial role in this cycle. Among those hormones, the gut hormones ghrelin and glucagon-like protein 1 (GLP-1), and the adiposity signal hormones insulin and leptin, are highlighted as particularly important for the testis. As its receptors are expressed in brain and testicular cells, these hormones can coordinate both the metabolic state of the individual and the energetic demands of the reproductive system. In a healthy individual, the HPG axis, gut and adiposity signal hormones

act together to maintain the male reproductive tract energy homeostasis (Oliveira et al., 2017). In the following sections, how these hormones modulate testicular energy homeostasis will be discussed.

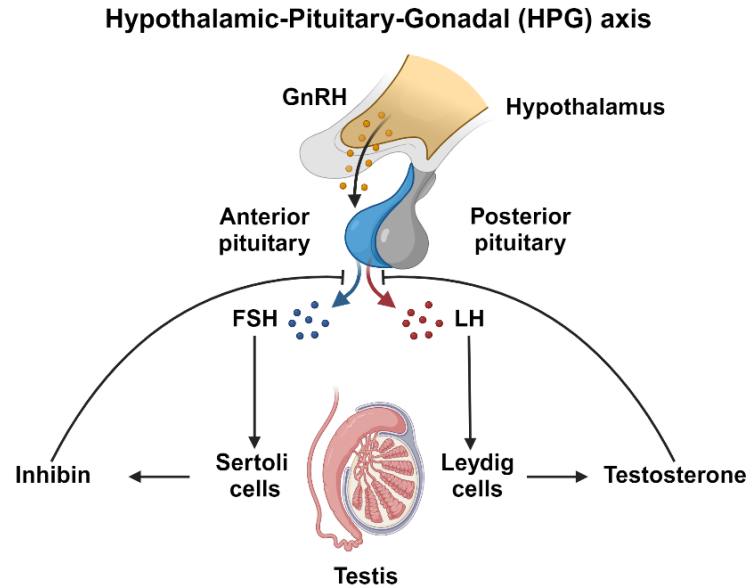


Fig. 1.3 – Schematic illustration of the hypothalamic-pituitary-gonadal (HPG) axis. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which stimulates the anterior pituitary to secrete the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH stimulates the production of testosterone by Leydig cells. FSH regulates the function of Sertoli cells, which produce inhibin in response. Both inhibin and testosterone inhibit the secretion of LH and FSH by the anterior pituitary, in a negative feedback mechanism.

1.2.2.1. Gut hormones

Glucagon-like protein 1 (GLP-1)

Glucagon-like protein 1 (GLP-1) is a peptide hormone encoded by the proglucagon gene and secreted into the bloodstream after tissue specific post-translational processing. GLP-1 has been extensively studied for its weight loss and glucose-lowering effects, as it plays a prominent role in regulating glucose homeostasis, gastrointestinal motility, and appetite. GLP-1 is predominantly released by the small gut in response to the presence of nutrients, particularly sugars, and fats, in the small intestine, or through direct contact between nutrients and L-cells (Shah & Vella, 2014). The intrinsic significance of GLP-1 in orchestrating appetite regulation is widely acknowledged. GLP-1 mediates its function through the GLP-

1 receptor (GLP-1R), which can activate different signaling pathways, including the phosphoinositide 3-kinases (PI3K)/protein kinase B (PKB, also known as Akt), cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathways, depending on its target cell (Buteau et al., 2001; Rowlands et al., 2018; Smith et al., 2019). Within the pancreas, it triggers insulin secretion while curbing glucagon release through the activation of the cAMP/PKA pathway (Lester et al., 1997; Meloni et al., 2013). Simultaneously, within the hypothalamus, GLP-1 fosters feelings of satiety, leading to a decrease in caloric consumption (MacDonald et al., 2002). Given these attributes, GLP-1 analogs were formulated for the management of diabetes and obesity (Hinnen, 2017).

Recent studies suggest that GLP-1 also plays a role in regulating male reproductive system, although its effects are not fully understood nor its mechanisms of action have been identified. In 2019, the GLP-1 receptor was firstly identified in human SCs (Martins et al., 2019). Using primary cultures of human SCs, Martins et al. demonstrated that exposure to GLP-1 decreased glucose consumption and increased lactate production, indicating a shift in its metabolic activity. However, the alternative energetic source that fuels lactate production or the signaling pathways that mediates the action of GLP-1 remains to be identified. Additionally, some evidence suggests that GLP-1 may have an anti-apoptotic effect on developing germ cells (Martins et al., 2019), although its role on spermatogenesis remains to be elucidated. Taken together, these results suggest that GLP-1 is a potential modulator of the testicular metabolism, further linking the nutritional status of the men to the quality of spermatogenesis. Nevertheless, functional studies using GLP-1R inhibitors or testis-specific knockout of GLP-1R are required to further elucidate the effects of this gut hormone in the control of spermatogenesis.

Ghrelin

Ghrelin, known as the hunger hormone, is another gut peptide hormone recognized for its wide physiological functions, including food intake, sleep, body weight, and inflammation (van der Lely et al., 2004). It is known that ghrelin induces the feeling of hunger and, therefore, promotes food intake whereas it also reduces insulin secretion both in rodents and in humans (Wren et al., 2001). Curiously, individuals with obesity are found to have significantly lower ghrelin levels, which supports the hypothesis that low ghrelin levels may have a counter-regulatory action on energy uptake (Tschöp et al., 2001). Ghrelin acts through the growth hormone secretagogue receptor (also known as ghrelin receptor, GHS-R). Two isoforms of the GHS-R have been identified, GHS-R1a and GHS-R1b, although only GHS-R1a seems capable of transducing ghrelin signaling (Yanagi et al., 2018). Ghrelin signaling

is mainly mediated through the phospholipase C (PLC)/inositol triphosphate (IP₃) pathway, which stimulates the intracellular release of Ca²⁺ from the endoplasmic reticulum (Hedegaard & Holst, 2020). However, the GSH-R1a is also able to form heterodimer receptors with other G-protein-coupled receptors (GPCR), leading to the activation of diverse signaling pathways according to its counterpart and cell type. For instance, the GSH-R1a can form a heterodimer with the somatostatin receptor 5 in pancreatic β -cells and prevent insulin release through the suppression of Ca²⁺ influx and the inhibition of cAMP formation (Dezaki et al., 2007; Park et al., 2012).

Although studies concerning ghrelin effects on the male reproductive function are scarce, ghrelin is hypothesized to be capable of modulating the testis either directly, or indirectly through regulation of the HPG axis in the central nervous system. In *in vitro* and *in vivo* studies, ghrelin was reported to inhibit the testicular secretion of testosterone and suppress LH and FSH secretion by the pituitary (Dupont et al., 2010; Lanfranco et al., 2008; Tena-Sempere et al., 2002; Vulliemoz et al., 2008). In the human male reproductive system, Gaytan et al. observed that ghrelin is expressed in both Leydig and Sertoli cells but not in the germ cell line (Gaytan et al., 2004). *In vitro* studies proposed that ghrelin can inhibit the proliferative activity of Leydig cells and immature germ cells, avoiding an excess build-up of germ cells, which is crucial for spermatogonia survival (Barreiro & Tena-Sempere, 2004; Kheradmand et al., 2012). In human SCs, the presence of the GSH-R was confirmed by Martins et al., who also demonstrated that ghrelin was a modulator of human SCs' metabolism (Martins et al., 2016). In this study, it was observed that the concentration of ghrelin reported in healthy men (100 pM) decreased glucose and pyruvate consumption and acetate production as compared to the concentration reported in men with obesity (20 pM). However, lactate production was not modified due to the increased lactate dehydrogenase (LDH) activity upon exposure to ghrelin levels observed in healthy men. These results led the authors to suggest that ghrelin can potentially act as an energy sensor for human SCs in a dose-dependent manner, although additional studies are required to further clarify the activated signaling pathways and the repercussions of these findings.

1.2.2.2. Adiposity signal hormones

In contrast to gut hormones, which are secreted after a meal, adiposity signal hormones are constantly active and translate the energy state of the organism. Leptin and insulin fulfill many of the criteria for being putative adiposity signals. Firstly, plasma leptin and insulin levels are positively correlated with body weight and with adipose mass (Benoit et al., 2004). Additionally, these adiposity signals can interact with several anabolic and catabolic

pathways, changing not only the brain's sensitivity to satiation signals but also altering energy-related pathways in multiple tissues, including the male reproductive system (Woods & D'Alessio, 2008). Since testicular function and spermatogenesis are metabolically regulated, adiposity signal hormones play an important role in its regulation.

Insulin

Insulin is an anabolic hormone that is mainly produced by the pancreatic β -cells. The main functions attributed to insulin comprise the regulation of glucose and lipid metabolism. In brief, insulin promotes the entry of glucose into cells through the translocation of GLUT4 to the plasma membrane, whilst it also promotes glycogenesis and lipogenesis (Kersten, 2001; Qaid et al., 2016). The action of insulin is mediated through the insulin receptor, which changes its conformation upon insulin binding to promote its tyrosine kinase activity and auto-phosphorylation. Then, the phosphorylation of the insulin receptor substrates (IRS) occurs, which activates the PI3K/Akt and MAPK signaling pathways to transduce its effects (Boucher et al., 2014; White & Kahn, 2021).

Insulin plays a crucial role in modulating the male reproductive system, both indirectly through the HPG axis and directly at the testis. In the central nervous system, insulin regulates the hypothalamus-pituitary axis in response to the metabolic and energy status of the organism. It does so by regulating GnRH secretion through direct action on GnRH neurons or indirectly through the regulation of neuropeptide Y neurons, which are able to modulate the function of GnRH neurons (Pralong, 2010). In the presence of low insulin levels or insulin resistance, GnRH, LH, and FSH secretion are impaired, which disrupts the HPG axis and highly impacts on the reproductive function (Boukhliq et al., 1996; Burcelin et al., 2003; Natah et al., 2013). At the testis, insulin is known for its role in testicular development and regulation of sex-determination genes (Nef et al., 2003; Pitetti et al., 2013). Besides, insulin mediates the metabolic control of spermatogenesis both directly, due to its action in germ cells, and indirectly through the regulation of SCs metabolism (Qaid et al., 2016). In SCs, the insulin receptor was first identified by Oonk and Grootegoed, who suggested that insulin had specific functions in these cells maturation and activity (B. Oonk & Grootegoed, 1987). Insulin is reported to regulate SCs viability, differentiation, and metabolism, particularly by influencing the production of lactate and acetate (Alves et al., 2012; Escott et al., 2013; Pitetti et al., 2013). Insulin-deprived SCs present decreased transcript levels of genes associated with lactate metabolism (*LDH* and *MCT4*) and decreased glucose consumption and acetate production, suggesting that insulin resistance can impair the metabolic support of spermatogenesis (Alves et al., 2012; Oliveira et al., 2012). Insulin has also been widely

studied for its capacity of modulating steroidogenesis. Notably, insulin resistance has been linked to a decline in testosterone secretion by Leydig cells, accomplished through the modulation of LH receptors or by affecting the population of robust and functional cells (Pitteloud et al., 2005). In addition, studies in rodent animal models showed that hyperglycemia is linked to hyperplasia of Leydig cells and increased risk of testicular tumors development, whereas insulin treatment decreases the number of hyperplastic cells and increases its viability and testosterone secretion (Leisegang & Henkel, 2018; Nakane et al., 2020). Nevertheless, these observations in animal models are yet to be demonstrated in humans.

Leptin

Leptin is a peptide hormone produced mainly by white adipocytes, whose function is to decrease food intake through the feeling of satiety (Friedman & Halaas, 1998). Although leptin cannot induce satiety by itself, it can interact with other hypothalamic hormone pathways associated with appetite regulation (Friedman & Halaas, 1998; Rogge et al., 2008). The action of this hormone is modulated by the leptin (or obesity) receptor (LepR) (Tartaglia, 1997). From the several isoforms of the LepR already identified, the long isoform (LepRb) is considered the main signaling transducer (Lee et al., 1996). This receptor is widely expressed in the human organism and its effects are dependent on the target tissue and cell type. The canonical signaling pathway attributed to LepR is the janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway (Evans et al., 2021).

Besides its regulatory role on energy intake, leptin also modulates the HPG axis both in the hypothalamus and the testes, which makes this hormone an important mediator between energy reserves, nutritional status, and the male reproductive system (Carrageta et al., 2019). In the hypothalamus, animal studies have demonstrated that leptin administration induces pulsatile GnRH release in a dose-dependent manner (Parent et al., 2000). Furthermore, leptin has been shown to increase LH and FSH secretion by stimulating the growth and differentiation of pituitary cells. This effect occurs independently of the presence or absence of GnRH (Tezuka et al., 2002). However, the effects of leptin for the reproductive system seem to be more pronounced than the regulation of the hypothalamus-pituitary axis. Animal studies demonstrated that leptin is essential for the reproductive system as inactivating mutations of the leptin gene, as observed in ob/ob mice, renders animals infertile (Barash et al., 1996). The causality has been demonstrated since leptin administration rescues the fertility of these animals, while a similar effect is not observed in mice with LepR inactivating mutations (db/db mice) (Hoffmann et al., 2016; Mounzih et al., 1997). In humans, congenital leptin deficiency was also associated with hypogonadism, puberty arrest,

and infertility both in male and female individuals (Strobel et al., 1998). In the same study, it was shown that administration of GnRH to the man with congenital leptin deficiency rescued FSH and LH levels, whereas administration of human chorionic gonadotropin (hCG) rescued testosterone levels, further suggesting the role of leptin in the regulation of the hypothalamus (Strobel et al., 1998). In a follow-up study, it was demonstrated that leptin replacement therapy greatly improved his body composition, metabolism, and endocrine and gonadal function (Licinio et al., 2004). Interestingly, the reproductive effects of leptin observed do not seem to be related with the canonical signaling pathway, since the knock-out of STAT3 and STAT5 in LepRb-expressing cells resulted in obesity but not impaired fertility (Singireddy et al., 2013), but rather through the insulin receptor substrate (IRS)/PI3K/Akt pathway (Garcia-Galiano et al., 2017).

The development of metabolic disorders is associated with leptin resistance, which results in high levels of circulating leptin (Enriori et al., 2007; Frederich et al., 1995). Clinical studies in humans have demonstrated that obesity in men is associated with higher circulating levels of leptin, which in turn are correlated with poorer sperm parameters (Hofny et al., 2010; Leisegang et al., 2014). Interestingly, leptin levels in the seminal fluid are also related to poorer sperm parameters. Guo et al. observed higher leptin concentrations in the seminal fluid of men with asthenozoospermia, although no differences were observed concerning serum leptin in this cohort (Guo et al., 2014). Seminal leptin levels were also negatively correlated with sperm progressive motility and serum total testosterone levels. In a meta-analysis, Mo et al. observed higher leptin levels in both serum and seminal fluid of infertile men. In addition, a negative correlation between both serum and seminal leptin levels and sperm counts and motility was reported (Mo et al., 2022). Animal studies support these findings, showing that leptin administration to normal-weight rodents leads to increased oxidative stress and poorer sperm parameters. These results suggest that high leptin levels are associated with poor male reproductive health and impaired spermatogenesis (Abbasihormozi et al., 2013; Almabhouh et al., 2015; Haron et al., 2010; Wang et al., 2018). In fact, compelling evidence highlights the regulatory role of leptin in the testis, although the molecular mechanisms are not fully disclosed (Soyupek et al., 2009). In human SCs, Martins et al. were the first to demonstrate the presence of the LepR (Martins et al., 2015). In this study, it was found that leptin at a concentration of 5 ng/mL, as usually detected in lean men, increased GLUT2 expression and LDH activity in SCs. However, this effect was not observed for leptin concentrations equivalent to circulating levels usually detected in men with obesity (25 and 50 ng/mL). While glucose consumption and lactate production did not show consistent alterations, exposure to leptin decreased acetate production in human SCs across all tested concentrations (Martins et al., 2015). There is also evidence from animal studies that exposure to high leptin levels disrupts the BTB. Wang et

al. treated seven-week-old male C57BL/6 mice with leptin (0.1, 0.5, or 3 mg/kg) and observed a dose-dependent disruption of the BTB through the downregulation of tight junction-related proteins in SCs. In addition, these authors also reported a dose-dependent decrease in sperm concentration and motility, whereas a higher percentage of morphologically abnormal sperm cells were observed after leptin treatment, suggesting impaired spermatogenesis (Wang et al., 2018). In support to these results, these authors observed that a 48 h treatment with leptin (100 nM, equivalent to 1.6 µg/mL) decreased the expression of tight junction-related proteins in TM4 cells (mouse SCs line). The expression of these proteins was partially rescued with the use of PI3K and MAPK signaling pathways chemical inhibitors, and to a lesser extent with the use of JAK2 chemical inhibitor, suggesting its involvement in the signaling transduction of leptin (Wang et al., 2018). Nevertheless, future studies are required to further elucidate which signaling pathways mediate the effects of leptin in SCs.

1.2.3. Conclusions

The testes are highly intricate organs that are strongly influenced by an individual's hormonal and metabolic status. Notably, testicular cells, especially SCs, exhibit heightened sensitivity to energy-related hormonal signals. This underscores that an individual's dietary choices and metabolic status wield a substantial and direct influence on male reproductive health (Fig. 1.4). As SCs directly translate these metabolic and hormonal cues to spermatogenesis, the characterization of SCs' metabolic function and hormonal regulation has been under intense research. As novel data arises from the effects of gut and adiposity signal hormones effects on testicular cells, it became imperative to disclose the signaling pathways involved. Besides, the future seems to reside on the deeper characterization of metabolic processes of SCs through the use of multi-omics technologies. Multi-omics approaches, including proteomic, lipidomic, and metabolomic analyses, will offer valuable data for evaluating metabolic-induced alterations in reproductive function. The conjugation and interpretation of all these data will, undoubtedly, shed some light on how metabolic diseases, which have increased to epidemic proportions, cause a huge toll in the human reproductive health. Additionally, these -omics can highlight not only biomarkers of metabolic-induced dysregulation but also identify novel therapeutic targets for improving assisted reproductive technology (ART) outcomes. By taking advantage of these emerging platforms, we can better understand the impact of metabolic disorders on male fertility and develop more effective strategies for managing and treating these conditions.

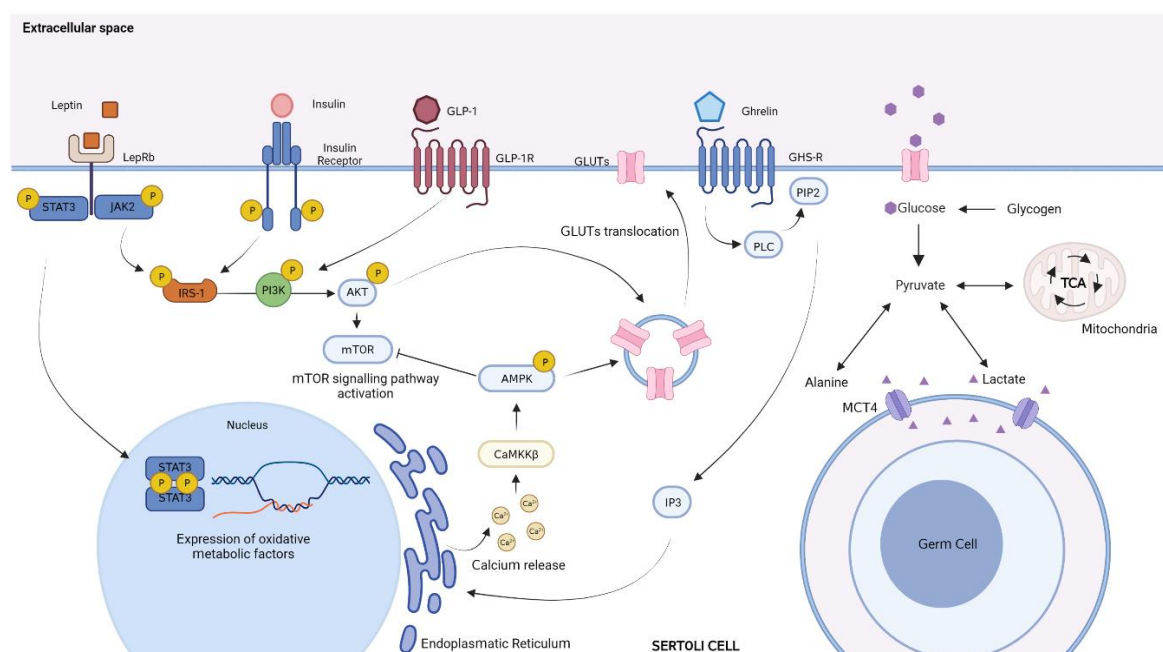


Fig. 1.4 – Putative signaling pathways involved in energetic regulation of human Sertoli cells (SCs) by gut and adiposity signal hormones. SCs are highly glycolytic, favoring the production of high quantities of lactate – the germ cell line preferential source of energy. For that purpose, SCs are able to orchestrate several metabolic and hormonal cues, indicating that the metabolic and hormonal status of the individual are reflected in his reproductive health. Among the energy-related hormones, gut and adiposity signal hormones are known regulators of SCs metabolism, although the signaling pathways are not fully characterized. Among the gut hormones, glucagon-like peptide 1 (GLP-1) and ghrelin are known regulators of SCs function and metabolism. GLP-1 mediates its function through the GLP-1 receptor (GLP-1R), which is hypothesized to activate the phosphoinositide 3-kinases/protein kinase B (PI3K/Akt) pathway. Ghrelin acts through the growth hormone secretagogue receptor (also known as ghrelin receptor, GHS-R). Ghrelin signaling is mediated through the phospholipase C (PLC)/inositol triphosphate (IP₃) pathway, which stimulates the intracellular release of Ca²⁺ from the endoplasmic reticulum and the activation of the Calcium/calmodulin-dependent protein kinase kinase beta (CaMKKβ). The later activates the AMP-activated protein kinase (AMPK). Among the adiposity signal hormones, leptin and insulin are the most widely studied. Leptin acts through the leptin receptor (LepRb), which canonically activates the janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway. However, leptin seems to modulate SCs function mainly through the insulin receptor substrate (IRS)/PI3K/Akt pathway, which is canonically associated with insulin and insulin receptor. All these pathways are hypothesized to promote the translocation of glucose transporters (GLUTs) into the plasma membrane, stimulating the glycolytic rate and controlling the supply of energy substrate for germ cells development. *Abbreviations:* MCT4 – monocarboxylate transporter 4; mTOR – mammalian target of rapamycin; PIP2 - Phosphatidylinositol 4,5-bisphosphate; TCA - tricarboxylic acid cycle.

1.2.4. References

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1.3. Metabolic and hormonal regulation of human spermatozoa

Spermatogenesis culminates with the release of spermatozoa into the lumen of the seminiferous tubules, in a process denominated spermiation. However, during this stage, spermatozoa remain non-motile, depending on peristaltic movements for transportation into the epididymis. Within the epididymis, a subsequent phase of maturation unfolds, enabling the spermatozoa to acquire motility and undergo further development. These matured spermatozoa are then stored, awaiting either ejaculation or degradation (Cornwall, 2009). However, the very last stage of spermatozoa maturation only occurs in the female reproductive tract. In the uterus and oviducts, spermatozoa encounter a specific ionic, energetic, and hormonal environment that stimulates complex biochemical and morphological modifications, which will allow these cells to fertilize the oocyte. The discovery and characterization of this process took place in the early 1950s, when Austin and Chang, two independent researchers, simultaneously described the requirements for spermatozoa to be able to fertilize an oocyte *in vivo* (Austin, 1951; Chang, 1951). This process was denominated as “capacitation” and was pivotal for the development of nowadays *in vitro* fertilization (IVF) techniques.

1.3.1. Capacitation of human spermatozoa

Capacitation comprises the biochemical and morphological alterations in spermatozoa that confer the ability to fertilize the oocyte. During capacitation, spermatozoa achieve hyperactivation (or hypermotility), gaining a new motility pattern characterized by an asymmetric beat and increased flagellar bend amplitude that improves sperm swimming ability and aids in the penetration of the oocyte's *zona pellucida* (Puga Molina et al., 2018a). Although intensely studied in the past decades, the molecular mechanisms that regulate human spermatozoa capacitation are still not completely understood. There are three known major triggers for human spermatozoa capacitation – HCO_3^- influx and cytoplasm alkalinization; Ca^{2+} influx and signaling; albumin and cholesterol efflux from the membrane (Fig. 1.5). It is hypothesized that capacitation is triggered by the high HCO_3^- concentrations, which spermatozoa encounter in the female reproductive tract (Puga Molina et al., 2018a). According to the gradient, HCO_3^- enters spermatozoa through sodium bicarbonate cotransporters (NBC) that upholds cytoplasm alkalinization (Puga Molina et al., 2018b). In addition, cytoplasm alkalinization is further enhanced by proton efflux from the cytoplasm through voltage-gated proton (Hv1) channels (Lishko & Kirichok, 2010). Cytoplasm alkalinization then promotes activation of the sperm cation channel (CatSper), a Ca^{2+} -permeable and pH- and low voltage-dependent channel that is specifically expressed in mammalian spermatozoa (Lishko

et al., 2012; Lishko & Mannowetz, 2018). Both HCO_3^- and Ca^{2+} influx activate the soluble adenylyl cyclase (sAC), increasing cAMP production and inducing PKA activation. In fact, cAMP/PKA signaling pathway is regarded as the predominant pathway responsible for the onset of mammalian capacitation (Puga Molina et al., 2018a). PKA phosphorylates the cystic fibrosis transmembrane conductance regulator (CFTR) and other $\text{Cl}^-/\text{HCO}_3^-$ cotransporters, leading to the continuous HCO_3^- influx that sustains cytoplasm alkalinization (Berger et al., 1993; Puga Molina et al., 2017; Tabcharani et al., 1991). In addition, PKA is responsible for the phosphorylation of several downstream proteins, in a sperm-specific process called protein tyrosine phosphorylation cascade, which activates several signaling pathways that culminate with the accomplishment of hypermotility and membrane restructuring (Chung et al., 2014; Naz & Rajesh, 2004).

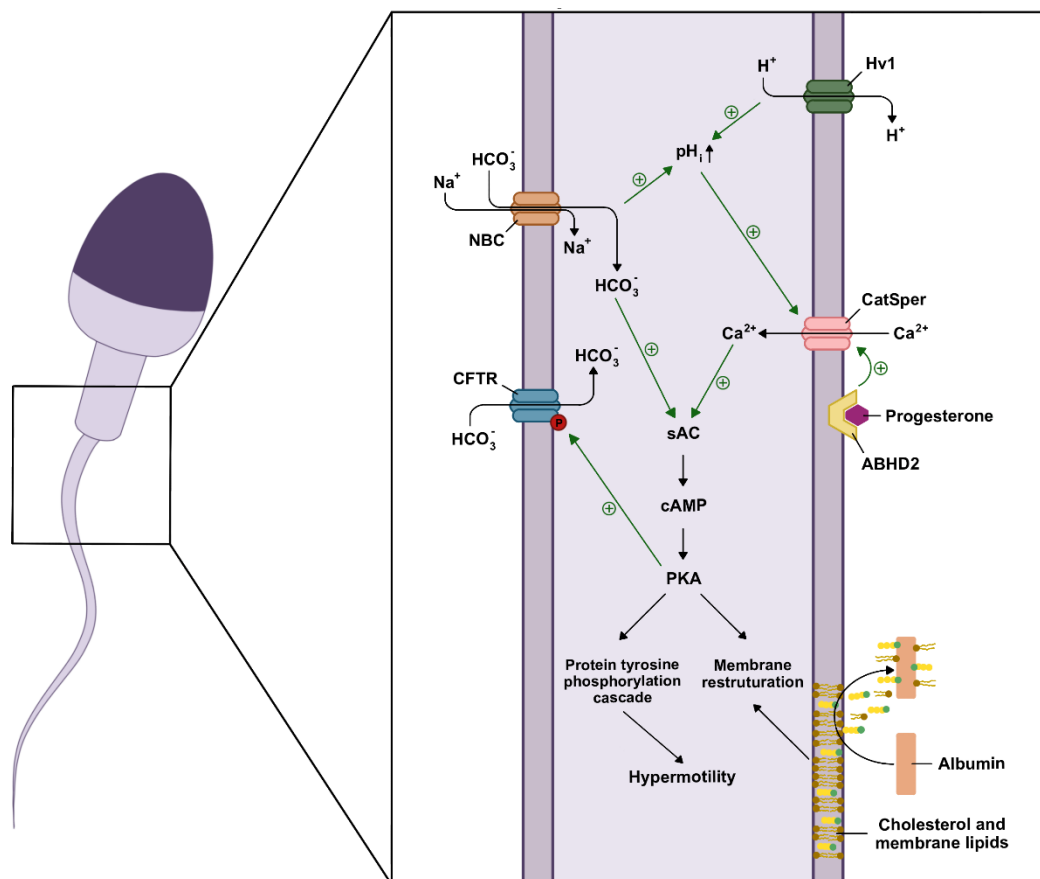


Fig. 1.5 – Schematic illustration of the human spermatozoa capacitation pathway. There are three known major triggers for human spermatozoa capacitation – HCO_3^- influx and cytoplasm alkalinization; Ca^{2+} influx and signaling; albumin and cholesterol efflux from the membrane. Due to the high concentrations found in the female reproductive tract, HCO_3^- enters spermatozoa through the sodium bicarbonate cotransporters (NBC) and according to the gradient. The influx of HCO_3^- is mainly responsible for the increase intracellular pH (pH_i), which is maintained due to the activity of voltage-gated proton (Hv1) channels. The cytoplasm alkalinization activates the sperm cation channel

(CatSper), which is the main transporter of Ca^{2+} into the cytoplasm. Besides, progesterone indirectly activates CatSper through its interaction with Abhydrolase Domain-Containing 2 Acylglycerol Lipase (ABHD2). The influx of both HCO_3^- and Ca^{2+} activate soluble adenylyl cyclase (sAC), increasing cAMP levels and inducing the protein kinase A (PKA) activity. Then, capacitation is mediated through the cAMP/PKA signaling pathway, which induces the protein tyrosine phosphorylation cascade that results in hypermotility activation, and in the plasma membrane restructuring, leading to the efflux of cholesterol and several other lipid species that confer increased membrane fluidity to spermatozoa.

Simultaneously to these intracellular processes, high albumin concentrations in the female reproductive tract allows the efflux of membrane cholesterol and other lipids, which also plays a role on membrane restructuring that culminates in increased membrane fluidity (Gadella, 2008; Puga Molina et al., 2018a). However, the crosstalk between membrane cholesterol efflux and intracellular alterations remains poorly understood. It is hypothesized that cAMP/PKA pathway activation facilitates cholesterol efflux (Flesch et al., 2001; Gadella & Harrison, 2000; Harrison & Miller, 2000), but cholesterol removal was also reported to be essential for HCO_3^- influx and cytoplasm alkalinization (Cross, 1998; Cross & Razy-Faulkner, 1997).

1.3.2. Acrosome reaction

The acrosome is an acidic vesicle located in the apical part of the head that is thought to be originated from the Golgi apparatus. Interestingly, recent data evidence that the acrosome may also incorporate condensed mitochondria (Ren et al., 2022). Upon capacitation, the fusion between the outer acrosome membrane with the plasma membrane occurs, releasing the hydrolytic enzymes stored inside the acrosome (including hyaluronidase and acrosin) through exocytosis – a process denominated as acrosome reaction (Fig. 1.6). Acrosome reaction is a fundamental process for fertilization, conferring spermatozoa the ability to penetrate and fuse with the oocyte, as the hydrolytic enzymes that are released aid in *zona pellucida* penetration (Aldana et al., 2021). Despite being identified decades ago, the molecular stimuli that triggers acrosome reaction in human spermatozoa is not fully understood, mainly due to ethical restrictions. From *in vitro* studies and the use of animal models, it is hypothesized that capacitation is a precondition, as the factors that seem to stimulate acrosome reaction are the Ca^{2+} influx and increased cAMP. The current model proposes that the membrane fusion that occurs during the acrosome reaction is due to the formation of complexes between SNARE proteins located in the plasma and outer acrosome membranes (Mayorga et al., 2007). Upon capacitation, the intracellular Ca^{2+} influx promotes the

entrance and accumulation of this cation inside the cytoplasm and acrosome. Simultaneously, the Ca^{2+} influx and cytoplasm alkalinization activates the adenylyl cyclase and the formation of cAMP, which will promote the acrosome swelling (Zanetti & Mayorga, 2009). Membrane fusion is arrested until the Ca^{2+} is released from the acrosome to the space between the plasma and the outer acrosome membranes, leading to the formation of protein complexes between the SNARE proteins and, finally, the fusion of both membranes and exocytosis of the hydrolytic enzymes (Sosa et al., 2016; Zanetti & Mayorga, 2009).

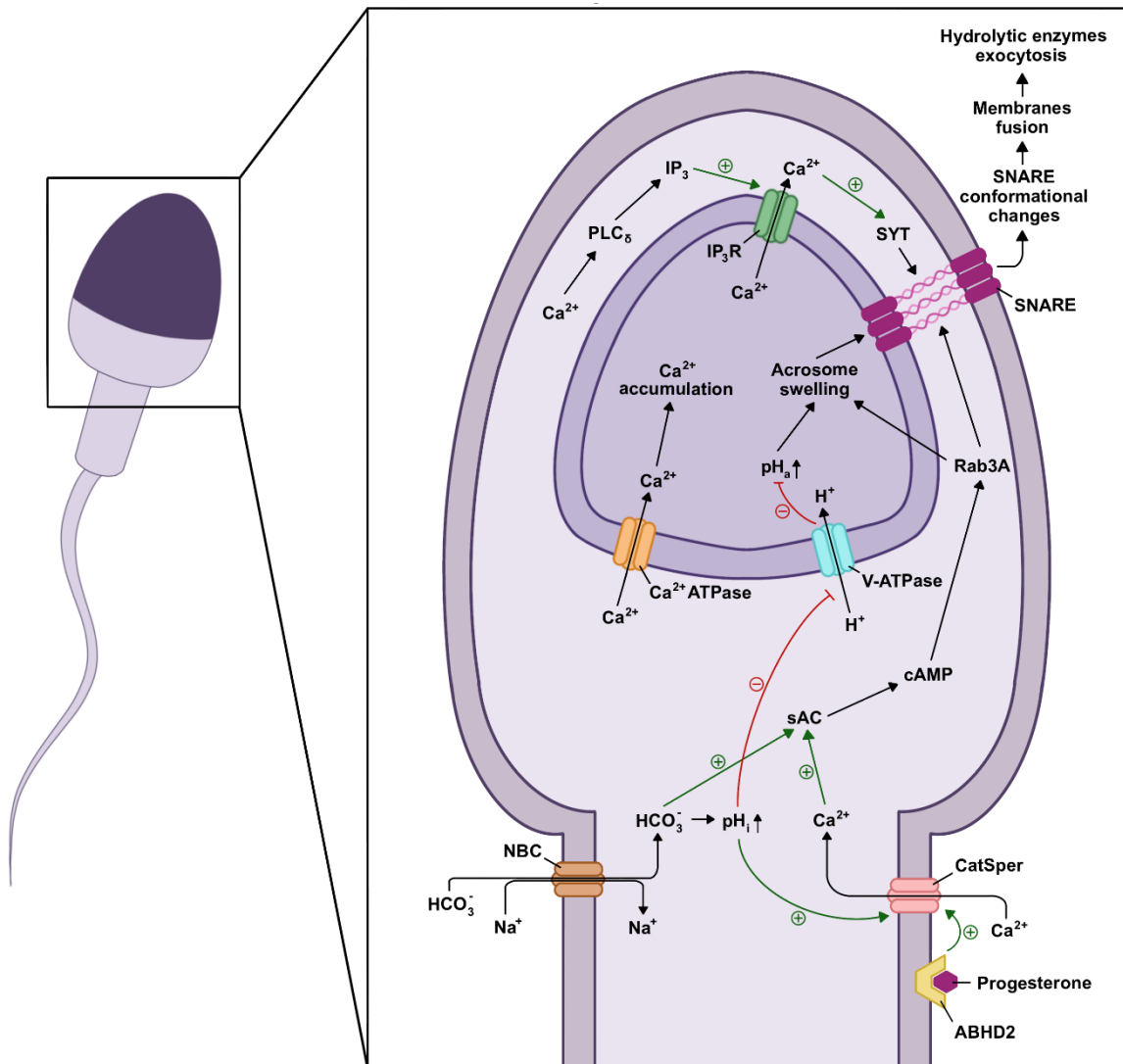


Fig. 1.6 – Schematic illustration of the putative mechanism responsible for the acrosome reaction in human spermatozoa. It is hypothesized that capacitation is a precondition for acrosome reaction. During capacitation, the Ca^{2+} influx and cytoplasm alkalinization activates the soluble adenylyl cyclase (sAC) and the formation of cAMP, which will promote acrosome swelling through Rab3A. In addition, acrosome swelling is stimulated by the increase on acrosome pH (pH_a), due to the inhibition of the proton transporters V-ATPase by the cytoplasmic pH (pH_i) alkalinization. Simultaneously, Ca^{2+} starts to accumulate in the acrosome through the activation of the Ca^{2+} ATPase.

Membrane fusion is then arrested until the accumulated Ca^{2+} is released from the acrosome to the space between the plasma and the outer acrosome membranes. The transport of Ca^{2+} is promoted by the inositol triphosphate receptor (IP_3R), which is activated through the formation of IP_3 by the Ca^{2+} -activated phospholipase C delta ($\text{PLC}\delta$). Finally, Ca^{2+} promotes the interaction between synaptotagmin-1 (SYT) which, together with Rab3A, leads to the changes in the conformation of SNARE protein complexes, causing the fusion of the plasma and outer acrosome membranes and the exocytosis of the acrosome-contained hydrolytic enzymes. *Abbreviations:* ABHD2 – Abhydrolase Domain-Containing 2 Acylglycerol Lipase; CatSper – sperm cation channel; NBC – sodium bicarbonate cotransporters.

The spatial location within the female reproductive tract where acrosome reaction occurs also remains to be disclosed. There is evidence that the human *zona pellucida* matrix contains glycoproteins (ZP1-4) that mediate the sperm-oocyte binding and stimulate acrosome reaction (Gupta, 2021). However, the fact that it is possible to stimulate this process *in vitro* without stimulating or simulating the binding suggests that other factors must be considered. In support, it is known that a fraction of spermatozoa is capable of undergoing acrosome reaction spontaneously, whereas others require progesterone or are not able to undergo acrosome reaction at all (Harper et al., 2006; Nakanishi et al., 2001). These observations suggest not only that acrosome reaction is a time-dependent process, but also that several subpopulations of spermatozoa simultaneously co-exist at the same time. However, its molecular characterization remains to be clarified.

The capacitation and acrosome reaction stand as the ultimate biochemical processes orchestrating the essential molecular and morphological modifications necessary for spermatozoa to effectively engage and fuse with the oocyte. Beyond the intricate ionic regulation previously mentioned, the processes of capacitation and acrosome reaction in human spermatozoa are also under the governance of hormonal and metabolic mechanisms. Consequently, the accomplishment of successful fertilization pivots significantly on the hormonal and metabolic cues encountered by spermatozoa within the female reproductive tract. The subsequent subsections will delve into the comprehensive exploration of the hormone and metabolic regulation governing human spermatozoa's capacitation and acrosome reaction.

1.3.3. Hormonal regulation of human spermatozoa

Human spermatozoa are sensitive to the concentration of several hormones present, not only in the seminal fluid, but also in the uterine and oviduct fluids, which regulate capacitation and acrosome reaction. These fluids include mainly steroid hormones (estradiol, progesterone, and testosterone), but also energy-related hormones such as adipose signal

(insulin and leptin), and gut hormones (GLP-1 and ghrelin). The regulation conferred by these hormones will be detailed in the following subsections.

1.3.3.1. Steroid hormones

Human spermatozoa are exposed to different steroid hormones throughout its journey from the seminal to the uterine and oviduct fluids. However, data on the role of steroid hormones on post-ejaculation spermatozoa is very limited. It is known that human spermatozoa express androgen and estrogen receptors (Guido et al., 2011; Zuccarello et al., 2009). Among steroid hormones, testosterone is the most abundant hormone found in the seminal fluid. Four decades ago, Ridley & Blasco found a negative association between testosterone concentration in seminal fluid and sperm quality, with infertile men having higher testosterone levels (750 ± 110 pg/mL) than fertile men (290 ± 30 pg/mL) (Ridley & Blasco, 1981). In contrast, higher testosterone levels in fertile men (690-710 pg/mL) were observed in later studies, refuting the earlier concept that higher testosterone in the seminal fluid could indicate poor reproductive parameters (Adamopoulos et al., 1976; Moreno-Escallon et al., 1982). The observation that high testosterone concentrations (1.5 ng/mL, which is even higher than those found in the seminal fluid) significantly reduced human spermatozoa motility *in vitro* provided the basis for the association between high testosterone levels in the seminal fluid and infertility (Ridley & Blasco, 1981). However, Aquila et al. reported that substantially higher concentrations (1 nmol/mL, which is equivalent to 288 ng/mL) improved human spermatozoa migration and capacitation (Aquila et al., 2003), challenging those results. Surprisingly, and to the best of our knowledge, no other studies focused on the effects of testosterone on the physiology or capacitation of human spermatozoa were performed. The application of substantially higher concentrations than those observed in physiological conditions warrants additional investigation for the physiological effects of testosterone on human spermatozoa.

In comparison to testosterone, 17β -estradiol levels were found to be much lower (30-70 pg/mL) in the seminal fluid of normozoospermic men (Santemma et al., 1991; Zhang et al., 2010). Data on 17β -estradiol's effects on spermatozoa are also scarce and studies have the same limitations regarding the use of substantially higher concentrations than those found in physiological fluids, lack of standardization on type of media, and incubation time. Aquila et al. demonstrated that 17β -estradiol (1 nmol/mL, which is equivalent to 272 ng/mL) improved migration and capacitation of human spermatozoa (Aquila et al., 2003). Using mouse spermatozoa, Adeoya-Osiguwa et al. found that 17β -estradiol (1 to 100 nmol/mL,

which is equivalent to 0.272 to 27.2 $\mu\text{g/mL}$) immediately improved capacitation and acrosome reaction but had no further effects after 30 min (Adeoya-Osiguwa et al., 2003). More recently, Kotwicka et al. demonstrated that 17β -estradiol (100 pmol/mL, which is equivalent to 27.2 ng/mL) rapidly improved mitochondrial membrane potential in human spermatozoa, but a higher concentration (1 nmol/mL, which is equivalent to 272 ng/mL) had the opposite effect. Additionally, 17β -estradiol increased mitochondrial superoxide production in a dose-dependent manner (Kotwicka et al., 2016). Overall, the data suggests that 17β -estradiol may play a role in the initial moments of capacitation, but further studies are needed to confirm this proposition.

Progesterone is arguably the most studied of the steroid hormones in the context of its effects on human spermatozoa. After ovulation, progesterone is abundant in the human female reproductive tract as it is produced by the *corpus luteum* and cumulus cells in the ovary and oviducts, respectively (Guidobaldi et al., 2008; Mirihagalle et al., 2022). During the luteal phase, progesterone levels in the oviduct of gilts were shown to be higher than in the serum (Hunter et al., 1983; Stefanczyk-Krzymowska et al., 1994). Progesterone levels were also found to be higher in the oviducts of bovines during the post-ovulatory and luteal phase (Lamy et al., 2016). Although the concentration of progesterone in the human oviduct after ovulation is unknown, it is expected that higher concentrations are also found near the oocyte and that a progesterone gradient is formed post-ovulation throughout the female reproductive tract (Cicinelli et al., 2009). Several *in vitro* studies highlight that progesterone stimulates capacitation, motility, and acrosome reaction in human spermatozoa (Bedu-Addo et al., 2007; Sagare-Patil et al., 2012; Sumigama et al., 2015). Interestingly, the mechanism of action of progesterone seems to be independent of the canonical progesterone receptor. In 2011, two independent groups reported simultaneously that progesterone induces Ca^{2+} influx via CatSper (Lishko & Mannowetz, 2018; Strunker et al., 2011). Both groups reported that either CatSper itself or an associated protein would function as a non-genomic progesterone receptor, but could not identify such protein. In support to these results, Smith et al. found that the spermatozoa of CatSper-deficient patients, which are infertile, are insensitive to progesterone and therefore a Ca^{2+} influx does not occur (Smith et al., 2013). This mysterious protein was identified just five years later, when Miller et al. demonstrated that progesterone binds to the Abhydrolase Domain-Containing 2 Acylglycerol Lipase (ABHD2), a serine hydrolase that depletes the membrane of 2-arachidonylglycerol, a known inhibitor of CatSper. Therefore, progesterone indirectly activates CatSper by removing its inhibitors, inducing a Ca^{2+} influx that triggers capacitation (Miller et al., 2016). This discovery led some groups to target ABHD2 for the potential development of male contraceptives, a task that proves to be challenging. Firstly, there is a lack of validated antibodies and pharmacological inhibitors. So far, only one specific inhibitor was identified

by Baggelaar et al. and the results have not been promising (Baggelaar et al., 2019). Although this undisclosed compound completely inhibited progesterone-induced acrosome reaction in mouse spermatozoa, it failed to prevent spontaneous acrosome reaction (about 20% of total spermatozoa), limiting the potential of targeting ABHD2 for male contraception (Baggelaar et al., 2019). Even though the discovery of the progesterone-induced Ca^{2+} influx mechanism was a major breakthrough in the field, these findings emphasize the importance for identifying the different mechanisms responsible for acrosome reaction.

1.3.3.2. Gut and adiposity signal hormones

Besides steroid hormones, which are classically associated with the reproductive function, energy-related hormones, such as gut and adiposity signal hormones, are hypothesized to play a role on the regulation of human spermatozoa capacitation and acrosome reaction. Among those, the adiposity signal hormone leptin is probably the most well studied. In 2003, Jope et al. identified the expression of leptin and its receptor in human spermatozoa (Jope et al., 2003). Interestingly, these authors identified the LepR both in the tail of human spermatozoa and a soluble isoform in the seminal fluid. Later, Aquila et al. observed that leptin is mainly expressed in the equatorial region of the head and midpiece in non-capacitated human spermatozoa (Aquila et al., 2005b). These authors also reported that leptin is released during capacitation, being responsible for both cholesterol efflux and protein tyrosine phosphorylation in an autocrine/paracrine fashion. Due to the pattern of expression of the leptin receptor, it was hypothesized that leptin can regulate human spermatozoa motility, however, conflicting results are reported in the literature. Lampiao and Plessis reported that leptin (20 nM, or 30.6 ng/mL) improved human spermatozoa total and progressive motility, and stimulated the acrosome reaction (Lampiao & du Plessis, 2008). Contrastingly to these findings, Li et al. observed no effects on spermatozoa motility, capacitation, or acrosome reaction upon incubation with leptin (10-1000 ng/mL) (Li et al., 2009). Interestingly, these authors found that highly motile spermatozoa expressed higher levels of leptin than the low motility fraction. In addition, no correlation was observed between leptin or leptin receptor seminal fluid levels and sperm parameters (Li et al., 2009).

Insulin, another adiposity signal hormone, is also reported to promote human spermatozoa capacitation, motility, and acrosome reaction. Lampiao and Plessis observed that the addition of insulin (0.35 ng/mL) to the media improved human spermatozoa total and progressive motility and acrosome reaction (Lampiao & du Plessis, 2008). Conversely, Wang et al. reported that higher concentrations of insulin (10 and 100 $\mu\text{g/mL}$) did not produce any effect on human spermatozoa motility (Wang et al., 2015). Interestingly, although the data

is scarce and conflicting, there is evidence that the human germ cell line is able to produce insulin, notwithstanding its function being poorly understood (Aitken et al., 2021; Aquila et al., 2005a).

Although less studied, the gut hormones may also play a role on human spermatozoa capacitation and acrosome reaction. For instance, GLP-1 was highlighted as a potential regulator of human spermatozoa capacitation. In 2020, Rago et al. identified the GLP-1R in human spermatozoa (Rago et al., 2020). These authors treated human spermatozoa with a GLP-1 analog, exendin-4 (Ex-4), and found that progressive motility and cholesterol efflux increased 1 h after incubation with 300 pM of Ex-4. The beneficial effects of Ex-4 were reversed by incubation with Ex-4 plus H89, a PKA inhibitor, suggesting that Ex-4 acts via the GLP-1 receptor to regulate capacitation through a PKA-dependent signaling pathway (Rago et al., 2020). In addition, Ex-4 increased LDH, glucose-6-phosphate dehydrogenase (G6PDH), lipase, and acyl-CoA dehydrogenase activities, while sperm triglyceride content was decreased, suggesting that this hormone may have a lipolytic effect in human spermatozoa (Rago et al., 2020). These findings suggest that spermatozoa are a potential GLP-1 target, unveiling the perspective of an additional pharmacological use for this hormone analogs on *in vitro* capacitation of spermatozoa. Ghrelin, another gut hormone that plays an important role in the metabolic regulation of SCs, may also promote capacitation. Łukaszyk et al. reported that GHSR-1a is expressed in the acrosome region of rat spermatids and epididymal spermatozoa (Łukaszyk et al., 2012). In the same study, these authors demonstrated that 1 μ M of ghrelin increased intracellular Ca^{2+} concentration and improved progressive motility, suggesting a potential role on sperm capacitation and acrosome reaction (Łukaszyk et al., 2012). However, to the best of our knowledge, studies concerning ghrelin's role on human spermatozoa capacitation were never conducted.

1.3.4. Metabolic regulation of human spermatozoa

During the capacitation, human spermatozoa achieve the biochemical alterations that confer fertilization capacity. Among these processes, protein tyrosine phosphorylation cascade is necessary for the activation of several signaling pathways that culminate with the accomplishment of hypermotility and membrane restructuring (Chung et al., 2014; Naz & Rajesh, 2004). The maintenance of both phosphorylation cascade and hypermotility have high energy demands. Therefore, high rates of ATP production are essential for the maintenance of these processes (Vivenes et al., 2009). The mobility of spermatozoa hinges on the functionality of dynein ATPases situated within the flagellum. These ATPases represent a category of motor proteins with the remarkable ability to transform the chemical energy stored

in ATP into kinetic energy. When dyneins are phosphorylated, these proteins apply mechanical force into flagellar microtubules, promoting its sliding and flagellar beating responsible for spermatozoa motility (Pereira et al., 2017). Therefore, spermatozoa prioritize metabolic pathways that produce energy in the form of ATP.

Glucose is traditionally regarded as essential for spermatozoa to undergo capacitation. Unsurprisingly, glycolysis and oxidative phosphorylation are also highlighted to be the two main energetic pathways for ATP production in human spermatozoa (Goodson et al., 2012). Due to its morphology, the spermatozoa metabolism is compartmentalized – oxidative phosphorylation is restricted to the midpiece, where the mitochondria are located; and glycolysis mainly occurs in the fibrous sheath of the flagellum, although there are smaller quantities of glycolytic enzymes in the sperm head (du Plessis et al., 2015b; Ferramosca & Zara, 2014). Spermatozoa are known to have the greatest glycolytic activity and the least Krebs cycle activity of all germ cells (Bajpai et al., 1998). In fact, some authors proposed glycolysis to be the exclusive ATP source of human spermatozoa (Nascimento et al., 2008; Williams & Ford, 2001), while others defend that oxidative phosphorylation in the mitochondria is also essential for supporting human sperm capacitation and hyperactivated motility (Ferramosca et al., 2012; Gallon et al., 2006; Marchetti et al., 2002). Oxidative phosphorylation is regarded to be the most efficient source of energy due to large yield of ATP molecules per metabolized molecule of glucose (du Plessis et al., 2015b). In support, an increase in mitochondrial respiration occurs during capacitation, which is positively associated with hyperactivated motility (Hicks et al., 1972; Stendardi et al., 2011).

Other studies have focused on alternative energetic sources that are able to sustain human spermatozoa motility. For instance, human spermatozoa are also able to metabolize pyruvate and lactate, which are present in high concentrations at the oviductal fluid (Ferramosca & Zara, 2014). Evidence demonstrates that spermatozoa also have the capacity for metabolizing fatty acids, amino acids, glycerol, and even glycogen, suggesting a great metabolic plasticity to meet its metabolic requirements (Ballester et al., 2000; Jones & Bubb, 2000; Mohri & Masaki, 1967; White, 1957). More recently, metabolomics and proteomics studies have grown in the field, highlighting the potential importance of several metabolic pathways for human spermatozoa motility and capacitation (Amaral et al., 2013; Amaral et al., 2014; Engel et al., 2019; Paiva et al., 2015). However, further studies are required to characterize these hypotheses.

1.3.5. Mitochondria and reactive oxygen species production and signaling

The mitochondria of spermatozoa exhibit unique features. Its location is very specific, as mitochondria are compartmentalized in the midpiece of mammalian spermatozoa. In addition, it is reported that the mitochondria of human spermatozoa suffer morphological changes during capacitation. Using X-ray microscopy, Vorup-Jensen et al. demonstrated that the mitochondria were initially compact and tightly wrapped around the axoneme and would then distend upon capacitation (Vorup-Jensen et al., 1999). The main function attributed to mitochondria is ATP production through oxidative phosphorylation. However, reactive oxygen species (ROS) is another mitochondrial product that is required for a successful capacitation. Oxidative phosphorylation is not perfectly coupled to ATP synthesis and some of the energy present in the electrochemical force is dissipated due to the re-entry of protons in the mitochondrial matrix independently of ATP synthase. Indeed, some electrons escape to oxygen (0.2–2%), essentially in complexes I and III, leading to the formation of ROS (Ferramosca & Zara, 2014; Ramsden et al., 2012; Zhao et al., 2019). In fact, spermatozoa are described as professional generators of ROS (Aitken & Drevet, 2020), since these molecules play a fundamental role in the induction of capacitation. The main pro-capacitation effects of ROS are thought to involve the stimulatory activity on adenylyl cyclase and the simultaneous inhibition of tyrosine phosphatases, enhancing the tyrosine phosphorylation cascade (Aitken et al., 1995; Du Plessis et al., 2015a; Rivlin et al., 2004). In addition, ROS increase cholesterol efflux from the membrane, promoting membrane fluidity, acrosome reaction, and ability of spermatozoa to bind to the *zona pellucida* (Aitken, 2000; Aitken et al., 1995).

Conversely, high levels of ROS are also responsible for oxidative damage. ROS production and oxidative stress are a hot topic in the study of human fertility that has been discussed for decades. The link between oxidative stress and male factor infertility is well documented since it was first described by MacLeod in 1943, where a connection between H_2O_2 production and loss of spermatozoa mobility was observed (MacLeod, 1943). In fact, clinical studies have reported that 30% to 80% of infertile men exhibit supraphysiological levels of seminal ROS (Agarwal et al., 2006; Iwasaki & Gagnon, 1992; Ochsendorf et al., 1994; Shekarriz et al., 1995; Tremellen, 2008; Zini et al., 1993). Even though infertile men present lower seminal plasma antioxidant capacity (Ko et al., 2014; Martin-Hidalgo et al., 2019), oxidative damage in spermatozoa is mostly associated with high levels of ROS as opposed to impaired superoxide scavenging activity (Zini et al., 1993). The aetiology of oxidative stress is multifactorial and can be attributed to conditions such as chronic inflammation, metabolic disorders, ageing, and exposure to drugs and pollutants (Forrester et al., 2018; Martin-Hidalgo et al., 2019; Montano et al., 2022; Moreira et al., 2022). Oxidative

stress on the testes has been associated with several harmful and irreversible damage, leading to lower sperm counts and higher number of spermatozoa morphologically defective and immotile (Aitken et al., 2010; Athayde et al., 2007). Besides impaired spermatogenesis, oxidative stress is also associated with DNA fragmentation and genomic instability, which are positively associated with poorer fertilization ability, abnormal embryo development, and higher miscarriage rates (Cissen et al., 2016; McQueen et al., 2019; Tarozzi et al., 2007). Loss of motility is also one of the major consequences of oxidative stress in spermatozoa (Athayde et al., 2007), arising from a combination of lower membrane fluidity, due to dysregulated lipid peroxidation (Hosseinzadeh Colagar et al., 2013), and defective axonemal phosphorylation (de Lamirande & Gagnon, 1992a, 1992b; Kurkowska et al., 2020). Taken together, ROS can be considered a double-edged sword for spermatozoa. Moderate levels of ROS are regarded as essential to achieve a successful fertilization, whereas higher levels of ROS are responsible for oxidative damage. The threshold or the mechanisms that regulate its levels, however, are still a hot topic in the field.

1.3.6. Conclusions

Human spermatozoa acquire fertilizing ability in the female reproductive tract, where it faces specific hormonal, energetic, and ionic conditions that stimulates its final biochemical and morphological modifications – capacitation and acrosome reaction. Although the ionic requirements for capacitation are comparatively well-established, the metabolic and hormonal regulation of these processes require further investigation. Throughout its journey – from the seminal fluid to the immediacy of the oocyte – spermatozoa encounter different metabolic and hormonal conditions, triggering alterations that confer adaptability and fertilizing ability. In fact, it is reported that its metabolism changes upon capacitation (Balbach et al., 2020; Hidalgo et al., 2020; Tourmente et al., 2022), revealing that time and location are important factors for the study of spermatozoa physiology. Despite advancements, our understanding of the effects of the majority of hormones in the female reproductive tract remains limited. Similarly, the intricate metabolic pathways governing the capacitation and acrosome reaction of human spermatozoa, as well as its adaptations to various energetic substrates, warrant additional exploration. Moreover, the role of mitochondria remains unclear, and ongoing debates surround the threshold or mechanisms regulating ROS levels. One potential regulatory mechanism, although barely explored in the context of male fertility, are the mitochondrial uncoupling proteins (UCPs). However, little is known concerning its expression and function in the male reproductive system. In the following subsection, the potential role of UCPs on male (in)fertility will be detailed.

1.3.7. References

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1.4. The potential role of mitochondrial uncoupling proteins in male (in)fertility

UCPs are integral transmembrane proteins of the inner mitochondrial membrane and members of the mitochondrial anionic transporter family SLC25 (Chan et al., 2006; Hinz et al., 1999; Pecqueur et al., 2001; Ramsden et al., 2012). UCPs are constituted by three repetition domains, each composed of two α -helix regions (Arsenijevic et al., 2000; Hinz et al., 1999; Mao et al., 1999; Ramsden et al., 2012; Sanchis et al., 1998). The carboxyl and amino-terminal regions are on the intermembrane space, while the α -helix regions are linked by long loops that reside on the matrix side of mitochondria (Eckerskorn & Klingenberg, 1987; Miroux et al., 1993; Ramsden et al., 2012).

Currently, six homologs of mammalian UCPs (UCP1-6) have been identified (Fig. 1.7). Among those, only UCP1 is, in fact, thought to have a genuine uncoupling activity, although the molecular mechanisms for the transport of protons are not completely understood. There are two main hypotheses: the flip-flop model and the cofactor model (Hoang et al., 2012; Ricquier & Bouillaud, 2000). The flip-flop model postulates that in the presence of high concentrations of fatty acids, in which the protonated form of the fatty acid freely crosses the membrane towards the matrix and dissociates the proton due to a higher pH in the matrix. The anionic fatty acid returns to the intermembrane space with the aid of UCP1, thus completing the cycle. The cofactor model postulates that protons can be transported independently or, at a higher rate, linked to the carboxyl group of fatty acids, which operate as prosthetic groups. Due to the transport of protons from the intermembrane space into the matrix, the proton gradient is dissipated. Mitochondrial respiration is then stimulated, through the increased activity of the mitochondrial complexes I and III, which generates heat in a process denominated as thermogenesis (Cannon & Nedergaard, 2004; Carrageta et al., 2020). The remaining homologs, however, do not seem to share the same uncoupling properties. More recently, the hypothesis that UCPs, except for UCP1, could act as small molecules transporters instead of pure respiratory uncouplers has gained further support in the field (Hirschenson et al., 2022). One of the main contributors to this hypothesis was the work conducted by Voza et al., who reported that UCP2 can exchange C4 metabolites, including malate, oxaloacetate, and aspartate, for phosphate and a proton, from the mitochondria to the cytosol (Voza et al., 2014). Considering these findings, it was proposed that UCP2 controls the oxidation of Krebs cycle intermediates, the redox pressure of oxidative phosphorylation, and consequently, ROS production. The UCPs activity on ROS regulation and redox homeostasis will be discussed on the following topics.

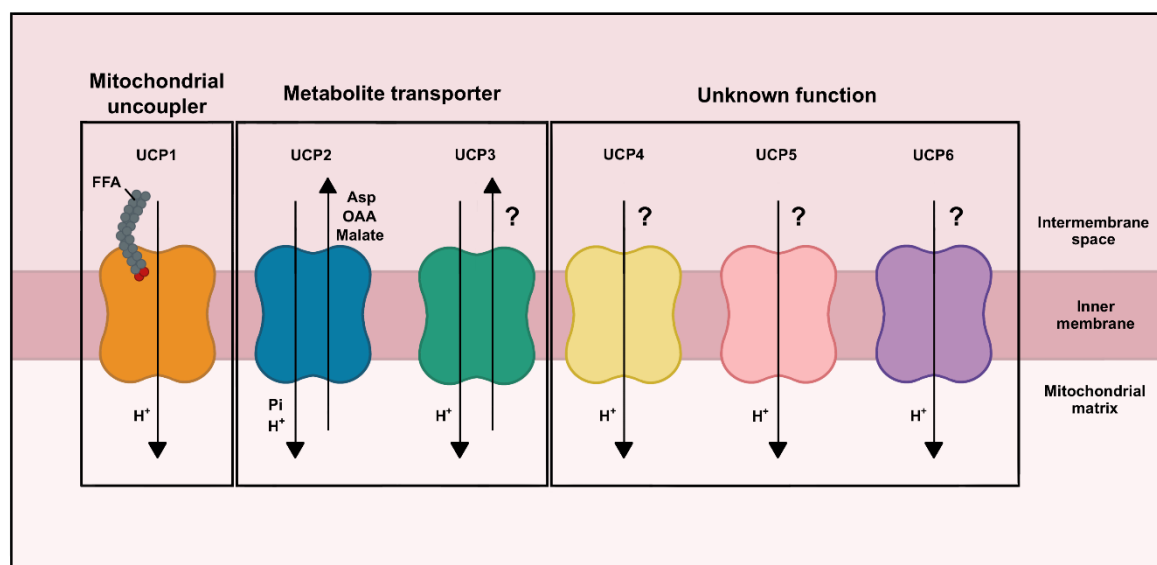


Fig. 1.7 – Schematic illustration of the mammalian mitochondrial uncoupling proteins (UCPs) and putative function. Six homologs of UCPs (UCP1-6) have been identified. Among the homologs, only UCP1 is known to have a true uncoupling activity, which is hypothesized to use free fatty acids (FAA) as cofactor. However, the other homologs do not seem to exhibit an uncoupling activity, but rather act as transporters. For instance, UCP2 is reported to exchange aspartate (Asp), oxaloacetate (OAA), or malate for a phosphate (Pi) and a proton (H^+). Based on its homology, it is predicted that UCP3 would also have a role as a transporter, even though the molecular substrates remain to be identified. Also based on its homology, it is predicted that UCP4, UCP5, and UCP6 also are capable of transporting protons but data on these proteins is scarce and its function remains unknown.

1.4.1. Mitochondrial uncoupling proteins are key regulators of reactive oxygen species production and redox homeostasis

ROS are a natural byproduct of mitochondria's oxidative phosphorylation, however, the unregulated production of free oxygen radicals renders the cellular antioxidant system incapable of mitigating its harmful effects, causing a shift in the homeostasis of the redox balance towards oxidative stress (Basria S.M.N. Mydin & I. Okekpa, 2019; Papa & Skulachev, 1997). All UCPs homologs are well known for regulating ROS homeostasis, although UCP1 antioxidant role remains debatable. Given the extensively described uncoupling activity of UCP1 and thermogenic role, this protein would be expected to stimulate the production of ROS (Yau & Yen, 2020). However, there are some conflicting data that highlights that the association between UCP1, thermogenesis, and ROS production may be even more complex. For instance, Dlasková et al. observed that brown adipose tissue (BAT) mitochondria from both wild-type C57BL/6J and *Ucp1* knockout mice treated with guanosine diphosphate

(GDP), an UCP1 inhibitor, exhibited greater H₂O₂ production and higher mitochondrial membrane potential (Dlaskova et al., 2010). In accordance with these results, ablation of *Ucp1* in BAT mitochondria of mice exposed to cold stress is associated with higher superoxide production, and subsequently oxidative stress, when compared to wild-type mice (Oelkrug et al., 2010; Stier et al., 2014). Besides, ROS were shown to be capable of modulating UCP1 activity via sulfonylation of the Cys253 residue, increasing thermogenesis (Chouchani et al., 2016). Conversely, Shabalina et al. reported that UCP1-dependent proton leak is not affected by the oxidative stress byproduct 4-hydroxy-2-nonenal (4-HNE), and *Ucp1* deletion in mice does not predisposes to oxidative damage (Shabalina et al., 2006). In a subsequent study, also using BAT mitochondria from wild-type and *Ucp1* knockout mice, the same authors demonstrated that UCP1 activity had no impact on ROS reduction, except when succinate was exogenously added. Interestingly, the authors also inferred that low membrane potential may not necessarily correlate with lower oxidative stress, challenging the claim that mild uncoupling protects mitochondria against oxidative damage (Shabalina et al., 2014).

UCP2 and UCP3 role as modulators of ROS production is extensively described, although the molecular mechanisms remain poorly understood. UCP2 and UCP3 do not display a clear uncoupling activity, so it is unlikely that these UCPs regulate superoxide production in a mitochondrial uncoupling-dependent manner. The current consensus in the field arises from the work of Voza et al., who demonstrated that UCP2 is able to transport C4 metabolites in exchange for phosphate and protons, regulating Krebs cycle, oxidative phosphorylation, and also the glutathione system (Voza et al., 2014). The redox balance mediated by UCP2 was first demonstrated by Nègre-Salvayre et al., who have shown that mitochondria fractions containing high levels of UCP2 treated with the inhibitor GDP significantly increased H₂O₂ generation, whereas in mitochondria with low expression of UCP2 the same treatment led to no changes in the oxidative state (Negre-Salvayre et al., 1997). Other studies focusing on the overexpression of UCP2, using an adenoviral vector demonstrated that UCP2 plays a protective role against oxidative stress, shielding both cardiomyocytes and endothelial cells from ROS-induced cell death (Lee et al., 2005; Teshima et al., 2003). UCP2 neuroprotective role against oxidative stress has also been reported (Chan et al., 2009; Jun et al., 2015; Liu et al., 2009). Compelling evidence highlights that ablation of *Ucp2* in multiple mice strains induces chronic high levels of ROS in pancreatic cells (Lee et al., 2009; Pi et al., 2009; Robson-Doucette et al., 2011). Elevated ROS levels in *Ucp2*-deficient mice also results in higher levels of oxidative stress markers in the liver and delayed hepatic regeneration (Horimoto et al., 2004), as well as aggravated atherosclerosis injury (Blanc et al., 2003). On another note, Arsenijevic et al. observed that *Ucp2* knockout

mice infected with *Toxoplasma gondii* presented a stronger fighting capacity against infection, when compared to wild type mice, due to higher ROS production by macrophages (Arsenijevic et al., 2000). Due to its ROS suppressing activity, targeted UCP2 inhibition has been proposed as a potential anti-cancer treatment (Baffy, 2010; Derdak et al., 2008; Mailloux et al., 2010).

Akin to UCP2, it was hypothesized that the overproduction of ROS activates UCP3 through a negative feedback mechanism, diminishing oxidative damage (Anderson et al., 2007). The presence of superoxide from both endogenous (Anderson et al., 2007; Talbot & Brand, 2005; Talbot et al., 2004) and exogenous sources (Echtay et al., 2002a; Echtay et al., 2002b), along with byproducts of lipid peroxidation (Echtay et al., 2005; Murphy et al., 2003) have been shown to induce UCP3 activity, which was firstly hypothesized to be due to its mild uncoupling activity. Considering the results obtained by Vozza et al., the potential role of UCP3 as a metabolite channel instead of an uncoupler requires further investigation. Studies with *Ucp3* knockout mice demonstrate higher rates of ROS production, leading the mice to be more vulnerable to oxidative damage (Brand et al., 2002; Ozcan et al., 2013; Toime & Brand, 2010; Vidal-Puig et al., 2000). Toime and Brand studied isolated energized skeletal muscle mitochondria from wild-type and *Ucp3* knockout mice and reported that UCP3 decreases the rate of ROS production (Toime & Brand, 2010). Interestingly, these authors treated isolated mitochondria with FCCP, a well-known chemical uncoupler, which mirrored the UCP3 antioxidant activity. However, inhibition with GDP was ineffective at reducing UCP3-dependent ROS suppression and no differences in the mitochondrial membrane potential were observed, which suggests that UCP3 can modulate ROS production through a membrane potential-independent mechanism. In support to these results, MacLellan et al. demonstrated that moderate overexpression of UCP3 in L6 myocytes does not interfere with basal cellular oxygen consumption or membrane potential despite potentiating its antioxidant activity (MacLellan et al., 2005). UCP3 is also reported to be upregulated in myocytes upon muscle/fiber contraction, suggesting a protective role against physical exercise-related ROS production (Anderson et al., 2007; Jiang et al., 2009). Overexpression of UCP3 has also been associated with a protective effect against age-related mitochondrial oxidative damage (Nabben et al., 2008). On a final note, UCP3-mediated translocation of lipid peroxides to the extra-mitochondrial medium has also been reported to be a competent strategy for minimizing oxidative stress (Goglia & Skulachev, 2003).

Although fewer studies focus on UCP4-6, compelling evidence highlights its role on the regulation of ROS production. Higher levels of *Ucp4* and *Ucp5* mRNA and respective proteins were found to be protective of neurons against oxidative stress damage (Huang et al.,

2017). In addition, other studies have shown that UCP5 expression decreased the production of superoxide radicals in mouse neurons (Kim-Han et al., 2001), whereas it protected cells against oxidative stress in hyperoxic conditions (Pichiule et al., 2003). UCP6 represents an intriguing case. Although UCP6 does not display an uncoupling function, this homolog can be upregulated in response to oxidative stress (Haguenauer et al., 2005). It was hypothesized that its antioxidant effect must be due to the regulation of H₂S levels. Although H₂S whenever present in high concentrations exerts cytotoxic effects, at low concentrations it exhibits beneficial and protective effects (Xie et al., 2016). One of the beneficial effects of H₂S in low concentrations is the inhibition of mitochondrial ROS production through the stimulation of cellular antioxidants (Gorgoglione et al., 2019; Xie et al., 2016). Hence, UCP6 seems to contribute to the maintenance of low ROS levels by keeping H₂S concentrations low.

1.4.2. The potential role of mitochondrial uncoupling proteins on male (in)fertility

Given the role of oxidative stress in male reproductive function and fertility, further understanding on the harmful impact of ROS production and antioxidant mechanisms in the male reproductive tract will most likely contribute for the understanding of the mechanisms responsible for male factor infertility and unravel more accurate diagnosis, as well as targets for intervention, to improve the outcomes of fertility treatments for subfertile and infertile men. Hence, UCPs by virtue of its antioxidant activity may represent a clinically relevant target to improve reproductive outcomes. However, little is known about its function in the male reproductive tract. In fact, most of the existent studies are focused on UCP2. Zhang et al. reported that UCP2 protects germ cells from hyperthermia-derived ROS production. Furthermore, *Ucp2* mRNA expression was demonstrated to rise in testis of mice with experimental cryptorchidism. Hyperthermia in the testis arises from multiple pathological conditions such as varicocele, cryptorchidism, or obesity, and correlates with exacerbated spermatozoa apoptosis (Zhang et al., 2007). In another study, it was found that ROS production is the main mechanism responsible for hyperthermia-induced sperm cell apoptosis. Acute exposure of mouse testis to heat (43°C for 5 min) was sufficient to induce a 6-fold increase in UCP2 expression. Furthermore, overexpression of UCP2 in mouse GC-2 cell line, a pre-meiotic spermatocyte cell line, improved the survival rate after exposure to menadione, a ROS-inducing agent (Zhang et al., 2007). Moreover, older mice exhibit increased UCP2 activity and proton leak in the testis. Testicular ageing is associated with mitochondrial dysfunction and increased oxidative stress, therefore, UCP2 upregulation in the testis of older

mice might represent a protective mechanism against ageing-related oxidative damage (Amaral et al., 2008). In humans, UCP2 expression was found to be significantly increased in spermatozoa from donors with normal sperm motility when compared to men with asthenozoospermia (Wang et al., 2018). Additionally, treatment of spermatozoa with H₂O₂ was shown to induce UCP2 expression (Wang et al., 2018). Overall, the experimental data on the role of UCPs in the male reproductive function is scarce. Given its presence in the testis and established function in other tissues, we hypothesize that UCPs may act as important modulators of testicular bioenergetics and redox state, which remain to be fully disclosed.

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Chapter 2

Aims



2. Aims

Infertility is currently one of the most challenging health and societal issue worldwide. Male fertility is thought to be steadily decreasing, as the quality of human spermatozoa was found to be reducing. Infertility attributed to male factor is most frequently labeled as idiopathic, since an etiology for the deficient sperm quality or defective fecundation cannot be identified. One of the main reasons for this phenomena is the fact that there is an overall lack of knowledge on human spermatozoa physiology and bioenergetics, which is also a moving target, as it differs according to the stage of cell development and spatial location within the male and female reproductive tract.

The male gamete embarks on an extensive voyage before achieving the feat of fertilizing the oocyte and thereby commencing the creation of new life. This journey commences within the seminiferous tubules with the assistance of Sertoli cells, progresses through maturation in the epididymis, and ultimately capacitates within the female reproductive tract. This sequence of changes culminates in the attainment of the spermatozoon's fertilization prowess. From the very beginning (spermatogenesis) to the end (capacitation and acrosome reaction), the development and function of the germ cell line is highly regulated by its environment. Depending on the stage of development or maturation, the ionic, metabolic, and hormonal requirements of the germ cell line are variable. Simultaneously, the spatial location of these cells has its own influence due to the different fluid compositions. Therefore, there is a complex interaction between the stage of maturation of the male gamete and the characteristics of the fluid where these cells are located at a given moment. Most of the molecular mechanisms responsible for human spermatozoa development and capacitation are, in fact, currently unknown.

In this work, my overarching objective was to delve into the intricate metabolic and hormonal orchestration governing human spermatozoa and the functioning of Sertoli cells. A pronounced emphasis was directed towards scrutinizing the regulation of mitochondrial activity and the production of ROS. In detail, the following specific goals were outlined to guide this pursuit:

- **Study the role of glucose for the metabolic profile and mitochondrial activity of human spermatozoa during capacitation.** Glucose is widely acknowledged as a vital component in instigating the capacitation process of human spermatozoa. Yet, the exact contribution of glucose to the molecular mechanisms that instigate this phenomenon remains partially elucidated. The focus of my research was to comprehensively examine the impact

of various glucose concentrations, as well as its absence, on the metabolic dynamics, mitochondrial function, and ROS generation during the *in vitro* capacitation of human spermatozoa.

• **Study the role of mitochondrial uncoupling proteins (UCPs) in human Sertoli cells and spermatozoa.** Glycolysis and oxidative phosphorylation are central in human spermatozoa bioenergetics and the main ATP sources that sustain motility. During capacitation, the mitochondrial activity and ROS production increases in spermatozoa. However, ROS are a double-edge sword not only for spermatozoa but also for spermatogenesis and testicular function. ROS are necessary for human spermatozoa physiology and capacitation, but uncontrolled ROS production and oxidative stress are a hallmark of testicular dysfunction and male infertility. The threshold or the mechanisms that regulate ROS levels are still not completely understood. A potential mechanism of ROS and redox status regulation are the mitochondrial uncoupling proteins (UCPs), but the knowledge on its expression and function in the male reproductive tract is very scarce. Thus, I aimed to study the expression and characterize the function of UCPs in human spermatozoa and Sertoli cells.

• **Study the potential secretion of insulin by human spermatozoa and its role during capacitation.** Testicular cells and male gametes are highly sensitive to the metabolic status of the individual. Energy-related hormones, such as gut and adiposity signal hormones, play an important role in the metabolic and hormonal regulation of the testicular function. Insulin is one of the most important anabolic hormones, stimulating the uptake of glucose. However, data concerning the role of insulin on human spermatozoa is limited. Evidence suggests that human spermatozoa contain insulin vesicles, although the mechanism underlying insulin production and its role on spermatozoa physiology is not understood. With this objective in mind, my aim was to discern the presence and role of insulin, alongside the enzymes PC1 and PC2 in human spermatozoa. These enzymes play pivotal roles in the biosynthesis of insulin, facilitating the cleavage of proinsulin into insulin and C-peptide. Furthermore, my focus extended to unravelling the influence of insulin and its associated signaling pathway in the context of human spermatozoa capacitation.

In sum, this dissertation aims to unravel the metabolic mechanisms related to the glycolytic pathway and mitochondrial activity, with particular emphasis on UCPs and insulin, that orchestrate the function of human spermatozoa and Sertoli cells. The essential role of glucose and mitochondria for human spermatozoa capacitation is highlighted. The discovery of UCPs expression and its characterization on human spermatozoa and Sertoli cells

will pinpoint its role as modulators of the mitochondrial ROS production and overall metabolic function. Lastly, the validation of insulin release by human spermatozoa will demonstrate that the male gamete can be classified as an insulin-producing cell, suggesting a collaborative teamwork to achieve the fertilization of the oocyte. Overall, the implications of this dissertation pave the way for the further understanding of the bioenergetic mechanisms that govern male reproduction, which may be applied for the improvement of current Assisted Reproductive Techniques (ART) and/or the development of methods for contraception.

Chapter 3

Evaluation of human spermatozoa mitochondrial membrane potential using the JC-1 dye



This chapter was adapted from the published work:

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Abstract

Mitochondria are fundamental for human spermatozoa motility and fertilizing ability. Mitochondria participate not only in ATP production, but also in ROS production, redox equilibrium, and calcium regulation, all of which are central for human spermatozoa motility, capacitation, acrosome reaction, and ultimately, oocyte fertilization. Mitochondrial membrane potential is a key indicator of mitochondrial health and activity. Most used methods for the study of mitochondrial membrane potential, however, cannot be applied to human spermatozoa due to its unique characteristics, including its high motility and time-dependent decay of quality, limiting the study of this important parameter in these cells. Here, we describe an easy, fast, and cheap protocol for the quantitative evaluation of human spermatozoa mitochondrial membrane potential, using the fluorescent cationic dye, 5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 is a sensitive marker for mitochondrial membrane potential, exhibiting a potential-dependent accumulation in the mitochondria. At high mitochondrial membrane potential, JC-1 forms J-aggregates, which emit red fluorescence, whereas, at low mitochondrial membrane potential, JC-1 remains at its monomer state, which emits green fluorescence. We first describe how to obtain this data using a fluorescence plate reader, for high throughput studies. The calculation of the JC-1 ratio (indicative of the J-aggregates/monomers ratio) is then used to quantitatively evaluate mitochondrial health and activity. In addition, we describe an imaging protocol for the qualitative evaluation of human spermatozoa mitochondrial membrane potential using a fluorescence microscope. This allows for a visual analysis of the results that can complement the quantitative data. These protocols can be used to study the effects of spermatozoa exposure to compounds of interest and alterations due to diseases or different conditions. While these protocols are illustrated with human spermatozoa, these can be adapted and used on spermatozoa of different species.

3.1. Introduction

Human spermatozoa are dependent on high levels of ATP production to support their motility and function. Most of the ATP is used by dynein ATPases located in the flagellum, which promotes sperm motility (Guerra-Carvalho et al., 2022). Spermatozoa also need high levels of ATP for capacitation, which is a finely regulated and complex process involving structural and functional changes necessary for fertilization. For capacitation to occur, a specific ionic and energetic environment is necessary to support the mass phosphorylation that leads to hyperactivated motility (Carrageta et al., 2020). Although human spermatozoa are highly glycolytic cells, mitochondrial oxidative phosphorylation also plays a major role in ATP generation from glucose in these cells (Ferramosca & Zara, 2014). In spermatozoa,

the mitochondria are exclusively located in the midpiece. Increased mitochondrial respiration occurs during capacitation, which is positively associated with hyperactivated motility (Stendardi et al., 2011). In addition to being a major source for ATP production, mitochondrial oxidative phosphorylation is also the most significant source of reactive oxygen species (ROS) in spermatozoa, which, at low concentrations, are known to play key physiological roles, including their participation in signaling pathways responsible for capacitation (Monteiro et al., 2021). Thus, healthy and highly functional mitochondria are essential for human spermatozoa function and fertilization capacity.

The measurement of mitochondrial membrane potential is an important indicator of the mitochondrial health and activity of human spermatozoa. High membrane potential suggests highly functional and healthy mitochondria, which can support the high demand for ATP necessary for motility. In contrast, low membrane potential is associated with unhealthy cells; for instance, to low motility and unhealthy spermatozoa (Appell et al., 1977; Peirce et al., 2015; Zhou et al., 2015). Given the importance of mitochondrial function for spermatozoa, studying mitochondrial membrane potential in these cells is of high relevance. The study of human spermatozoa, however, is tricky – spermatozoa are highly sensitive to environmental changes, including temperature, pH, and composition of the media. In addition, the study of spermatozoa has a short time window, as its function decays rapidly over time. Experiments are recommended to be performed up to 1-2 h after sample collection, and experiments performed on the same sample at different time periods might not be comparable (Pinto et al., 2021).

There are several methods available to evaluate mitochondrial membrane potential. Commercial and hands-on kits are a reliable and simple option to evaluate mitochondrial respiration and membrane potential, however, the cost per sample can be high and prohibitive for certain groups. Other techniques to study mitochondrial function, for instance, analysis of respiration by determining Oxygen Consumption Rate (OCR), are highly informative but depend on expensive equipment or are limited by time factors. As only one sample can be analyzed at a time, results across conditions may not be comparable, due to spermatozoa's functional decay. In addition, protocols involving the use of the Seahorse XF technology, which measures oxygen consumption rate and extracellular acidification rate, have not yet been reported for use in human spermatozoa.

An additional tool to study membrane potential involves the JC-1 dye. JC-1 (5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) is a lipophilic and cationic fluorescent dye that works as a sensitive marker for mitochondrial membrane potential, which exhibits a potential-dependent accumulation in the mitochondria. JC-1, which naturally exhibits green fluorescence (530 ± 15 nm), enters the mitochondria, where it accumulates according to the mitochondrial membrane potential. In energized and negatively

charged mitochondria (i.e., actively respiring mitochondria with high membrane potential), JC-1 accumulates, and due to its high concentration, spontaneously forms reversible aggregates (J-aggregates), which exhibit red fluorescence (590 ± 17 nm). In less energized mitochondria (i.e., low membrane potential), the concentration of JC-1 dye inside the mitochondria is reduced and fewer J-aggregates are formed. Therefore, JC-1 remains in its monomer state, exhibiting green fluorescence. The quantification of the J-aggregates/monomers ratio (revealed by the red/green fluorescence ratio) is indicative of the state of the mitochondrial membrane potential (Reers et al., 1995): the more J-aggregates are formed, the higher the ratio, which is indicative of a higher mitochondrial membrane potential. In contrast, the fewer J-aggregates are formed, the lower the ratio, which is indicative of mitochondrial depolarization. Since the mitochondrial membrane potential depends on the transport of protons from the matrix to the mitochondrial intermembrane space during oxidative phosphorylation and this potential is used for ATP production, the mitochondrial membrane potential reflects mitochondrial activity (Zorova et al., 2018). A higher JC-1 ratio is indicative of higher mitochondrial activity; and *vice-versa*. It has also been suggested that a lower JC-1 ratio, when compared to a control condition, is indicative of unhealthy or apoptotic cells (Akhtar et al., 2020; Sivandzade et al., 2019). This assay, therefore, can also be used to evaluate cell health under different conditions.

The JC-1 ratio can be used to evaluate mitochondrial activity during the *in vitro* study of pathological conditions or in response to various compounds (i.e., pharmacological compounds, pesticides, heavy metals, etc.). The major advantages of this method, besides its simplicity and low cost, are that the JC-1 ratio is only dependent on the membrane potential, and is not affected by factors such as mitochondrial size, density, or the number of spermatozoa per sample (Sivandzade et al., 2019). The JC-1 assay can be performed in both a quantitative form through the measurement of the fluorescence intensity with a fluorescence plate reader or flow cytometry or qualitatively, through observation by fluorescence or confocal microscopy.

Here, we describe an easy and cheap method for both the quantitative and qualitative evaluation of the mitochondrial membrane potential of human spermatozoa using the JC-1 dye. In Basic Protocol 1, we describe the quantitative assay, using a fluorescence plate reader, and in Basic Protocol 2, we describe the qualitative assay, using a fluorescence microscope. In addition, in Support Protocol 1, we describe the preparation of the JC-1 working solution. The quantitative JC-1 assay should be considered the primary protocol, due to the quantitative nature and objectivity of the data. The qualitative JC-1 should be considered complementary to the quantitative protocol, as it allows for a visual analysis of the results. Figure 3.1 outlines the steps that must be followed to perform the quantitative

or qualitative JC-1 assay, using a multi-well microplate reader or a fluorescence microscope, respectively.

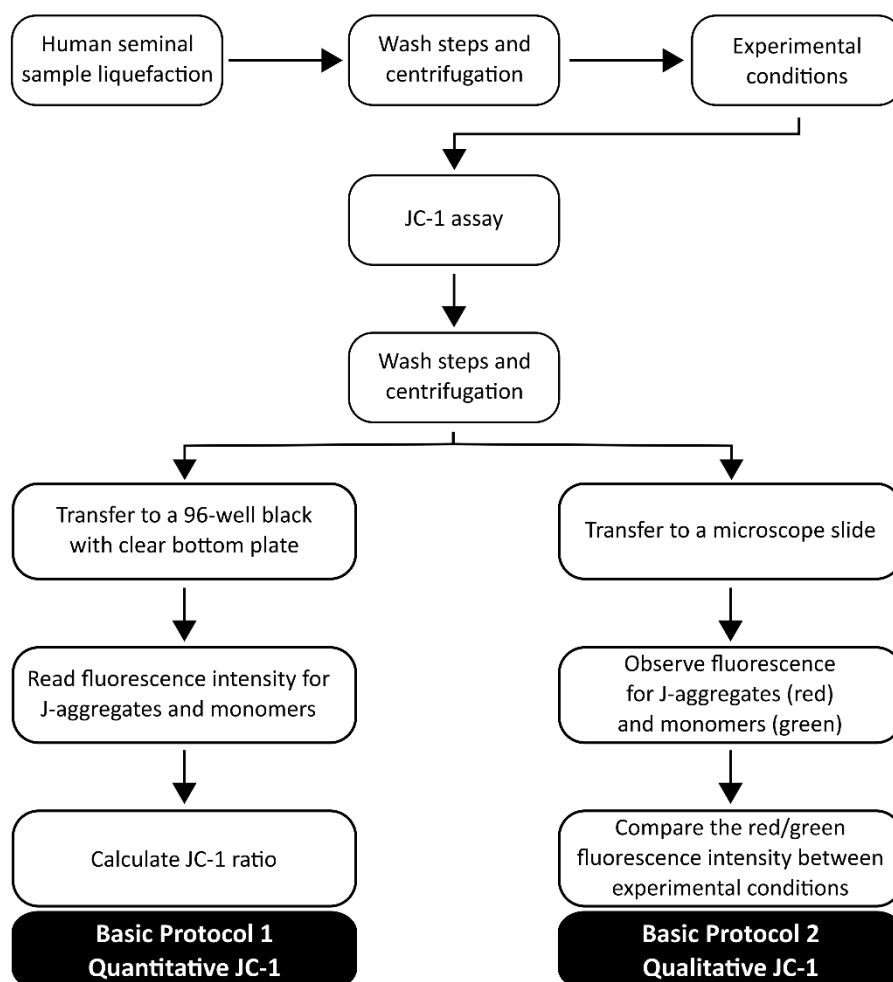


Fig. 3.1 - Schematic workflow for the quantitative (Basic Protocol 1) and qualitative (Basic Protocol 2) evaluation of mitochondrial membrane potential of human spermatozoa using the JC-1 dye. The JC-1 assay allows for the evaluation of the mitochondrial membrane potential through the calculation of the JC-1 ratio (J-aggregates/monomers). The comparison of the JC-1 ratio between control and experimental conditions allows for the assessment of mitochondrial activity and health state. The assay involves processing human seminal samples, subjecting to experimental conditions of interest, washing, and then transferring to a plate or a microscope slide, for analysis. While the qualitative JC-1 method (Basic Protocol 2) is complementary to the quantitative method (Basic Protocol 1), we recommend its execution to help visualize and interpret results.

3.2. Strategic planning

Human seminal samples should be collected according to the World Health Organization (WHO) guidelines (WHO, 2021), unless the aim of the study requires alterations. In brief,

seminal samples should be obtained by masturbation into sterile containers 3 to 5 days after sexual abstinence. Human seminal samples should be processed 1 to 2 hours upon its collection to avoid loss of quality. We recommend preparing the necessary setup for the experiment during the liquefaction period. Spermatozoa should always be washed with PBS (or with a commercial sperm medium) to remove the seminal fluid before performing any treatment or assay unless the objective of the study states otherwise. Never wash spermatozoa with water, as it will cause irreversible loss of motility. Temperature is also important as spermatozoa are highly sensitive to temperature changes. We recommend performing all steps at room temperature (23-25°C) unless stated otherwise.

Experimental design and exposure to compounds of interest should be clearly defined before performing the JC-1 assay on human spermatozoa. Pilot experiments should be performed to test for viability and motility alterations for the compound concentrations and exposure time of interest before performing the JC-1 assay. The user should expect high variability between samples from different donors. We recommend dividing each sample to all defined conditions to improve the consistency and reproducibility of the data. Even if the quantitative evaluation of the mitochondrial membrane potential (Basic Protocol 1) requires a low concentration of spermatozoa, we recommend to not using that sample if the concentration of spermatozoa is too low to test all conditions.

Note: Before performing these protocols, the user should be aware of the particularities of working with human seminal samples. It is important to highlight that all procedures should be conducted in accordance with the Declaration of Helsinki and the user should always consult the Ethics Committee to seek approval for conducting the project. The study of human seminal samples can be performed on either donated or on surplus semen samples collected for diagnostic purposes. An informed and written consent should always be obtained from all individual donors included in the study. All samples should be used under strict individual anonymity and should be destroyed when the study is completed.

3.3. Basic Protocol 1: Quantitative evaluation of human spermatozoa mitochondrial membrane potential using the JC-1 dye and a fluorescence plate reader

This protocol describes the methodology used to perform the JC-1 assay for the quantitative evaluation of mitochondrial membrane potential in human spermatozoa using a fluorescence plate reader. Herein, users will assess the mitochondrial membrane potential, following the treatment conditions of interest, through the incubation of spermatozoa with the JC-

1 dye. The fluorescence of emitted by J-aggregates and monomers will be quantified. Then, the JC-1 ratio will be calculated. By comparison to a control condition, the evaluation of the JC-1 ratio will be used to assess mitochondrial membrane potential and the state of mitochondrial health and activity in the experimental conditions.

3.3.1. Materials

Fresh human seminal samples (see Strategic Planning)

Filtered phosphate-buffered saline (PBS) solution (see recipe)

Test compounds of interest (e.g., pharmacological compounds, pesticides, heavy metals)

JC-1 working solution (1 μ M; see Support Protocol)

Depolarization control: dimethyl sulfoxide (DMSO; Labkem, cat. no. DMSO-00A-1K0) or

FCCP (100 μ M, Sigma-Aldrich, cat. no. C2920-000010MG)

Graduated conical tubes (15 mL)

Pipettes (1000, 200, and 20 μ L) and pipettes tips

Benchtop centrifuge (suitable for microtubes)

Neubauer chamber (BLAUBRAND®, cat. no. BR718605)

Microtubes (1.5 mL)

Thermoblock for microtubes (1.5 mL, Labnet, cat. no. LA-D1302)

96-Well Black/Clear Bottom Plate (Corning®, cat. no. 3904)

Multi-well fluorescence plate reader (BioTek Synergy H1, wavelength range 250-700 nm)

Recipient for liquid residues

3.3.2. Steps and annotations

1. Transfer the human seminal sample to a labeled graduated conical tube (15 mL). Place the tube at 37°C until viscosity is reduced and the sample is mostly liquid (15-30 min).
2. Measure sample volume and add an equal volume of filtered PBS to the sample. Gently mix by pipetting up and down.

Vortexing at a slow speed can also be used. If the sample is still highly viscous, please see the Troubleshooting section for details.

3. Centrifuge samples 5 min at 300 $\times$ g, room temperature.

4. Carefully remove and discard the supernatant, then add 1 mL of filtered PBS. Gently mix by pipetting up and down.

The appearance of the pellet can be cloudy or diffuse (instead of compact), due to spermatozoa motility. If the supernatant is not quickly removed, the pellet will disperse.

5. Assess spermatozoa concentration using a Neubauer chamber.
6. Transfer 1×10^6 spermatozoa to labelled microtubes (1.5 mL), one per condition to be tested plus two for the control groups (untreated and depolarization controls). Add 1 mL of filtered PBS to each sample and gently mix.
Proper mixing of the samples is very important. The depolarization control sample will be used in step 10.
7. Centrifuge samples 5 min at $500 \times g$, room temperature. Carefully remove and discard the supernatant.
8. Expose spermatozoa to the conditions (e.g. capacitation) or compounds of interest for the desired time.
9. Centrifuge samples 5 min at $500 \times g$, room temperature. Carefully remove and discard the supernatant.
10. Add 1 mL of filtered PBS and gently mix by pipetting up and down. Centrifuge once again for 5 min at $500 \times g$, room temperature. Carefully remove and discard the supernatant.
11. Add 750 μ L of fresh JC-1 working solution (1 μ M) to each pellet. For the depolarization control sample, add 600 μ L of 1 μ M JC-1 working solution plus 150 μ L of DMSO. Gently mix by pipetting up and down.
FCCP (100 μ M) can be used as a depolarization control instead of DMSO.
12. Incubate the samples in a thermoblock at 37°C for 30 min. Protect from light exposure.
13. Centrifuge samples 5 min at $500 \times g$, room temperature. Carefully remove and discard the supernatant.
The user should observe a pinkish pellet due to JC-1 (Fig. 3.2A).
14. Add 1 mL of filtered PBS and gently mix by pipetting up and down. Centrifuge once again for 5 min at $500 \times g$, room temperature. Carefully remove and discard the supernatant.

The color of the pellet should be lighter (light pink, Fig. 3.2B).

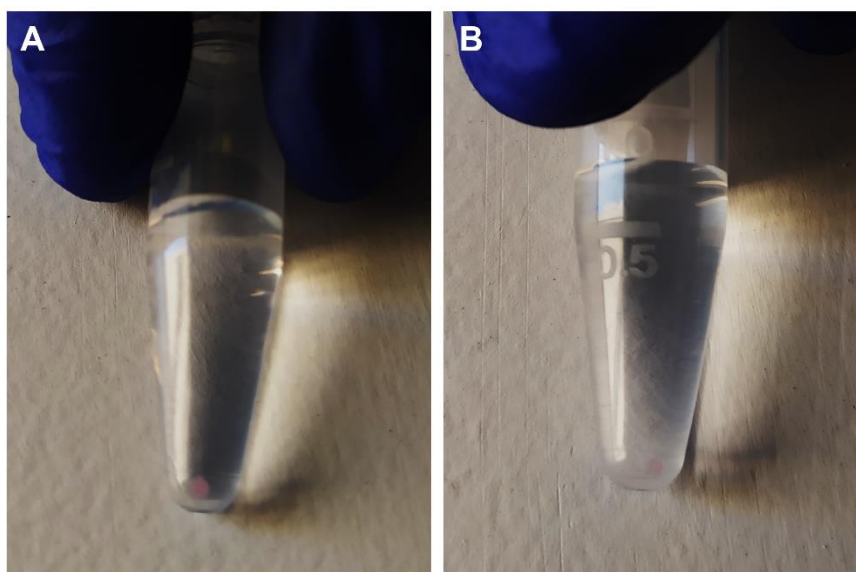


Fig. 3.2 – Resulting pellet of the JC-1 assay performed on human spermatozoa. (A) Upon incubation with the fresh JC-1 working solution and centrifugation, the user should observe a pinkish pellet of spermatozoa. **(B)** After the wash step with filtered PBS and centrifugation, the user should observe a lighter pinkish pellet of spermatozoa.

- 15.** Add 750 μL of filtered PBS to each sample. Gently mix by pipetting up and down.
- 16.** Transfer 200 μL of each sample to a 96-well black, clear-bottom plate. Use 200 μL of filtered PBS as blank. Analyze triplicates for each sample.
- 17.** Read fluorescence at 485/530 nm (excitation/emission) and 535/590 nm (excitation/emission) for monomer and J-aggregates, respectively, using a multi-well fluorescence plate reader. Export data to a spreadsheet.
- 18.** Calculate the JC-1 ratio (J-aggregates/monomers) as follows:
 - a. Calculate the mean value for the blank triplicates at both wavelengths.
 - b. Calculate the JC-1 ratio (J-aggregates/monomers) for each replicate using the following formula:

$$\text{JC-1 Ratio}_{(\text{J-aggregates/monomers})} = \frac{\text{Abs}_{535/590 \text{ nm}} - \overline{\text{Abs}}_{\text{blank } 535/590 \text{ nm}}}{\text{Abs}_{485/530 \text{ nm}} - \overline{\text{Abs}}_{\text{blank } 485/530 \text{ nm}}}$$

c. Calculate the mean value of the JC-1 ratio for each sample and controls.

The JC-1 ratio for the depolarization control should be lower (< 25%) than the control (untreated) group of experiment, as almost no J-aggregates should be formed due to mitochondria uncoupling. Sample data is shown in Figure 3.3.

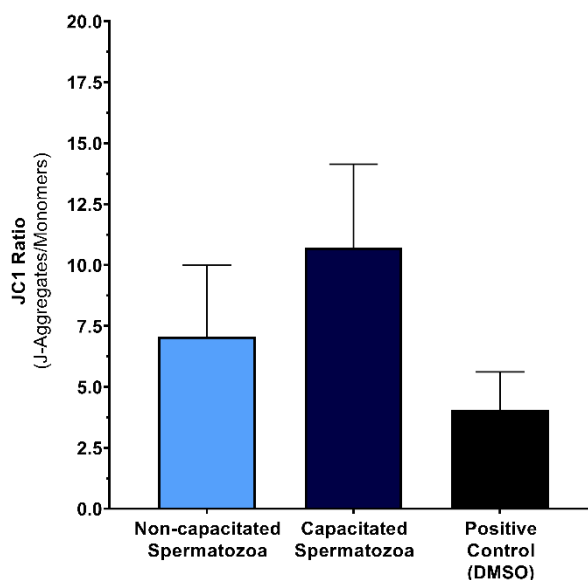


Fig. 3.3 – JC-1 results in human spermatozoa following incubation for 6 h in capacitating conditions. DMSO was used as depolarization control. Results are expressed as mean $\pm$ standard deviation ($n = 15$). Sample data adapted from (Carrageta et al., 2020).

3.4. Basic Protocol 2: Qualitative evaluation of human spermatozoa mitochondrial membrane potential using the JC-1 dye and fluorescence microscopy

This protocol concerns the qualitative evaluation of the JC-1 ratio using a fluorescence microscope. Here, human spermatozoa samples stained with the JC-1 dye are placed on microscope slides and analyzed via fluorescence microscopy. With this protocol, pictures of J-aggregates (red fluorescence) and monomers (green fluorescence) are obtained and visually compared for their intensity. The images of the JC-1 assay obtained here provide a visual representation of the data obtained during Basic Protocol 1 and should be used as complementary to that data. Quantitative data can also be obtained from this protocol, although we do not recommend it, as the fluorescence signal of JC-1 decays rapidly, which can compromise results. In addition, the use of fluorescent nuclear stains may interfere with the quantification of the JC-1 signal, which would also compromise results. Fluorescent nuclear stains are useful for the spatial localization of spermatozoa, as mitochondria are

found in the midpiece, separated from the nucleus. Moreover, the use of fluorescent nuclear stains is helpful to distinguish between the very small spermatozoa midpiece and potential JC-1 artifacts found in the background. If the user intends to quantify the fluorescence signal, we do not recommend including fluorescence nuclear stains. This protocol, therefore, should only be used as a complement to Basic Protocol 1.

3.4.1. Additional Materials (also see Basic Protocol 1)

Fresh human seminal samples (see Strategic Planning)

Hoechst 33342 (10 mg/mL, Invitrogen™, cat. no. H3570; or another nuclear stain)

Pipettes (100 and 2.5 µL) and pipette tips

Microscope slides (Biosigma, cat. no. VBS650)

Stainless steel spatula

Fluorescence microscope (Nikon Eclipse E400 microscope equipped with a DS-Fi3 microscope camera, a Y-FL Epi-Fluorescence Attachment, DAPI filter (395/425 nm; excitation/emission), FITC filter (480/510 nm; excitation/emission), G-2A filter (510/575 nm; excitation/emission), and HB-10103AF Super High Pressure Mercury Lamp Power Supply)

3.4.2. Steps and annotations

1. Follow Basic Protocol 1, steps 1-16.
2. Add 0.75 µL of Hoechst 33342 (10 mg/mL) for staining the nuclei.
Other nuclear and chromosome counterstain dyes (for example, DAPI) can be used.
3. Carefully transfer 30 µL of each sample to a clean and labeled microscope slide (Fig. 3.4A). Add one sample per slide. Prepare duplicates for each sample.
4. Carefully place a rectangular coverslip over the sample. Protect microscope slides from direct light exposure (Fig. 3.4B).
Avoid the formation of air bubbles when pipetting and placing the coverslip.
5. Immediately observe the microscope slides under a fluorescence microscope using the appropriate fluorescence filters for observing JC-1 monomers and J-aggregates.
We recommend FITC filter (480/510 nm; excitation/emission) for JC-1 monomer (green fluorescence), and G-2A filter (510/575 nm; excitation/emission) for JC-1 J-aggregates (red fluorescence).

6. Take pictures using 400× or 1000× magnification. All pictures should be captured with the same exposition time and gain.

Pictures should be taken as fast as possible as the fluorescence intensity will quickly decay. Sample data is show in Figure 3.5.

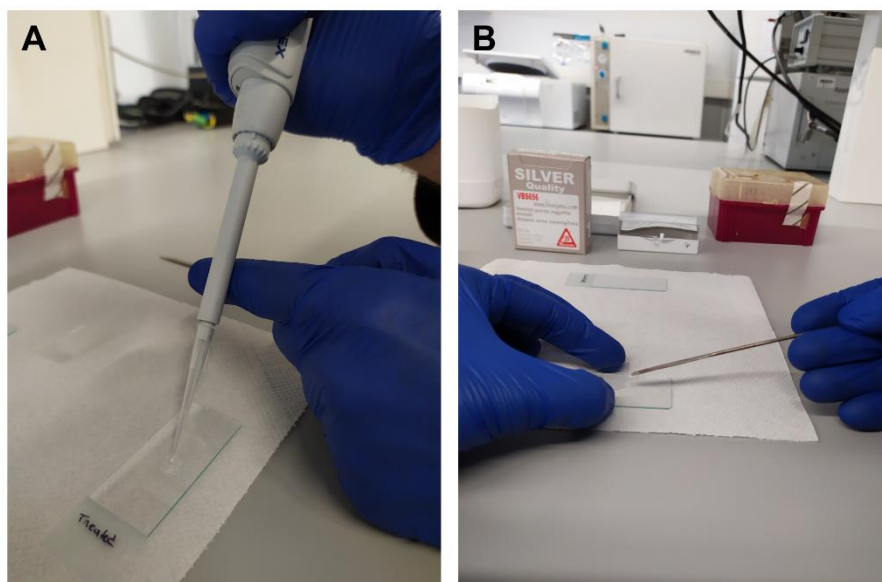


Fig. 3.4 – Preparation of microscope slides for qualitative JC-1 analysis. (A) Carefully transfer 30 μ L of each sample to clean and labeled microscope slides. Try to minimize light exposure as much as possible. (B) Carefully place a coverslip over the sample. Avoid the formation of air bubbles when pipetting and placing the coverslip.

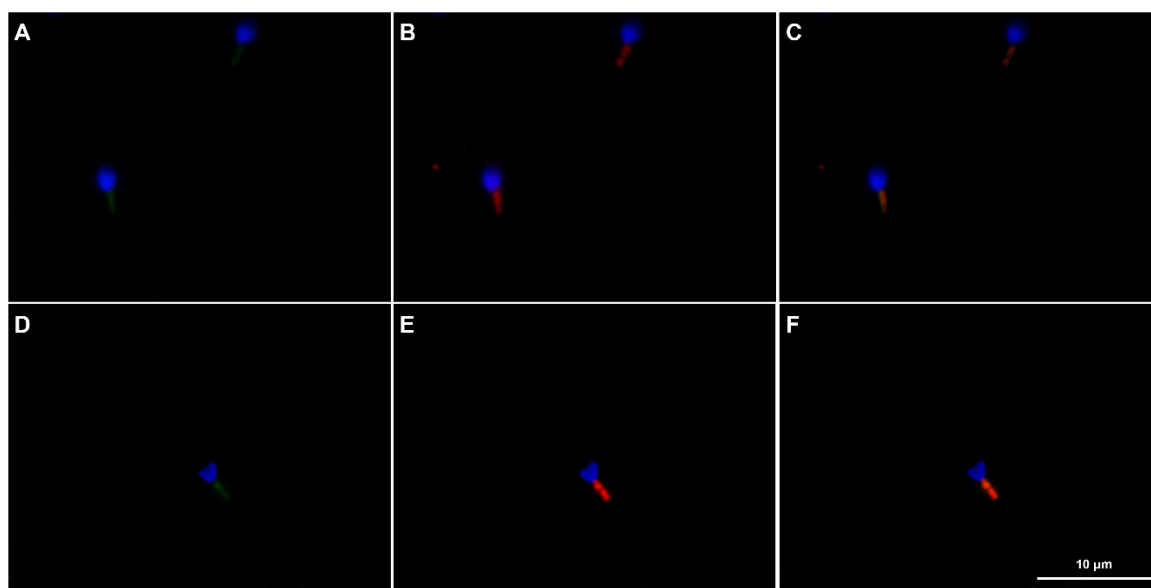


Fig. 3.5 – JC-1 imaging in human spermatozoa following incubation for 6 h in capacitating conditions. Hoechst 3342 was used to stain the nuclei. (A) Monomer (green) or (B) J-aggregates

(red) observed in the mitochondria of non-capacitated human spermatozoa. **(C)** Merged images of both monomers and J-aggregates observed in the mitochondria of non-capacitated human spermatozoa. **(D)** Monomer (green) or **(E)** J-aggregates (red) observed in the mitochondria of capacitated human spermatozoa. **(F)** Merged images of both monomers and J-aggregates observed in the mitochondria of capacitated human spermatozoa. Merged images of monomers and J-aggregates allow for the qualitative evaluation of the JC-1 ratio, which is complimentary to the results obtained in Basic Protocol 1. Intense red fluorescence in the mitochondria of **(F)** capacitated compared to those in **(C)** non-capacitated human spermatozoa can be observed, which is indicative of increased mitochondrial membrane potential. Scale bar 10 μm . Sample data adapted from (Carrageta et al., 2020).

3.5. Support Protocol: Preparation of the JC-1 working solution

A fresh JC-1 working solution should be prepared for each assay. The preparation of the JC-1 working solution, which is a lipophilic dye, is not easy. JC-1 is usually supplied as a crystalline solid. A stock solution should be made by dissolving the JC-1 in an organic solvent, such as DMSO (also see recipe for JC-1 stock solution in Reagents and Solutions). The JC-1 working solution is then obtained through the dilution of the JC-1 stock solution in filtered PBS. However, the formation of JC-1 precipitates is a common issue due to undissolved particulate matter. Most protocols recommend the centrifugation of the JC-1 solution, although removing the undissolved JC-1 decreases its concentration. Here, we describe an easy and fast protocol to prepare the JC-1 solution and avoid this issue using Pluronic® F-127. Pluronic® F-127 is a nonionic detergent that facilitates the solubilization of water-insoluble dyes. The inclusion of this reagent in the preparation of the JC-1 solution greatly decreases the appearance of undissolved particles.

3.5.1. Materials

10% Pluronic® F-127 solution (see recipe)

JC-1 stock solution (5 mg/mL in DMSO, see recipe)

Filtered PBS solution (see recipe)

Graduated conical tubes (15 mL)

Pipettes (10 mL and 2.5 μL) and pipette tips

Vortex

Aluminum foil

3.5.2. Steps and annotations

1. Pipette 1.25 μL of 10% Pluronic® F-127 solution on to the wall of a 15 mL conical tube (Fig. 3.6A).

Heat the Pluronic® F-127 solution if it is solid at room temperature.

2. Pipette 1.25 μL of JC-1 stock solution (5 mg/mL in DMSO) and mix it with the 10% Pluronic® F-127 solution on the wall of the tube (Fig. 3.6B).

A red/pinkish foam should be observed.

3. Add 10 mL of filtered PBS to the tube and vortex to homogenize.

No undissolved particulate matter should be observed in the final solution.

4. Protect the tube from light exposure with aluminum foil (Fig. 3.6C).

5. Prepare fresh before Basic Protocol 1 and/or 2. Do not store.

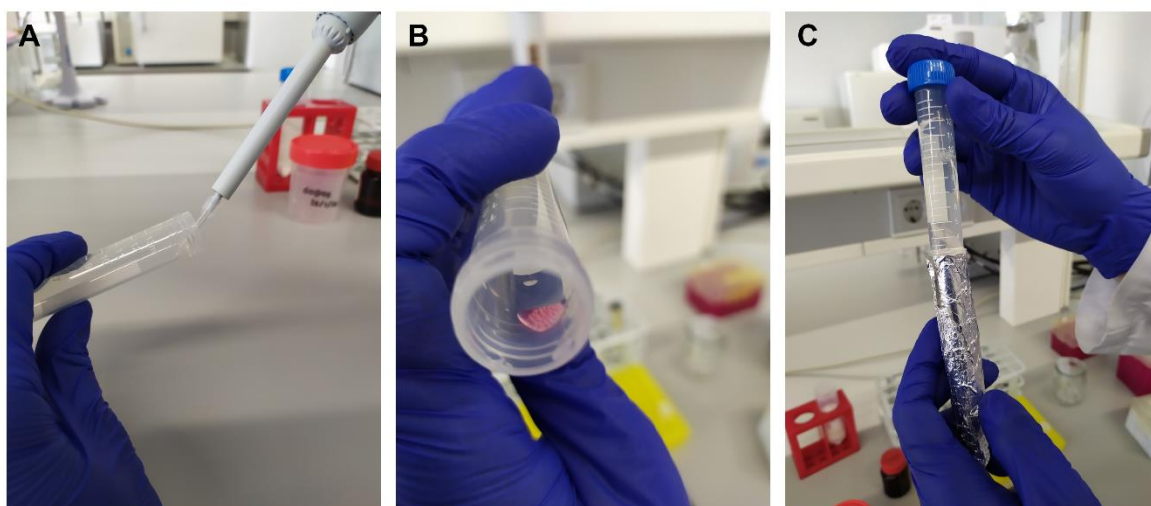


Fig. 3.6 – Preparation of the JC-1 working solution. (A) Add Pluronic® F-127 and HC-1 stock solution to the wall of the tube. (B) A red/pinkish foam should be observed after mixing both reagents. (C) After adding filtered PBS and homogenization, protect from light exposure with aluminum foil.

3.6. Reagents and solutions

3.6.1. Filtered PBS solution (50 mL, pH 7.4)

8 g sodium chloride (NaCl)

0.2 g potassium chloride (KCl)

1.44 g sodium phosphate dibasic (Na_2HPO_4)

0.24 g potassium phosphate monobasic anhydrous (KH_2PO_4)

Instructions: Dissolve all reagents in distilled water for a final volume of 1 L. Adjust pH to 7.4 using HCl or NaOH. Store at room temperature for up to 2 months. Filter using a syringe (0.2 μm) from the stock solution before use.

3.6.2. JC-1 stock solution (5 mg/mL in DMSO)

JC-1 dye (5 mg, Invitrogen™, cat. No. T3168)

1 mL of DMSO

Instructions: Add 1 mL of DMSO to dissolve the JC-1 dye. Divide the JC-1 stock solution into small portions (e.g. 20 μL) for storage. Store at -20°C until required to use.

3.6.3. Pluronic® F-127 solution, 10% (w/v)

0.1 g Pluronic® F-127 (Invitrogen™, cat. No. P6867)

1 mL of distilled water

Instructions: Add 1 mL of distilled water to dissolve Pluronic® F-127. Heat the solution if needed (40°C - 50°C should help solubilize). Store at room temperature. Do not refrigerate or freeze the Pluronic® F-127 solution because it may precipitate.

3.7. Commentary

3.7.1. Background information

Fluorescent probes for monitoring membrane potential have been under intense study over the years. Rhodamine and cyanine-based dyes are the most commonly used fluorescent membrane potential-dependent probes (Chen, 1988). These probes can permeate the mitochondrial membrane, and their characteristics are altered depending on the membrane potential. In 1936, it was observed for the first time that some dyes would form aggregates under certain conditions, resulting in a dramatic change in both their excitation and emission wavelengths, which is a violation of Beer's law (Jelley, 1936; Scheibe et al., 1937). The peak of the monomer spectrum (M-band) is usually at shorter wavelengths, whereas the peak of aggregates is at a longer wavelength (J-aggregates). In other words, when the dye aggregates, its absorption band moves to a longer wavelength. The fluorescence wavelength of monomers is similar to the absorption wavelength of J-aggregates, in a phenom-

enon termed resonance fluorescence (Wrigge et al., 2007). J-aggregates are formed spontaneously in response to high concentrations of the dye, which accumulates due to energized mitochondrial membranes. Therefore, membrane potential-dependent fluorescent probes can be used for studying several biological processes, including the energetic state of the mitochondria. Among several J-aggregate-forming dyes, JC-1 (5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) is highlighted as one of the most useful membrane potential-dependent probes. JC-1 was originally applied in the photography and film industry. Only in 1995, Reers et al. were able to demonstrate that JC-1 could be applied as a useful fluorescent probe for the study of the mitochondrial membrane potential (Reers et al., 1995). JC-1 is a lipophilic and cationic dye whose monomers exhibit green fluorescence (530 ± 15 nm), whereas the J-aggregates exhibit red fluorescence (590 ± 17 nm). In energized and negatively charged mitochondria, JC-1 spontaneously forms J-aggregates which exhibit red fluorescence, whereas only monomers are found in less energized mitochondria, resulting in the emission of green fluorescence. Thus, the quantification of the J-aggregates/monomer ratio (red/green fluorescence ratio) allows for the assessment of the state of the mitochondrial membrane potential (Sivandzade et al., 2019). The more J-aggregates are formed, the higher the ratio and the higher the mitochondrial membrane potential is, and *vice-versa*.

JC-1 has been applied to several living cells and tissues for the evaluation of the mitochondrial membrane potential (Chaoui et al., 2006; Martins et al., 2019; Salvioli et al., 1997). Here, we describe a specific protocol for the application of JC-1 to human spermatozoa. Human spermatozoa are cells with particular characteristics, such as high motility, and quick decrease of function and viability with time. The energy requirements of spermatozoa are high due to capacitation and hyperactivated motility. Mitochondria play an essential role to support the high demand for ATP, so its dysfunction can lead to harmful consequences for spermatozoa. The mitochondrial membrane potential is indicative of the mitochondria's health and activity (Zorova et al., 2018). Due to human spermatozoa characteristics, a specific protocol for using JC-1 as a mitochondrial membrane potential probe was considered necessary. Through the application of the protocol described here, our group observed that the mitochondrial membrane potential increases during human spermatozoa capacitation, regardless of glucose stimuli or the presence of any energetic substrate (Carrageta et al., 2020).

The application of JC-1 as a mitochondrial membrane potential probe for spermatozoa has several advantages when compared to other available methods. JC-1 also has desirable characteristics for membrane potential quantification, as the resultant J-aggregates are delocalized lipophilic cations that are independent of pH and ionic strength (Reers et al., 1995). Besides the simplicity of the protocol and low cost, the JC-1 ratio is only dependent

on the membrane potential, and no other factors, such as mitochondrial size, density, or the number of spermatozoa per sample, impact the assay (Sivandzade et al., 2019). In addition, the JC-1 assay can be performed simultaneously in multiple samples and conditions. These characteristics highlight the usefulness of JC-1 for the quantification of mitochondrial membrane potential as an indicator of mitochondria's health and activity. Moreover, the JC-1 ratio can be used to evaluate mitochondrial activity during *in vitro* studies of pathological conditions or in response to compounds of interest (i.e. pharmacological compounds, pesticides, heavy metals, etc.). The main drawback of this method, however, is inherent to its simplicity, as little information can be obtained concerning the mechanisms that increase/decrease mitochondrial activity. In contrast, the evaluation of the mitochondrial function through OCR measurements, such as Seahorse XF or oxygraphy systems, is much more informative. Using some of these protocols for the study of human spermatozoa, however, is challenging. Oxygraph systems are limited to a reduced number of samples per assay, which is incompatible with the study of spermatozoa due to their time-dependent decay of motility and viability. An alternative would be the Seahorse XF technology, which allows for simultaneous comparison of multiple samples, but its cost is very high and a protocol for the study of mitochondrial function of human spermatozoa is yet to be reported. For this purpose, the method described here can be coupled with other experiments, including the assessment of ROS production, quantification of oxidative-stress related biomarkers (Carrageta et al., 2020), alternative OCR protocols (Shih et al., 2018), or apoptosis-related assays (Perelman et al., 2012).

3.7.2. Critical parameters

Both the quantitative evaluation and imaging of the mitochondrial membrane potential using the JC-1 dye represent easy and straight-forward assays. There are, however, some factors that might influence the success of these protocols. The first issue is the high viscosity of the seminal samples. Viscosity should be addressed, as it influences sample handling and the subsequent steps. Special attention should also be paid to the preparation of the JC-1 working solution. JC-1 is a lipophilic dye and its dilution in PBS could result in the occurrence of undissolved particulate matter. Please refer to Support Protocol for the preparation of the JC-1 working solution. The addition of Pluronic® F-127 greatly decreases the appearance of undissolved particles.

The inclusion of a depolarization control is essential – results are only valid when the JC-1 ratio for the depolarization control is much lower (at least < 25%) when compared to the control of the experiment, as almost no J-aggregates should be formed under the described conditions.

In addition, low-intensity signal could be a common issue during the imaging protocol (Basic Protocol 2). JC-1 dye fluorescence signal quickly decays over time, particularly upon light exposure. Protect samples from light exposure as much as possible.

3.7.3. Troubleshooting

See Table 3.1 for a list of common problems with the protocols, their causes, and potential solutions.

Table 3.1. Troubleshooting guide for the quantitative evaluation and imaging of the mitochondrial membrane potential using the JC-1 dye.

Problem	Possible Cause	Solution
The human seminal sample is too viscous	Low liquefaction time	Allow sample to liquefy up to 1 h at 37°C. Then, add filtered PBS and gently mix. If the sample remains viscous, repeated passage through a needle (23G) is recommended
Undissolved particulate matter observable in the working JC-1 solution	The working JC-1 solution was not correctly prepared	Repeat the preparation of the working JC-1 solution
Low-intensity signal in Basic Protocol 1/2	Loss of signal due to light exposure	Protect samples from light exposure using aluminum foil
Fluorescence signal overflow in Basic Protocol 1	The concentration of cells <i>per well</i> is too high	Decrease the concentration of cells to 0.75 or 0.5×10^6 spermatozoa
The value of the JC-1 ratio of the depolarization control is higher than of the control of the experiment	Error in the execution of the protocol	The results are not valid. Discard samples and repeat the protocol

3.7.4. Understanding results

The quantification of the J-aggregates/monomer ratio (red/green fluorescence ratio) is indicative of the state of the mitochondrial membrane potential. The more J-aggregates are formed, the higher the ratio and the higher the mitochondrial membrane potential. In contrast, the fewer J-aggregates are formed, the lower the ratio, which is indicative of mitochondrial depolarization. The higher the JC-1 ratio is when compared to control, the higher

the mitochondrial activity, and *vice-versa*. A diminished JC-1 ratio can also indicate unhealthy mitochondria or apoptotic cells (Zorova et al., 2018). As an example, the incubation of spermatozoa in *in vitro* capacitating conditions (using a capacitation medium containing glucose and a high concentration of calcium, albumin, and bicarbonate, as described in (Carrageta et al., 2020), for 6 h increases the JC-1 ratio as compared to non-capacitated spermatozoa, which suggests increased mitochondrial activity (sample data, Fig. 3.3). It should be highlighted that the assay is only valid when the depolarization control decreases as compared to the untreated condition (< 25%), as almost no J-aggregates should be formed due to mitochondria uncoupling. JC-1 assay data should always be interpreted in relative terms, as a comparison to a control condition of the same sample, as human spermatozoa exhibit high variability between samples. It is recommended to use the same sample for testing different conditions. The data generated by the quantitative protocol (Basic Protocol 1) should then be analyzed using paired statistical tests.

When performing the qualitative protocol using the fluorescence microscope (Basic Protocol 2), energized mitochondria appear red due to the intense red fluorescence, whereas de-energized mitochondria exhibit orange/yellowish fluorescence when the pictures from the green and red filters are merged. Using the aforementioned example, the incubation of spermatozoa in capacitating conditions for 6 h increases the accumulation of J-aggregates, leading to an intense red fluorescence (Fig. 5B/C) as compared to the non-capacitated spermatozoa (Fig. 5E/F). We recommend for the qualitative method (Basic Protocol 2) be performed as complementary to Basic Protocol 1. The fluorescence intensity of JC-1 quickly decays during fluorescence microscope observation, which complicates its quantification and may compromise results. Therefore, this protocol is useful to visually demonstrate the results obtained with Basic Protocol 1. The users are not required to perform and report both protocols, but we recommend the execution of both protocols as it helps the reader to visualize and interpret results.

3.7.5. Time considerations

Human spermatozoa are highly motile cells whose function and viability decrease rapidly over time. Basic Protocol 1 should be performed in one session, and samples should be exposed to the dye immediately after the desired treatment is performed. Subsequently, the quantitative and/or qualitative analysis should be performed right after incubation with the JC-1 working solution. The incubation with the JC-1 working solution requires 30 min. Performing both protocols require an estimated total time of 2 h.

3.8. Authors contribution

David F. Carrageta – conceptualization, formal analysis, investigation, methodology, visualization, writing original draft, writing review and editing; **Laís Freire-Brito** – conceptualization, formal analysis, investigation, methodology, visualization, writing original draft, writing review and editing; **Pedro F. Oliveira** - conceptualization, funding acquisition, project administration, resources, supervision, visualization, writing review and editing; **Marco G. Alves** – conceptualization, formal analysis, funding acquisition, project administration, resources, supervision, writing review and editing.

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Chapter 4

Mitochondrial activation and reactive oxygen species overproduction during sperm capacitation are independent of glucose stimuli



This chapter was adapted from the published work:

Carrageta DF, Guerra-Carvalho B, Sousa M, Barros A, Oliveira PF, Monteiro MP, Alves MG. (2020). Mitochondrial activation and reactive oxygen species overproduction during sperm capacitation are independent of glucose stimuli. *Antioxidants*, 2020; 9(8):750. <https://doi.org/10.3390/antiox9080750>

Abstract

Spermatozoa capacitation is a complex process that requires specific ionic and energetic conditions to support biochemical alterations leading to motility hyperactivation. However, human sperm capacitation is still poorly understood. Herein, we studied the effects of glucose on human sperm capacitation. Healthy men seminal samples ($n = 55$) were submitted to a density gradient centrifugation and incubated in capacitating conditions in the absence or presence of increasing glucose concentrations (0, 5.5, 11 and 22 mM). Viability and total motility were assessed. Phosphotyrosine levels were measured. Mitochondrial activity and endogenous ROS production were evaluated. Oxidative stress-induced damage was analyzed. Culture media was collected and analyzed by ^1H -NMR. Our results show that glucose is essential for human sperm capacitation and motility. Notably, we observed that mitochondrial activity increased even in the absence of glucose. This increased mitochondrial activity was followed by a ROS overproduction, although no oxidative stress-induced damage was detected. Our results show that glucose is essential for capacitation, but mitochondrial activation is independent from its stimuli. ROS overproduction may take part on a finely regulated signaling pathway that modulates or even activates capacitation. Taken together, our results constitute a paradigm shift on human sperm capacitation physiology.

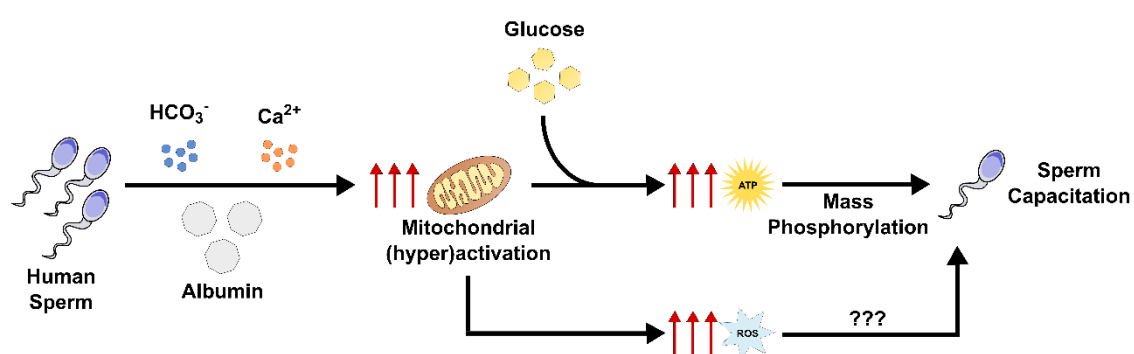


Fig. 4 – Graphical abstract. In this work, we provide evidence that glucose is essential for complete capacitation of human spermatozoa, although its stimuli seem to be dissociated from mitochondrial (hyper)activation. We were able to demonstrate that under capacitation conditions, the mitochondria are active and producing ROS even in the absence of glucose or any other energy substrates. We propose that human sperm capacitation occurs as a two-step process — on the first step, mitochondria become active and produce ROS as signaling intermediates; as a second step, glucose exposure leads to increased mitochondrial respiration, sustaining the high ATP demands necessary for mass protein phosphorylation and hyperactivated motility.

4.1. Introduction

Capacitation is a complex and finely regulated process of structural and functional alterations that occur in mammalian spermatozoa essential for oocyte fertilization. Sperm capacitation occurs naturally in the female reproductive tract and involves membrane modifications, several proteins phosphorylation and activation of signaling cascades. During capacitation, sperm acquires the ability to undergo acrosome reaction and achieves a state of hyperactivation, characterized by an increased motility with asymmetric beat and increased flagellar bend amplitude that improves sperm swimming ability and aids zona pellucida of the oocyte penetration (Puga Molina et al., 2018). A successful capacitation requires a specific ionic environment, a fine regulation of signaling cascades and energy management in order to support the biochemical alterations leading to the hyperactivation state (Bernardino et al., 2019; Ferramosca & Zara, 2014). The conditions for sperm capacitation were firstly described in 1951 and its discovery allowed the development of *in vitro* fertilization (IVF) (Austin, 1951; Chang, 1951; Steptoe & Edwards, 1978). In the female reproductive tract, human spermatozoa are exposed to a high concentration of HCO_3^- , albumin, Ca^{2+} , and an alkaline pH, which are essential for phosphorylation cascade activation and hyperactivated motility (Vyklícká & Lishko, 2020). On an energetic perspective, glucose is considered the preferential energy substrate for human sperm capacitation and hyperactivation (Williams & Ford, 2001). Sperm hyperactivation and subsequent increased motility have high ATP demands, whereas intracellular ionic balance maintenance also requires active transport and, consequently, increased ATP production for its sustainability (Bernardino et al., 2019; Kirichok & Lishko, 2011).

In human spermatozoa, glycolysis and mitochondrial oxidative phosphorylation are the two major metabolic pathways that are activated for ATP generation from glucose (Goodson et al., 2012). Human sperm are very specialized and unique cells, exhibiting a unique metabolism regulated by specific enzyme isoforms (Piomboni et al., 2012). For instance, glycolysis and mitochondrial respiration occur in distinct subcellular locations. Mitochondria are localized exclusively in the sperm midpiece, whereas glycolytic enzymes predominate in the flagellum (Ferramosca & Zara, 2014). However, there is no consensus concerning which one is the main ATP source in human spermatozoa. While some authors proposed glycolysis to be the exclusive ATP source (Nascimento et al., 2008), others defended that aerobic ATP production by sperm mitochondria to be essential for supporting human sperm capacitation and hyperactivated motility (Ferramosca et al., 2012; Gallon et al., 2006; Marchetti et al., 2002). In support of the latter, some studies reported an increase in mitochondrial respiration during capacitation, which was positively associated with hyperactivated motility (Hicks et al., 1972; Stendardi et al., 2011). Nevertheless, mitochondria are also a

source of reactive oxygen species (ROS), which at low concentrations play physiological roles (Murphy, 2009). On the other hand, high ROS concentration leads to protein and lipid damage (Ferramosca et al., 2013; Rivlin et al., 2004). Taken together, the physiology and bioenergetics of human sperm capacitation are still poorly understood. The interaction between glucose stimuli as energy source and mitochondria functioning, as well as the role of ROS remain to be clarified.

Herein, our aim was to study the effects of glucose on human sperm capacitation. For this purpose, human spermatozoa were incubated *in vitro* under capacitation conditions with increasing glucose concentrations (5.5, 11 and 22 mM) or without this energetic substrate. The effects of increasing glucose concentrations on human sperm viability and motility, protein phosphorylation levels and metabolism, were then characterized. As capacitation is associated with increased mitochondrial activity, we have also analyzed mitochondria membrane potential, ROS production and biomarkers for oxidative stress-induced damage.

4.2. Materials and methods

4.2.1. Reagents and chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

4.2.2. Ethical approval

Human seminal samples were obtained from men seeking infertility treatment at the Centre for Reproductive Genetics A. Barros (Porto, Portugal). Patients' selection, clinical study and diagnostic semen analysis were performed at the clinic. All procedures were conducted in accordance with the ethical guidelines for human samples research and informed and written consent was obtained from all individual donors included in the study. This study did not involve experiments on humans or animals, as only donated samples of surplus fresh ejaculated semen samples collected for diagnostic purposes were used. The approval of the Ethics Committee and the Declaration of Helsinki, revised in Tokyo 2004, on human experimentation does not apply to this work. The Centre for Reproductive Genetics A. Barros infertility clinic's procedure are under the provisions of the National Medically Assisted Procreation Act (Law of 2017) and overseen by the National Council for Medically Assisted Procreation (CNPMA-2018). According to these rules and guidelines, the use of clinical databases and patient biological material for diagnosis and research may be used without

further ethical approval, under strict individual anonymity and after patient written informed consent. Regarding the use of semen samples for laboratory experimentation at ICBAS-UP, the Ethics Committee authorization number is Project: 2019/CE/P017 (266/CETI/ICBAS).

4.2.3. Human sperm samples

Normozoospermic subjects were recruited among couples seeking infertility treatment at the medically assisted procreation clinic. Samples from healthy adult male donors (mean age of 36 ± 5 years old) were obtained by masturbation into specific sterile containers 3 days after sexual abstinence for a diagnostic semen analysis. Fresh ejaculated semen samples were left at 37°C until complete liquefaction, then analyzed according to the guidelines of the World Health Organization (WHO, 2010). Only samples of normozoospermic subjects ($n = 55$) were selected for this study. A standard discontinuous density gradient centrifugation was performed on the selected 55 samples using the Gems[®] Sperm Wash Gradient Set (45% and 90%) (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. Solutions were stabilized at room temperature before the gradient was constituted. All procedures were performed at room temperature. Only a maximum volume of 1.5 mL of seminal sample was placed on each gradient. Samples with higher volumes were separated in different gradients. The resultant pellet, containing the spermatozoa fraction with higher percentage of motility and viability (Jeyendran et al., 2019; Pinto et al., 2021), was collected and washed 2 times in sterile PBS followed by a centrifugation of $500 \times g$ for 5 min.

4.2.4. Experimental groups

For the purpose of studying the effects of increasing glucose concentrations on human sperm capacitation, retrieved spermatozoa from each sample isolated through density gradient centrifugation were equally separated into four groups. As a low concentration of spermatozoa is retrieved, not all samples were used for all experiments. Therefore, samples were randomly distributed among the experiments. A maximum of 15 million spermatozoa were incubated in a 5% CO_2 atmosphere at 37°C in 1 mL of a modified Biggers, Whitter and Whittingham (BWW) medium for the stimulation of sperm capacitation (in mM: 94.5 NaCl, 4.8 KCl, 1.7 CaCl_2 , 1.17 KH_2PO_4 , 1.22 MgSO_4 , 20 HEPES, 24 NaHCO_3 , 0.4 BSA, pH = 7.4) supplemented with increasing glucose concentrations (5.5, 11 and 22 mM). To study the

effects of glucose absence, a group was incubated in BWW medium only. After a 6 h incubation, samples were centrifuged and media was collected for ^1H -NMR analysis. Spermatozoa were then washed 2 times in PBS and a fraction of treated spermatozoa was collected either for viability, total motility, mitochondrial membrane potential, or endogenous ROS content analysis. The remaining spermatozoa were centrifuged and the pellet collected for protein analysis. A spermatozoa fraction obtained following the gradient density centrifugation was collected and used as reference group ($T = 0$ h). Results concerning the reference group were obtained 90 min after obtaining the samples.

4.2.5. Sperm viability and total motility analysis

Sperm membrane integrity was analyzed using the eosin/nigrosin staining protocol as viability indicator ($n = 15$ for each condition), as previously described (Rato et al., 2013). A total of 5 μL of spermatozoa suspensions obtained before and after treatments were mixed with 5 μL of 0.5% eosin-nigrosin stain and smeared on a glass microscope slide. The percentage of viable and nonviable spermatozoa was determined by counting a total of 200 spermatozoa per slide in continuous random fields under a light microscope (1000 $\times$ magnification). White spermatozoa were considered viable whereas pink-stained spermatozoa, indicating loss of membrane integrity, were considered nonviable.

Sperm total motility ($n = 20$ for each condition) was assessed using a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), according to the manufacturer's recommendations. A drop of spermatozoa suspensions (10 μL) was placed in the center of the chamber and carefully covered to ensure an evenly sperm distribution. The experiment was performed by the same user and the same criteria was applied between samples. Sperm motility was then assessed in 20 squares using an optical microscope (200 $\times$ magnification). New drops were placed on the chamber and the process was repeated until a total of 100 sperm cells per sample were counted. Results are expressed in percentage of motile spermatozoa.

4.2.6. Proton nuclear magnetic resonance (^1H -NMR)

^1H -NMR spectra were acquired using a 600 MHz Varian spectrometer (Varian Inc., Palo Alto, CA, USA) equipped with a 3-mm indirect detection probe, as previously described (Moreira et al., 2020). A total of 180 μL of medium of each condition ($n = 15$) was analyzed. Samples were diluted with a sodium fumarate and deuterated water solution (final concentration of 2 mM in 225 μL), which was used as internal reference (6.50 ppm) to quantify the

following metabolites present in the medium (multiplet, ppm): lactate (doublet, 1.33), acetate (singlet, 1.90), and malate (double doublet, 2.37). Spectra were manually phased and baseline corrected. Chosen metabolites peaks were integrated using the NUTS-Pro NMR software (Acorn NMR, Inc, Fremont, CA). Results are expressed as nmol/10⁶ spermatozoa.

4.2.7. Sperm capacitation analysis

Human spermatozoa lysates were prepared from the resulting pellets. Pellets were resuspended in RIPA buffer and incubated for 15 min at 4°C. The samples were then centrifuged (14000× g for 20 min) and total protein concentration was quantified using a BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

The phosphorylation state of spermatozoa proteins was used as a biomarker to evaluate sperm capacitation (Calle-Guisado et al., 2019). Protein samples were diluted to a final concentration of 50 ng/μL and transferred to a PVDF membrane using a slot blot technique in a Hybri-slot manifold system (Biometra, Göttingen, Germany). A Ponceau S staining solution (MB19201, NZYTech, Lisboa, Portugal) was used for total protein normalization. The resulting membranes (n = 6 for each condition) were incubated overnight at 4°C with a mouse anti-phosphotyrosine monoclonal antibody (1:1000, Clone 4G10, #05-321, Merck Millipore, Temecula, USA). Membranes were then incubated with a goat anti-mouse IgG antibody (1:5000, AP308P, Sigma-Aldrich, St. Louis, MO, USA). Blots were visualized with Clarity™ Western ECL Substrate (Bio-Rad, California, USA) and read using a Bio-Rad ChemiDoc XR system (Bio-Rad, California, USA). Densities from each band were quantified using Image Lab Software (Bio-Rad, California, USA).

4.2.8. JC-1 assay for mitochondrial membrane potential

The mitochondrial membrane potential of spermatozoa was evaluated using the fluorogenic dye JC-1 (T3168, Invitrogen™, California, USA). In brief, 10⁶ spermatozoa were collected before and after treatments and incubated in a JC-1 solution (1 μg/mL in PBS) at 37°C for 30 min. Spermatozoa incubated in a 20% DMSO solution were used as a positive control. Then, cells were centrifuged at 500× g for 5 min and washed 2 times with PBS. Sperm cells were then transferred into a 96-well plate and monomers (485/530 nm; excitation/emission) and J-aggregates (535/590 nm; excitation/emission) fluorescence read on a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, Vermont, USA). The ratio between JC-1 J-aggregates/monomers was calculated (n = 15 for each condition) and used as an indicator

of mitochondrial membrane polarization, where an increased/decreased ratio was considered indicative of hyperpolarization/depolarization, respectively (Dias et al., 2019). Results are expressed in fold variation to the reference group (T = 0 h).

JC-1 J-aggregates/monomers fluorescence was also observed in a Nikon Eclipse E400 microscope equipped with a DS-Fi3 microscope camera, a Y-FL Epi-Fluorescence Attachment and HB-10103AF Super High Pressure Mercury Lamp Power Supply (Nikon, Shinagawa, Tokyo, Japan). Nuclei were stained with Hoechst 33342 (H3570, Thermo Scientific, Waltham, MA, USA). Pictures were captured with a 1000× magnification and a DAPI filter (395/425 nm; excitation/emission) was used for observing the nuclei, FITC filter (480/510 nm; excitation/emission) for JC-1 monomer, and G-2A filter (510/575 nm; excitation/emission) for JC-1 J-aggregates. All pictures were captured with the same exposition time and gain. The obtained pictures were processed using the NIS-Elements Br 4.60.00 software (Nikon, Shinagawa, Tokyo, Japan).

4.2.9. Detection of intracellular reactive oxygen species (ROS)

Total ROS produced by spermatozoa were evaluated with the fluorogenic dye CM-H₂DCFDA (C6827, Invitrogen™, California, USA), according to the manufacturer's instructions. CM-H₂DCFDA is a chloromethyl derivative of H₂DCFDA, whose oxidation originates a fluorescent adduct that can be used as an indicator for intracellular ROS in cells. Briefly, 10⁵ spermatozoa were collected before and after glucose treatments and incubated in a CM-H₂DCFDA solution (5 μM in PBS) at 37°C for 30 min. Spermatozoa incubated in a 0.1% H₂O₂ solution was used as a positive control. Sperm cells were then transferred into a 96-well plate and fluorescence (495/529 nm; excitation/emission) read on a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, Vermont, USA). Results are expressed in fold variation to the reference group (T = 0 h).

4.2.10. Evaluation of oxidative stress-related damage

The potential oxidative stress-related damage was evaluated on the spermatozoa through the analysis of protein oxidation (n = 15 for each condition), lipid peroxidation (n = 15 for each condition), and protein nitration (n = 8 for each condition) by the slot blot technique and using specific antibodies, according to methods previously described (Carrageta et al., 2018). Briefly, for the analysis of protein oxidation the content of carbonyl groups was assessed. Protein samples of 5 μg were derivatized using 2,4-dinitrophenylhydrazine (DNPH) to obtain 2,4-dinitrophenyl (DNP) and then diluted to a final concentration of 1 ng/μL. For

lipid peroxidation and protein nitration analysis, protein samples were diluted to a concentration of 50 ng/ μ L. The slot blot technique was performed using a Hybri-slot manifold system (Biometra, Göttingen, Germany). A Ponceau S staining solution (MB19201, NZYTech, Lisboa, Portugal) was used for total protein normalization. The resulting PVDF membranes were incubated overnight at 4°C with a rabbit anti-DNP antibody (1:5000, D9656, Sigma-Aldrich, St. Louis, MO, USA), goat anti-4-hydroxynonenal antibody (1:5000, AB5605, Merck Millipore, Temecula, USA), and rabbit anti-nitro-tyrosine antibody (1:1000, 9691S, Cell Signaling Technology, Netherlands), respectively. Membranes were then incubated with a rabbit anti-goat IgG antibody (1:5000, AP107P, Sigma-Aldrich, St. Louis, MO, USA) or goat anti-rabbit IgG antibody (1:5000, AP307P, Sigma-Aldrich, St. Louis, MO, USA). Blots were visualized with Clarity™ Western ECL Substrate (Bio-Rad, California, USA) or Vistra ECF™ Substrate (RPN5785, GE Healthcare, Illinois, USA) and read using a Bio-Rad ChemiDoc XR system (Bio-Rad, California, USA). Densities from each band were quantified using Image Lab Software (Bio-Rad, California, USA). Results are expressed in fold variation to the reference group (T = 0 h).

4.2.11. Statistical analysis

Experimental results are presented as mean $\pm$ standard deviation (SD) or Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). Statistical analysis was performed in GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Data were tested for normal distribution with a Kolmogorov-Smirnov test. Statistical differences were determined by a repeated measures ANOVA or one-way ANOVA. $p < 0.05$ was considered significantly different.

4.3. Results

4.3.1. Glucose is essential for human sperm viability maintenance and capacitation

Following the 6 h incubation with increasing glucose concentrations (5.5, 11 and 22 mM) or absence of exogenous energy substrates in capacitating conditions, the percentage of viable spermatozoa were assessed through an eosin-nigrosin staining (Fig. 4.1A). We observed that in the absence of glucose (as well as any other exogenous energy substrate) the percentage of viable spermatozoa decreased ($76 \pm 15\%$) as compared with the T = 0 h group ($89 \pm 5\%$, $p = 0.003$). No differences were observed between glucose exposed groups when compared to T = 0 h or between glucose concentrations ($79 \pm 15\%$; $80 \pm 15\%$; $84 \pm 13\%$

after treatment with 5.5, 11 and 22 mM of glucose, respectively). Interestingly, spermatozoa treated with the highest glucose concentration (22 mM) presented a higher viability when compared with the group treated in the absence of glucose ($p = 0.046$).

Tyrosine residues phosphorylation is a common biomarker to evaluate human sperm capacitation. Tyrosine residues phosphorylation levels were determined through slot blot technique (Figure 4.1B and 4.1C). Glucose treatment increased human spermatozoa tyrosine residues phosphorylation at all concentrations (0.412 ± 0.295 arbitrary units; 0.434 ± 0.270 arbitrary units; 0.491 ± 0.329 arbitrary units, after treatment with 5.5, 11 and 22 mM of glucose, respectively) when compared to T = 0 h group (0.048 ± 0.074 arbitrary units; $p = 0.021$, $p = 0.016$, $p = 0.005$, respectively) and to no glucose group (0.035 ± 0.037 arbitrary units; $p = 0.017$, $p = 0.012$, $p = 0.004$, respectively). No differences were observed between the glucose groups or between the group with no glucose and the T = 0 h group.

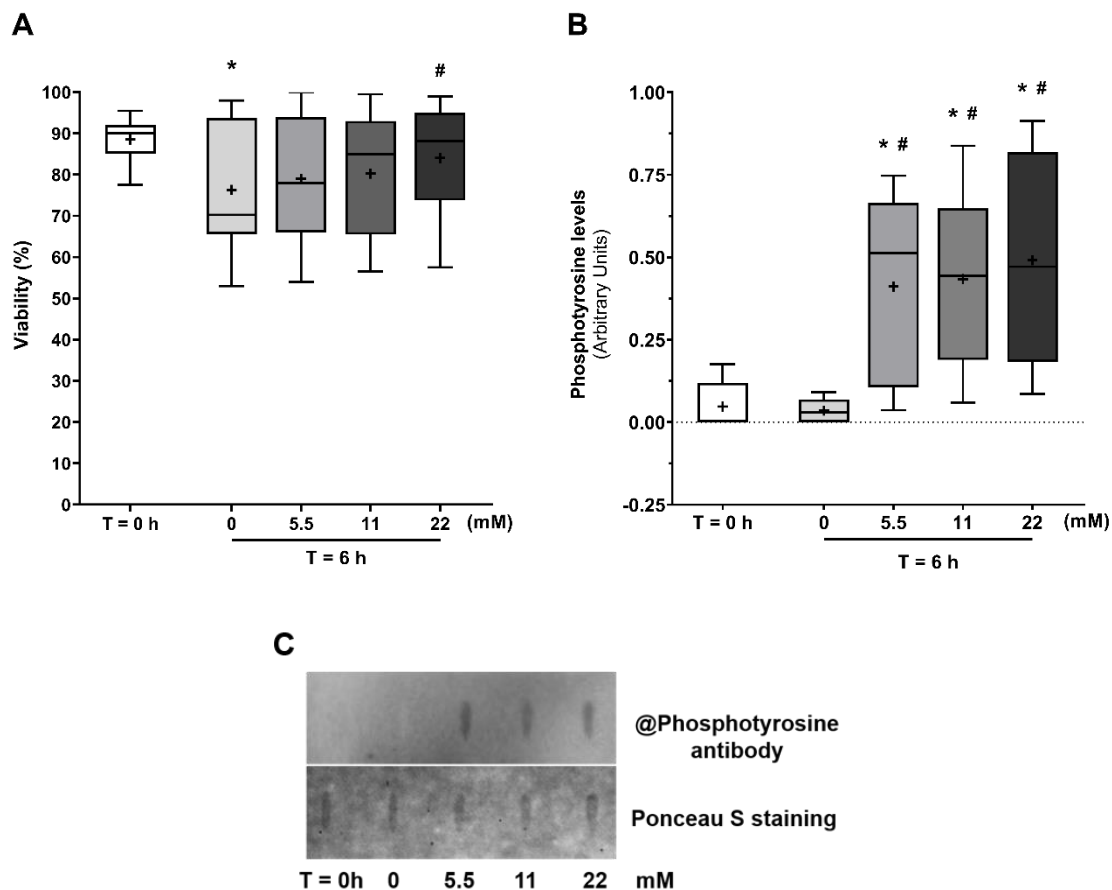


Fig. 4.1 – Evaluation of human spermatozoa viability as determined by eosin-nigrosin staining (A) and mean tyrosine residues phosphorylation levels (B) at baseline (T = 0 h) and without or with glucose (0, 5.5, 11 and 22 mM) stimuli for 6 h in capacitation conditions. (C) Representative slot blot membrane. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles ± 1.5 IQR; (A) $n = 15$ for each condition, (B) $n = 6$ for each condition). + – Represents group

average. Significantly different results are expressed as: * – Relative to T = 0 h group; # – Relative to 0 mM glucose group (T = 6 h).

4.3.2. Glucose exposure increases human sperm total motility

Human sperm capacitation is characterized by the increased conversion of energy substrates into mechanical energy, resulting in increased motility. In order to study the effects of increasing glucose concentrations (5.5, 11 and 22 mM) or absence of exogenous energy substrates on human spermatozoa motility during capacitation, we evaluated the percentage of motile spermatozoa using a Makler chamber (Fig. 4.2). We observed that the exposure to glucose increased the percentage of motile spermatozoa at all tested concentrations ($83 \pm 7\%$; $82 \pm 11\%$; $79 \pm 12\%$, after treatment with 5.5, 11 and 22 mM of glucose, respectively) when compared to T = 0 h group ($67 \pm 12\%$; $p < 0.001$, $p < 0.001$, $p = 0.024$, respectively) and to the no glucose group ($66 \pm 15\%$; $p = 0.004$, $p = 0.002$, $p = 0.017$, respectively). No differences were observed in the percentage of motile spermatozoa after treatment between all tested glucose concentrations. No differences were observed in the percentage of motile spermatozoa between those without glucose and the T = 0 h group.

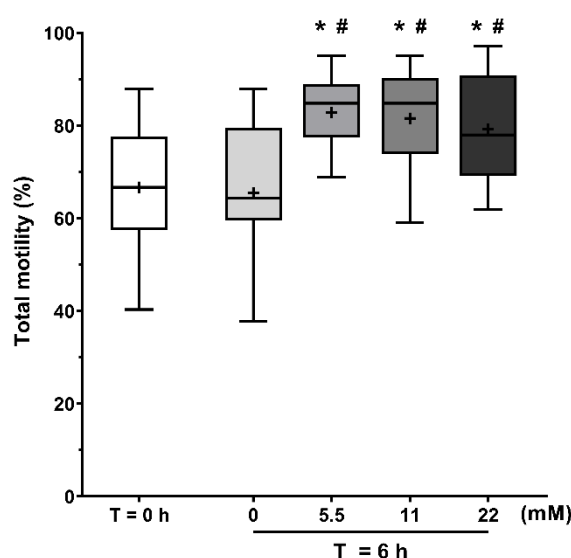


Fig. 4.2 – Evaluation of human spermatozoa total motility at baseline (T = 0 h) and without or with glucose (0, 5.5, 11 and 22 mM) stimuli following 6 h incubation in capacitation conditions. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR; $n = 20$ for each condition). + – Represents group average. Significantly different results are expressed as: * – Relative to T = 0 h group; # – Relative to 0 mM glucose group (T = 6 h).

4.3.3. The main metabolites produced during human sperm capacitation are lactate, acetate, and malate

We analyzed ^1H -NMR spectra of the media in order to evaluate the metabolic response of human spermatozoa to glucose stimuli and in the absence of exogenous energy substrates under capacitation conditions. Lactate (Figure 4.3A), acetate (Figure 4.3B), and malate (Figure 4.3C) were the main metabolites identified.

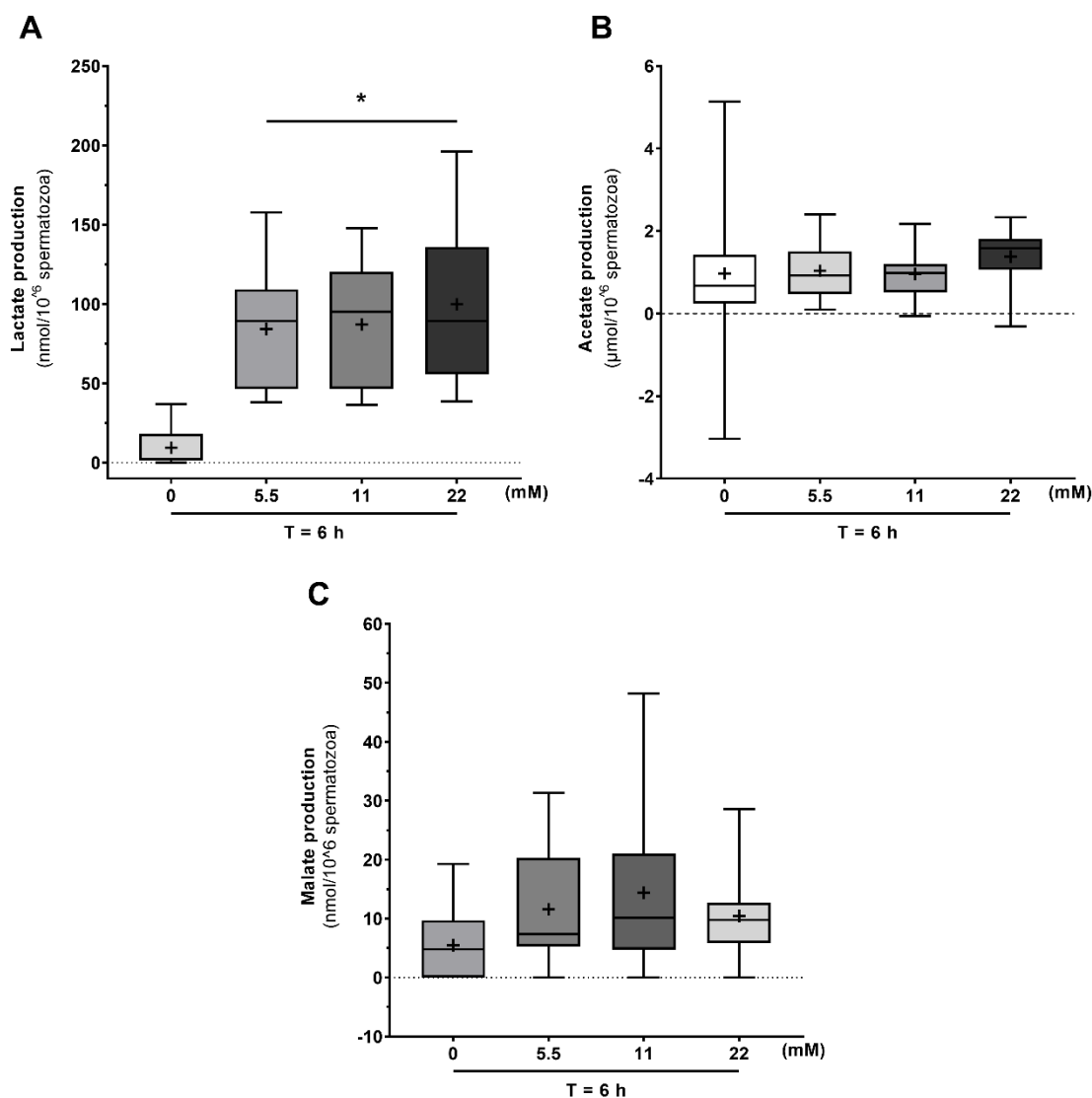


Fig. 4.3 – Production of lactate (A), acetate (B), and malate (C) by human spermatozoa 6 h after incubation without or with glucose (0, 5.5, 11, and 22 mM) under capacitation conditions. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR; $n = 15$ for each condition). + – Represents group average. Significantly different results are expressed as: * – Relative to 0 mM glucose group (T = 6 h).

All glucose concentrations led to spermatozoa lactate production (84.3 ± 35.8 nmol/ 10^6 spermatozoa; 87.2 ± 36.7 nmol/ 10^6 spermatozoa; 48.4 ± 13.4 nmol/ 10^6 spermatozoa, after treatment with 5.5, 11 and 22 mM of glucose, respectively). Interestingly, human spermatozoa produced lactate even in the absence of glucose or other exogenous energetic substrates, though at a lower concentration (9.5 ± 13.1 nmol/ 10^6 spermatozoa; $p < 0.001$). No differences were observed concerning acetate production (1.0 ± 2.0 nmol/ 10^6 spermatozoa; 1.0 ± 0.7 nmol/ 10^6 spermatozoa; 1.0 ± 0.6 nmol/ 10^6 spermatozoa; 1.4 ± 0.7 nmol/ 10^6 spermatozoa after treatment with 0, 5.5, 11 or 22 mM of glucose, respectively) or malate (5.5 ± 5.7 nmol/ 10^6 spermatozoa; 11.6 ± 10.6 nmol/ 10^6 spermatozoa; 14.4 ± 14.9 nmol/ 10^6 spermatozoa; 10.4 ± 7.5 nmol/ 10^6 spermatozoa after treatment with 0, 5.5, 11 or 22 mM of glucose, respectively) among studied groups.

4.3.4. Mitochondrial activation during human sperm capacitation does not requires glucose presence

The increased motility observed during human sperm capacitation is reported to be supported by increased mitochondrial activity. In order to study the effects of increasing glucose concentrations (5.5, 11 and 22 mM) or absence of glucose or other exogenous energy substrates on human spermatozoa mitochondrial activity during capacitation, we used the JC-1 dye. JC-1 J-aggregates/monomers ratio was used as an indicator of mitochondrial membrane polarization, where an increased/decreased ratio was considered indicative of hyperpolarization/depolarization, respectively. The fluorescence of JC-1 J-aggregates or monomers was firstly observed and captured on a Nikon Eclipse E400 microscope at a 1000 $\times$ magnification (Fig. 4.4). Herein, we observed that all tested conditions exhibited a more intense fluorescence of J-aggregates (red) in comparison to monomers (green). To confirm this observation, the ratio of JC-1 J-aggregates/monomers was measured using a microplate reader (Fig. 4.5A). We observed an increased JC-1 ratio on all tested glucose concentrations (1.52 ± 0.48 ; 1.57 ± 0.59 ; 1.51 ± 0.48 after treatment with 5.5, 11 or 22 mM of glucose, respectively) as compared to the T = 0 h group (1.00 ± 0.41 ; $p < 0.001$), which indicates mitochondrial hyperpolarization. No differences were observed in mitochondrial membrane potential among all tested glucose groups. We also observed an increased JC-1 ratio on the group treated in the absence of glucose or any other exogenous energetic substrates (1.74 ± 0.51) as compared to the T = 0 h group ($p < 0.001$).

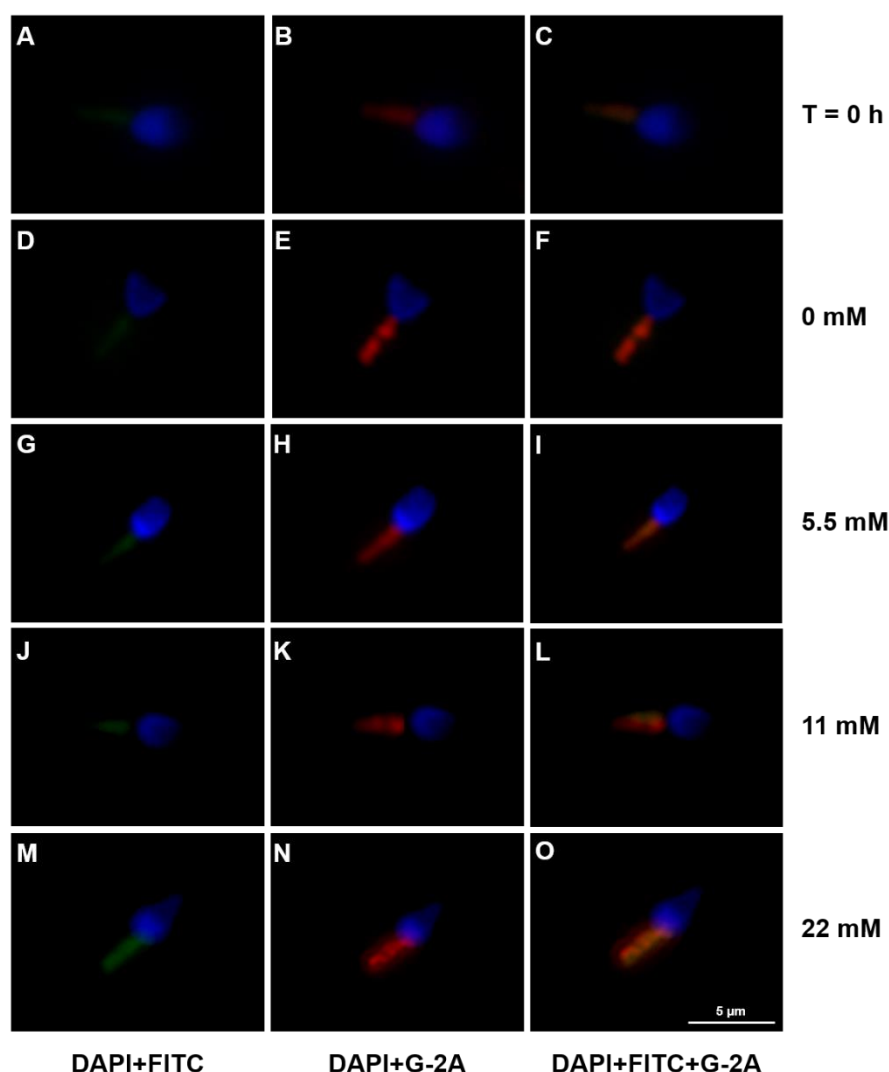


Fig. 4.4 – Analysis of JC-1 J-aggregates (red) or monomers (green) in human spermatozoa at baseline (T = 0 h) and without or with glucose (0, 5.5, 11, and 22 mM) stimuli following 6 h in capacitation conditions, by fluorescence microscopy. In (A,D,G,J,M) are represented JC-1 monomers (green, FITC filter) and nuclei (DAPI filter) for T = 0 h group and glucose concentrations (0, 5.5, 11 and 22 mM), respectively. In (B,E,H,K,N) are represented JC-1 J-aggregates (red, G-2A filter) and nuclei (DAPI filter) for T = 0 h group and glucose concentrations (0, 5.5, 11 and 22 mM), respectively. In (C,F,I,L,O) are represented the overlap between JC-1 J-aggregates and monomers for T = 0 h group and glucose concentrations (0, 5.5, 11 and 22 mM), respectively.

4.3.5. Human sperm capacitation leads to increased endogenous ROS production without inducing oxidative stress-related damage

Since increased mitochondrial activity was observed, we sought to evaluate the endogenous ROS production using the fluorogenic dye CM-H₂DCFDA (Fig. 4.5B). Increased endogenous ROS production was observed on all tested glucose concentrations (1.49 ± 0.61 ;

1.52 ± 0.72 ; 1.52 ± 0.65 after treatment with 5.5, 11 or 22 mM of glucose, respectively) as compared to the T = 0 h group (1.00 ± 0.48 ; $p < 0.001$). No differences in spermatozoa endogenous ROS production after treatment with all tested glucose concentrations were observed. Nevertheless, an increase in endogenous ROS production was detected on spermatozoa treated in the absence of glucose or other exogenous energy substrates (1.61 ± 0.70) as compared to the T = 0 h group ($p < 0.001$).

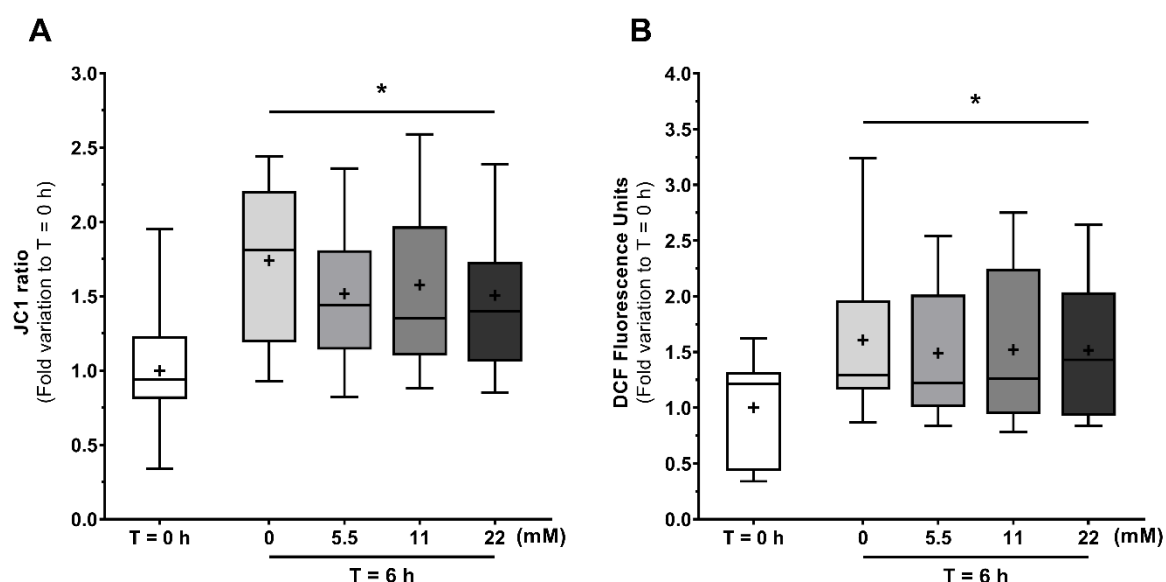


Fig. 4.5 – Evaluation of human spermatozoa mitochondrial membrane polarization and endogenous ROS production at baseline (T = 0 h) and without or with glucose (0, 5.5, 11 and 22 mM) stimuli following 6 h in capacitation conditions. (A) Evaluation of mitochondrial membrane polarization using the dye JC-1. **(B)** Evaluation of endogenous ROS production using the fluorogenic dye CM-H₂DCFDA. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR; n = 15 for each condition). + – Represents group average. Significantly different results are expressed as: * – Relative to 0 mM glucose group (T = 6 h).

Increased ROS production can be associated with oxidative stress-induced damage. Thus, we evaluated the levels of lipid peroxidation (Figure 4.6A), protein oxidation through determination of protein carbonyl groups content (Figure 4.6B) and protein nitration (Figure 4.6C) in human spermatozoa by slot blot technique. Although increased endogenous ROS production was observed, no alterations were detected in spermatozoa lipid peroxidation (0.91 ± 0.62 ; 0.76 ± 0.63 ; 1.12 ± 0.87 and 1.25 ± 0.76 after treatment with 0, 5.5, 11 or 22 mM of glucose, respectively), protein oxidation (1.09 ± 0.86 ; 0.83 ± 0.48 ; 0.77 ± 0.44 ; 0.83 ± 0.48 after treatment with 0, 5.5, 11 or 22 mM of glucose respectively), or protein nitration (0.65 ± 0.22 ; 0.88 ± 0.40 ; 1.43 ± 1.54 ; 1.02 ± 0.87 after treatment with 0, 5.5, 11 or 22 mM

of glucose, respectively) as compared with the levels detected in spermatozoa of the T = 0 h group (1.00 ± 0.60 , 1.00 ± 0.39 , 1.00 ± 0.26 , respectively).

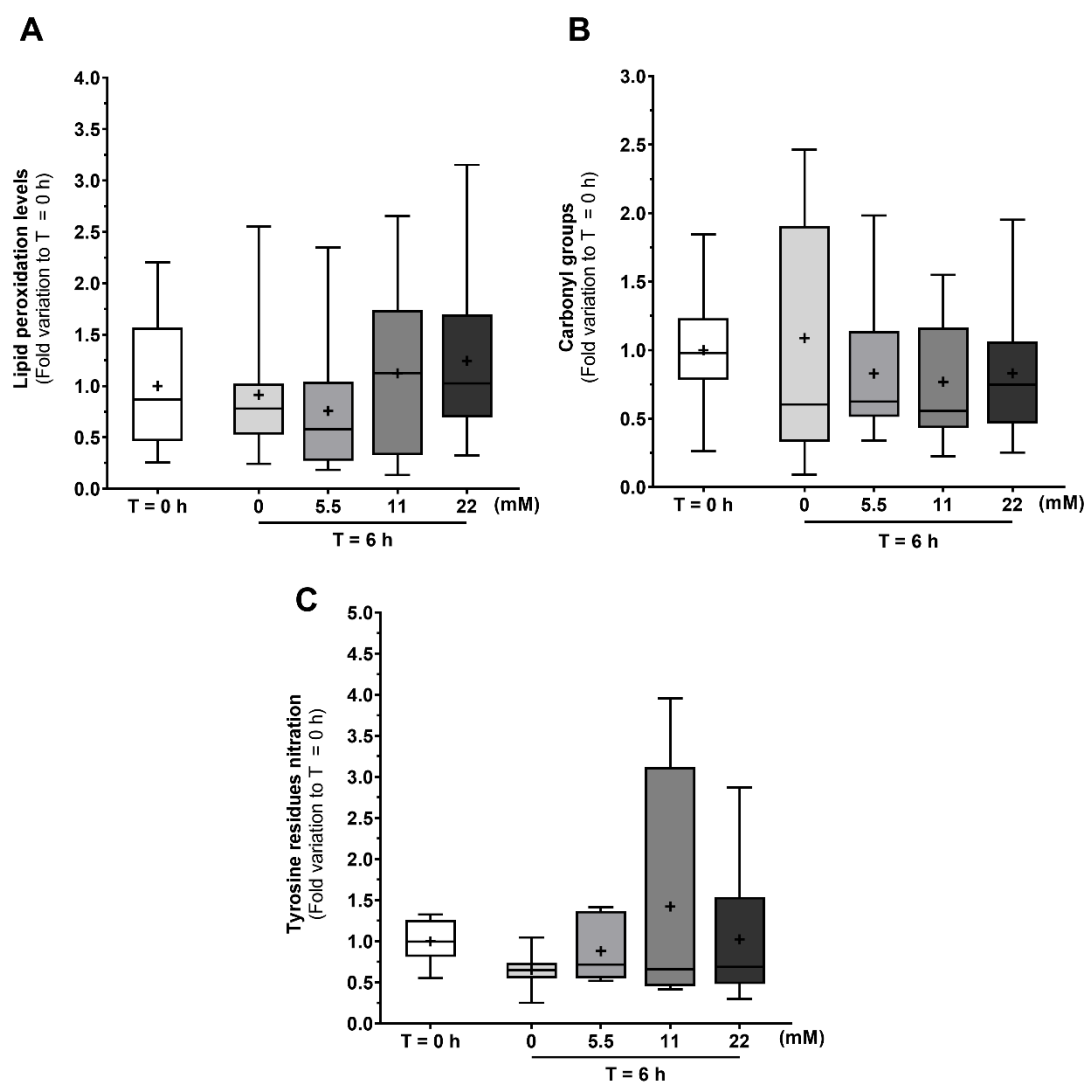


Fig. 4.6 – Oxidative stress-induced damage in human spermatozoa at baseline (T = 0 h) and after 6 h incubation without or with glucose (0, 5.5, 11, and 22 mM) under capacitation conditions on lipid peroxidation (A), carbonyl group formation (B), and tyrosine residues nitration (C) in human spermatozoa. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR; **(A,B)** n = 15 for each condition, **(C)** n = 8 for each condition). + – Represents group average. Significantly different results are expressed as: * – Relative to 0 mM glucose group (T = 6 h).

4.4. Discussion

Human sperm capacitation is a complex step necessary for a successful oocyte fertilization. Capacitation is characterized by numerous functional alterations in spermatozoa. Those are

mostly characterized by mass phosphorylation of proteins involved in signal transduction and increased oxidation of energetic substrates. In order to achieve this state, it is reported that spermatozoa require glucose as an energetic substrate (Williams & Ford, 2001). Glucose is regarded as the essential hexose for human spermatozoa optimal capacitation and fertilization, supporting their hyperactivated motility (Williams & Ford, 2001). For instance, fructose is present at higher concentrations in the human seminal fluid (mean concentration of 13.88 mM (Buckett & Lewis-Jones, 2002)) but does not lead to a complete sperm capacitation (Fraser & Quinn, 1981; Hoppe, 1976). Although human seminal fluid contains low glucose amounts (mean concentration of 0.41 mM (Povoa et al., 1986)), in the female reproductive tract sperm cells are exposed to higher glucose concentrations. The human cervical mucus presents a mean 5.2 mM of glucose, which may peak 8.25-11 mM during ovulation (Ehrstrom et al., 2006; Weed & Carrera, 1970). Together with a high concentration of HCO_3^- , albumin and Ca^{2+} , human sperm capacitation is then promoted (Puga Molina et al., 2018).

In this work, we studied the effects of increasing glucose concentrations or the absence of glucose or any other energetic substrate, on human sperm capacitation. For this purpose, human spermatozoa were incubated under *in vitro* capacitation conditions for 6 h without glucose or with increasing glucose concentrations (0, 5.5, 11 and 22 mM) in the absence of any other energy substrate. We observed that exposure to glucose maintained human spermatozoa viability over time after treatment with all studied glucose concentrations, whereas the group without glucose had a reduced percentage of viable spermatozoa. Thus, our results support the literature data highlighting glucose as essential for human spermatozoa viability maintenance over time (Amaral et al., 2011). In addition, we observed that elevated tyrosine residues phosphorylation levels was only present in groups exposed to glucose, which is a common biomarker of sperm capacitation (Naz & Rajesh, 2004). These results also support the literature data that suggests glucose to be as essential to promote human sperm capacitation (Ferramosca & Zara, 2014). Thus, our data corroborates previous studies findings that glucose is pivotal for human spermatozoa viability and capacitation. Notably, no differences were observed between the several glucose concentrations, which lead us to conclude that glucose stimuli is necessary to trigger human spermatozoa capacitation but this phenomenon is not glucose concentration-dependent.

As a direct consequence of sperm capacitation, there is an increased conversion of energy substrates into mechanical energy, resulting in sperm hyperactivation and increased motility. Hyperactivation is essential for human spermatozoa to swim through the endometrium and oviductal mucus, while is also necessary for oocyte zona pellucida penetration upon acrosome reaction (Vyklícká & Lishko, 2020). We analysed total motility and observed

that the percentage of motile spermatozoa increased in groups exposed to glucose in comparison to the reference group (baseline) or to the no glucose group. These results corroborate the previous results on capacitation, rendering glucose as necessary to initiate the process leading to increased sperm motility. Although we observed an increased motility, we were not able to identify hyperactivated sperm cells with this method. Analysis of hyperactivated human spermatozoa and their motility with a Computer Assisted Sperm Analyzer (CASA) system will be addressed in future research. Interestingly, the absence of energy substrates during capacitation did not decrease spermatozoa motility after 6 h, which is reported to occur when spermatozoa are not incubated under capacitation conditions (Valsa et al., 2019). Thus, our results may suggest the likely presence of other endogenous energy source able to sustain human spermatozoa motility and whose metabolism could be activated through the ionic environment conferred by the capacitation medium. Notably, our results also suggest that high glucose concentrations, such as two times the peak concentrations found in the female reproductive tract, are not detrimental for human spermatozoa. This is in agreement with studies suggesting that human spermatozoa are highly resistant to glucose toxicity, supporting concentrations up to 50 mM without noticeable adverse effects on sperm parameters (Portela et al., 2015). Nonetheless, the mechanisms by which human spermatozoa are able to support such high glucose concentrations without inducing toxic effects remains to be explored.

Both sperm capacitation and hyperactivation require increased energy substrate oxidation for maintenance. Herein, we analysed human spermatozoa metabolism through quantification of metabolites in spermatozoa media by ^1H -NMR. We were able to identify and quantify the production of lactate, acetate, and malate by spermatozoa. Lactate was the main metabolite produced by human spermatozoa and it was produced in higher amounts after glucose exposure, though there were no differences across glucose concentrations. Lactate was previously reported as the main metabolite produced by human spermatozoa (Calvert et al., 2019). In addition, human spermatozoa are able to further metabolize lactate into pyruvate and acetate (Calvert et al., 2019; Reynolds et al., 2017), which is corroborated by our results that show spermatozoa acetate production after glucose treatment. We were also able to identify and quantify malate in the media. Malate is a citric acid intermediate that also takes part on the malate-aspartate shuttle. Malate is known to increase mitochondrial uptake of carboxylic substrates, leading to an increased mitochondrial activity (Bobyleva-Guarriero & Lardy, 1986). Interestingly, the addition of malate to the capacitation media is reported to improve lactate and pyruvate oxidation, while also being required for maximal oxygen uptake (Ferramosca et al., 2008; Gerez de Burgos et al., 1994; Storey & Kayne, 1978). Although the reason for human spermatozoa to secrete malate during capacitation remains to be elucidated, we hypothesize that its secretion and uptake may be

necessary for optimal mitochondrial activity of the surrounding spermatozoa. We also observed that human spermatozoa were able to produce lactate, acetate, and malate even in the absence of glucose or any other energy substrate. It is possible that some energy substrates from the seminal plasma could still be present in the samples even after the washing procedure. Conversely, these results could also further suggest the hypothesis that human spermatozoa are likely to rely on other endogenous energy sources to sustain motility over time. Taken together, our results support the current hypothesis that human spermatozoa metabolism is highly dynamic and dependent on the energy substrate available (Piomboni et al., 2012).

Sperm bioenergetics and the main contributor for ATP production are still under debate. Both glycolysis and mitochondrial respiration are activated and contribute for spermatozoa capacitation and motility (Ruiz-Pesini et al., 2007). Still, some authors defend glycolysis to be the main ATP source in human spermatozoa (Nascimento et al., 2008), while others defend that mitochondrial respiration plays a major contribution for ATP production (Ferramosca et al., 2012; Gallon et al., 2006; Marchetti et al., 2002). Human sperm mitochondria are dynamic organelles that exhibit some peculiarities. For instance, mitochondria suffer morphological changes during capacitation. Vorup-Jensen et al. reported that under capacitation conditions, mitochondria of human spermatozoa increase their volume and change towards a more loosely wrapped morphology (Vorup-Jensen et al., 1999). Additionally, several studies reported that mitochondrial respiration highly increases during capacitation (Boell, 1985; Fraser & Lane, 1987). Based on these data, some authors hypothesized that the increase in mitochondrial respiration is associated with the presence of oxidizable substrates in the capacitation medium. In this work, we observed an increase in JC-1 J-aggregates/monomers ratio when human spermatozoa were exposed to glucose in capacitation conditions, which indicates mitochondria membrane hyperpolarization. These results are supported by data in the literature, reporting that *in vitro* human spermatozoa capacitation led to a peak in O₂ consumption and increased mitochondrial respiration capacity (Stendardi et al., 2011). In the same study, Stendardi et al. also reported that the high mitochondrial respiratory efficiency remained stable after capacitation, suggesting that mitochondria activity supported hyperactivated motility. However, we also observed an increase JC-1 J-aggregates/monomers ratio after 6 h incubation without glucose or any other energy substrate in the capacitation medium, which still indicates mitochondria membrane hyperpolarization. As the best of our knowledge, this is the first time that human sperm mitochondria activation is described to occur in capacitation medium even in the absence of glucose or any other known exogenous energy substrate. These results suggest that mitochondria activation during human sperm capacitation is dissociated from exogenous glucose stimuli. Thus, we hypothesize that human sperm capacitation could occur as a two-step process.

The classic description of sperm capacitation highlights that a high concentration of HCO_3^- stimulates the activity of adenylyl cyclase, which cause an increase in cAMP. Then, cAMP activates protein kinase A (PKA) and triggers spermatozoa capacitation, in the presence of high amounts of ATP produced through glucose oxidation (Liu et al., 2012). However, our results provide evidence that spermatozoa exhibit mitochondria activation in a way independent of glucose stimuli. Thus, we hypothesize that mitochondrial activation is the first step to occur during human sperm capacitation, which is mostly supported by the ionic environment established. Glucose would only be necessary for a second step, where high ATP production through glycolysis and mitochondrial respiration occurs and there is a subsequent mass phosphorylation of proteins needed for spermatozoa capacitation and hyper-activated motility. In support to our hypothesis, we also observed increased endogenous ROS production in all tested conditions, even in the absence of glucose. These results provide further evidence for an increased mitochondrial activity independently of glucose or any other energy substrate. Moreover, our results shed some light on possible sperm mitochondria functions other than ATP production through oxidative phosphorylation. Mitochondria are a known source of ROS, being reported to convert 0.2-2% of the oxygen uptake into ROS (Harper et al., 2004; Murphy, 2009). In small concentrations, ROS are hypothesized to play a physiological role and intervene as regulators in multiple signaling pathways (Dias et al., 2020; Sena & Chandel, 2012). On the other hand, increased ROS production may lead to oxidative stress-induced damage, which is reported to be a common cause for male infertility (Nowicka-Bauer & Nixon, 2020). For instance, increased ROS production causes DNA fragmentation or lipid peroxidation, which compromises the membrane and impairs mitochondrial activity (Moustafa et al., 2004; Nowicka-Bauer & Nixon, 2020). In fact, antioxidants have been proposed as adjuvants for sperm culture medium, being beneficial for spermatozoa viability and motility maintenance (Ali et al., 2021; Governini et al., 2020; Martin-Hidalgo et al., 2019). Since increased ROS production can lead to oxidative stress-induced damage, we also analyzed lipid peroxidation, protein oxidation and protein nitration. Interestingly, we were unable to find any evidence of oxidative stress-induced damage, although increased endogenous ROS production was observed. As increased oxidative stress-related damage is reported as the main cause for hyperglycemia-induced damage on pancreatic beta cells (Kawahito et al., 2009), the absence of high oxidative stress-induced damage could further explain the high glucose tolerance of human spermatozoa. Taken together, our results suggest that increased ROS production is a product of increased mitochondrial activity and may take part of a finely regulated signaling pathway that modulates or even activates human sperm capacitation. Although the role of endogenous ROS in sperm during capacitation is still a matter of debate, it is reported that spermatozoa

incubation under low H₂O₂ concentrations improves capacitation, hyperactivation and acrosome reaction (Griveau et al., 1994). In addition, there is data evidencing the role of nitric oxide as a signaling molecule for capacitation (Balercia et al., 2004; Herrero et al., 1999). Still, the role of endogenous ROS as signaling agents for the fertilization process are still poorly understood and more data are needed to clarify these molecular mechanisms.

In conclusion, our results provide further evidence that glucose is essential for complete capacitation of human spermatozoa, although its stimuli seem to be dissociated from mitochondrial (hyper)activation. We were able to demonstrate that under capacitation conditions, human spermatozoa mitochondria are active and producing ROS even in the absence of glucose or any other energy substrates. Taken together, we propose that human sperm capacitation occurs as a two-step process. On the first step, sperm mitochondria become active and produces ROS as signaling intermediates. As a second step, and when high glucose concentrations are available, such as occurs within the female reproductive tract, mitochondrial respiration increases to sustain the high ATP demands necessary for mass protein phosphorylation and hyperactivated motility. Although our hypothesis constitutes a paradigm shift on the physiology of human sperm capacitation, further studies are needed for its clarification. In addition, the role of the mitochondria and ROS as signaling intermediates during capacitation demands further research.

4.5. Authors contribution

David F. Carrageta – methodology, formal analysis, investigation, writing – original draft preparation, writing – review and editing; Bárbara Guerra-Carvalho – methodology, formal analysis, writing – review and editing; Mário Sousa – writing – review and editing; Alberto Barros – resources, writing – review and editing; Pedro F. Oliveira – conceptualization, formal analysis, investigation, resources, writing – review and editing, funding acquisition; Mariana P. Monteiro – conceptualization, formal analysis, investigation, writing – review and editing; Marco G. Alves – conceptualization, formal analysis, investigation, resources, writing – review and editing, project administration, funding acquisition.

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Chapter 5

Inhibition of mitochondrial uncoupling proteins arrests human spermatozoa motility without compromising viability



This chapter was adapted from the published work:

Carrageta DF, Freire-Brito L, Guerra-Carvalho B, Ribeiro JC, Monteiro BS, Barros A, Oliveira PF, Monteiro MP, Alves MG. (2023). Inhibition of mitochondrial uncoupling proteins arrests human spermatozoa motility without compromising viability. *Antioxidants*, 2023; 12(2):409. <https://doi.org/10.3390/antiox12020409>

Abstract

Mitochondrial uncoupling proteins (UCPs) are central in the regulation of mitochondrial activity and reactive oxygen species (ROS) production. High oxidative stress is a major cause of male infertility; however, UCPs expression and function in human spermatozoa are still unknown. Herein, we aimed to assess the expression and function of the different homologs (UCP1-6) in human spermatozoa. For this purpose, we screened for the mRNA expression of all UCP homologs. Protein expression and immunolocalization of UCP1, UCP2, and UCP3 were also assessed. The expression of all UCP homologs (*UCP1-6*) mRNA by human spermatozoa is herein reported for the first time. UCP1-3 are predominant at the head equatorial segment, whereas UCP1 and UCP2 are also expressed at the spermatozoa midpiece, where mitochondria are located. Highly motile spermatozoa were isolated from human normozoospermic seminal samples ($n = 16$) and incubated with genipin, an inhibitor of UCPs (0, 0.5, 5, and 50 μM) for 3 h at 37 °C. The inhibition of UCPs by 50 μM genipin, resulting in the UCP3 inhibition, did not compromise sperm cell viability but resulted in irreversible total motility loss that persisted despite washing or incubation with theophylline, a cAMP activator. These effects were associated with decreased mitochondrial membrane potential and lactate production. No differences concerning UCP3 expression, however, were observed in spermatozoa from normozoospermic versus asthenozoospermic men ($n = 6$). The inhibition of UCPs did not increase ROS production, possibly due to the decreased mitochondrial activity and genipin antioxidant properties. In sum, UCPs are major regulators of human spermatozoa motility and metabolism. The discovery and characterization of UCPs' role in human spermatozoa can shed new light on spermatozoa ROS-related pathways and bioenergetics physiology.

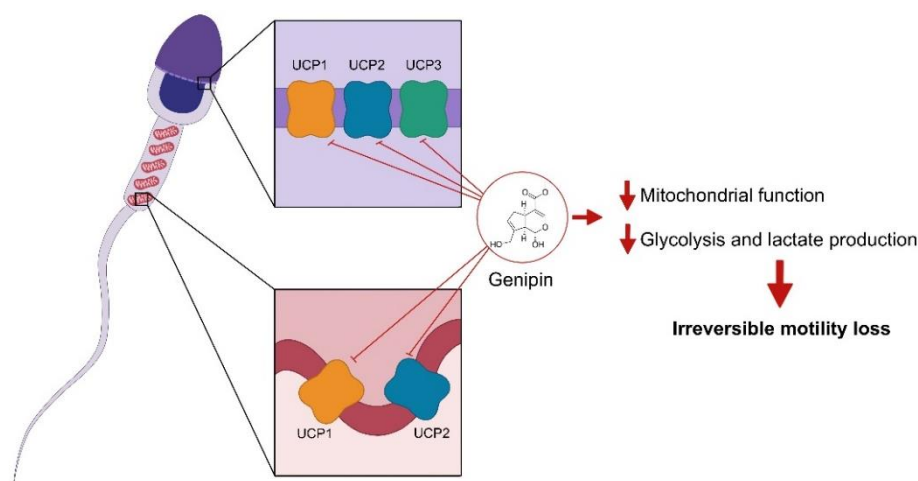


Fig. 5 – Graphical abstract. In this work, we provide evidence that UCP1, UCP2, and UCP3 are expressed in human spermatozoa. UCP1, UCP2, and UCP3 were found to be expressed at the equatorial segment of the head, besides the expression of UCP1 and UCP2 at the midpiece, where the mitochondria are located. UCPs regulate spermatozoa glycolytic metabolism and motility, as its inhibition by genipin leads to a relatively rapid and irreversible motility loss.

5.1. Introduction

Mitochondrial uncoupling proteins (UCPs) are transmembrane proteins of the mitochondrial anionic transporter family SLC25. Six homologs of mammalian UCPs (UCP1-6) have been so far identified (for review (Monteiro et al., 2021)). UCPs are expressed in the inner mitochondrial membrane, where they act as central regulators of mitochondrial membrane potential, oxidative phosphorylation, and reactive oxygen species (ROS) production (Ardalan et al., 2022; Hirschenson et al., 2022). UCPs have been known for its uncoupling activity, although this feature is absent in some of the homologs. UCP1, also known as thermogenin, is the main uncoupling homolog responsible for dissipating the proton gradient for heat production, *i.e.*, thermogenesis. Compared with UCP1, the remaining homologs (UCP2-6) either lack or exhibit less proton transport and uncoupling function (Nicholls, 2021). Nevertheless, all the homologs share the common characteristic of acting as regulators of redox balance, through regulation of ROS production (Hirschenson et al., 2022; Monteiro et al., 2021). This highlights a possible role for UCPs in male fertility, as high levels of oxidative stress are recognized as a major cause of infertility. Alarmingly, a temporal trend for a decrease in sperm counts was reported, leading to poorer semen quality and decreased male fertility worldwide (Levine et al., 2023). The decline in semen quality is positively linked to oxidative stress, as it is estimated that up to 80% of infertile men can have high ROS levels, which led to a novel terminology being proposed of Male Oxidative Stress Infertility (MOSI) (Agarwal et al., 2019). The origin of increased ROS production and oxidative stress is multifactorial. For instance, metabolic diseases, inflammation, exposure to certain drugs and pollutants, and ageing are all known factors that stimuli ROS production (Forrester et al., 2018; Martin-Hidalgo et al., 2019; Montano et al., 2022; Moreira et al., 2022). Oxidative stress leads to a myriad of harmful and irreversible consequences for male fertility, including impaired spermatogenesis, decreased spermatozoa concentration, and decreased numbers of motile spermatozoa (Athayde et al., 2007). The most highlighted phenomena associated with ROS are lipid peroxidation and consequently reduced membrane fluidity (Hosseinizadeh Colagar et al., 2013), and defective axonemal phosphorylation (Kurkowska et al., 2020). It should also be noticed that DNA fragmentation and genomic instability are common traits of oxidative stress-induced damage in spermatozoa (Aitken et al., 2010), which is linked to impaired oocyte fertilization, abnormal embryo development, and, ultimately, increased miscarriage rates (Cissen et al., 2016; McQueen et al., 2019; Tarozzi et al., 2007). Due to its activity on redox homeostasis, we hypothesized that UCPs could be a relevant target to understand MOSI and improve male reproductive outcomes. Although UCPs are widely expressed throughout the human body, data concerning its expression and role in the male gametes and reproductive system remains elusive. In this study, we

aimed to characterize the expression and function of UCPs in human spermatozoa. For this purpose, mRNA expression of UCP 1 to 6 homologs by human spermatozoa was screened. Spermatozoa expression and location of UCP1, UCP2, and UCP3 proteins, using commercially available and reliable antibodies, was also assessed. To study the function of UCPs in human spermatozoa, we used the UCPs inhibitor genipin (Kreiter et al., 2019). The effect of UCPs inhibition on human spermatozoa viability and motility were evaluated. In addition, the effect of UCPs inhibition on human spermatozoa mitochondrial membrane potential, total endogenous ROS production, as well as the spermatozoa culture media total antioxidant capacity and metabolic profile, were also evaluated.

5.2. Materials and methods

5.2.1. Reagents and chemicals

Unless stated otherwise, all reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.2.2. Patient recruitment and seminal samples preparation

Human spermatozoa were collected from patients attending the Medical Assisted Procreation Center, after approval by the Joint Ethics Committee CHUP/ICBAS (2021/CE/P002[P342/CETI/ICBAS]). Patient recruitment, clinical evaluation, and diagnostic semen analysis were performed at the clinic in compliance with the standards of care. All procedures were conducted according to the ethical guidelines for human research, under strict individual anonymity, and informed and written consent was obtained from all participants included in the study. Men with history of abusing drugs or alcoholism, were under treatment or exposed to drugs known to interfere with fertility, were diagnosed with an acute genital inflammatory disease before sperm collection, were a survivor of cancer treatment, or had any systemic disease known to affect fertility were excluded from the study. Smoking was not an exclusion criterion applied in this study. Sperm samples from otherwise healthy adult males were obtained by masturbation into specific sterile containers after 2-4 days of sexual abstinence, left at 37°C until complete liquefaction, and then analyzed according to the guidelines of the World Health Organization (WHO, 2021). Adult male subjects, normozoospermic ($n = 16$, mean age 38.3 ± 7.2) or asthenozoospermic (total motility $< 40\%$, $n = 6$, mean age 36.6 ± 3.8) were enrolled in this study (Fig. 5.1). A standard discontinuous density gradient centrifugation protocol was performed on selected 16 samples of normozoospermic men using the Gems® Sperm Wash Gradient Set (45% and 90%) (SWG-01,

Genea, Sydney, Australia) according to the manufacturer's instructions. The resultant pellet, containing the spermatozoa fraction with a higher percentage of motility and viability, was collected and washed two times in phosphate buffer saline (PBS) followed by centrifugation of 500× g for 5 min before being used for subsequent experiments. The asthenozoospermic samples and a fraction of the whole normozoospermic samples (n = 6 for each condition) were washed two times in PBS, centrifuged, and the pellet was collected for protein analysis.

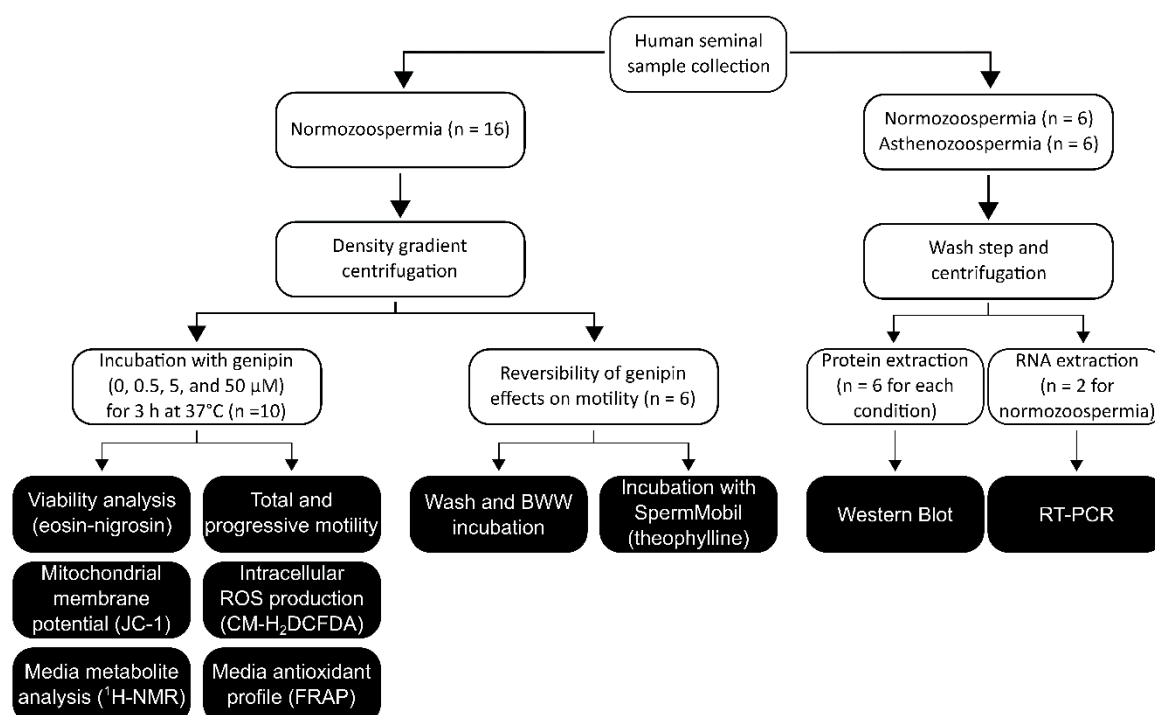


Fig. 5.1 – Schematic workflow of the experimental design applied in this study.

5.2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from pelleted spermatozoa of 2 normozoospermic men using the NZY Total RNA Isolation Kit (MB13402, NZYTech, Lisbon, Portugal) and accordingly to the manufacturer's instruction. Purified RNA concentration was determined using a Take3 microvolume plate on a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). RNA was reversely transcribed using the NZY M-MuLV Reverse Transcriptase Kit (MB083, NZYTech, Lisbon, Portugal). RT-PCR was performed to identify the expression of *UCP1*, *UCP2*, *UCP3* (*UCP3S* and *UCP3L* for short and long isoforms, respectively), *UCP4*, *UCP5*, and *UCP6* mRNAs in human spermatozoa using the resulting complementary DNA (cDNA) and specific primer sets (Table 5.1). Peripheral blood leukocytes (PBL) cDNA was used as a positive control.

Table 5.1. Oligonucleotides and cycling conditions for RT-PCR amplification of mitochondrial uncoupling proteins (UCPs) homologs.

Target (accession number)	Sequence 5'-3'	Annealing temperature	Number of cycles
UCP1 (NM_021833.5)	FWD: GCTCCAGGTCCAAGGTGAAT RVS: TTGCTTCCTAACTAGGTGCTG	59 °C	40
UCP2 (NM_001381943.1)	FWD: GAAGCCTCTACAATGGGCTGG RVS: CAGAGCCCTTGGTGTAGAACTG	63 °C	30
UCP3S (NM_022803.3)	FWD: CAACCTGGGATGTAGCGGTG RVS: GAGCGTGTGGAGACAGTGAG	60 °C	25
UCP3L (NM_003356.4)	FWD: TGGAACGTGGTGATGTTTCGTA RVS: AGACCAGAATCCCTCCTCCT	60 °C	33
UCP4 (NM_004277.5)	FWD: CGCACAGCTCTAGGGATCAT RVS: CGTGTCTGTAAATGGCGGGT	60 °C	33
UCP5 (NM_001282195.2)	FWD: GACTTTCCCTGTGGACCTTACC RVS: GCGCATGGAACATCCCTCTAT	60 °C	33
UCP6 (NM_001010875.4)	FWD: ACCCTGTTGATGTTGTGAGG RVS: TAGCCAGAACATCTGCCATCTC	63 °C	33

5.2.4. Western blot

Proteins from pelleted human spermatozoa were extracted using radioimmunoprecipitation assay (RIPA) buffer and then quantified using Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA). Western Blot protocol was performed to identify the expression of UCP1, UCP2, and UCP3 using standard methods (Bernardino et al., 2016). In brief, protein samples (30 µg) were denatured at 95°C for 5 min (UCP3) or 70°C for 10 min (UCP1 and UCP2). Protein samples were fractionated on a 12.5% polyacrylamide gel from TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad, Hemel Hempstead, UK) and transferred to a low fluorescence polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hemel Hempstead, UK) using a Trans-Blot® Turbo™ System (Bio-Rad, Hemel Hempstead, UK). Membranes were blocked with a solution of 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 solution. Then, membranes were incubated overnight at 4°C separately with primary antibodies rabbit anti-UCP1 (1:500, ab155117, Abcam, Cambridge, UK), rabbit anti-UCP2 (1:1000, PA5-77553, Invitrogen, Waltham, MA, USA), or rabbit anti-UCP3 (1:500, ab180643, Abcam, Cambridge, UK). Proteins were detected upon incubation with goat anti-rabbit IgG-AP (1:5000, A3687, Sigma-Aldrich, St. Louis, MO, USA) and reacted

with ECF Substrate (RPN5785, Cytiva, Marlborough, MA, US). Total protein content was evaluated using Ponceau S staining. Protein bands were detected using a Bio-Rad Chem-iDoc XR (Bio-Rad, Hemel Hempstead, UK).

5.2.5. Immunofluorescence staining

Immunofluorescence staining was performed to observe the cellular location of UCP1, UCP2, and UCP3 using standard methods (Pereira et al., 2021). In brief, human spermatozoa were fixed on glass coverslips with 4% paraformaldehyde in PBS for 20 min at room temperature. Spermatozoa were permeabilized with 0.1% Triton X-100 in PBS and blocked in 0.1% gelatin in PBS, both for 15 min at room temperature. Spermatozoa were then incubated with primary antibodies rabbit anti-UCP1 (1:50), rabbit anti-UCP2 (1:50), or rabbit anti-UCP3 (1:50) overnight at 4 °C. Spermatozoa incubated without primary antibodies were used as a negative control. Incubation with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1:1500, A-11008, Thermo Fischer Scientific, Waltham, MA, USA) occurred for 1 h at room temperature. Coverslips were mounted and nuclei were stained with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Fluorescence was observed in a Nikon Eclipse E400 microscope equipped with a DS-Fi3 microscope camera, a Y-FL Epi-Fluorescence Attachment, and HB-10103AF Super High-Pressure Mercury Lamp Power Supply (Nikon, Shinagawa, Tokyo, Japan). The pictures obtained were processed using the NIS-Elements Br 4.60.00 software (Nikon, Shinagawa, Tokyo, Japan).

5.2.6. Experimental groups

To study the role of UCPs in human spermatozoa physiology, we used genipin (Acros Organics™, Thermo Fisher Scientific, Waltham, MA, USA), a known UCPs inhibitor (Kreiter et al., 2019). Retrieved spermatozoa from each sample (n = 10) were isolated through density gradient centrifugation and allocated into four groups (3 million spermatozoa per condition). Spermatozoa were incubated at 37°C in 1 mL of modified Biggers, Whitter, and Whittingham (BWW) medium (in mM: 94.5 NaCl, 4.8 KCl, 1.7 CaCl₂, 1.17 KH₂PO₄, 1.22 MgSO₄, 20 HEPES, 25 NaHCO₃, 11 Glucose, pH = 7.4) in the absence (control group) or supplemented with increasing concentrations of genipin (0.5, 5, and 50 µM). The doses of genipin were selected based on studies reported in the literature. The highest concentration used in our study (50 µM) was chosen based on the IC₅₀ value reported for UCP3 inhibition (42 ± 12 µM) (Kreiter et al., 2019). A pilot study was performed (data not shown) using an even

higher concentration (100 μ M, based on the IC₅₀ value reported for UCP2 inhibition – 94 ± 24 μ M (Kreiter et al., 2019)) but since total loss of motility was observed with 50 μ M of genipin, we decided to exclude this concentration from further studies. The lower doses of genipin (0.5 and 5 μ M) were chosen according to the mitochondrial and metabolic effects previously reported (Zhang et al., 2006). To avoid the formation of genipin-albumin complexes, spermatozoa were incubated in the absence of bovine serum albumin (BSA) (Wang et al., 2021). After 3 h incubation, samples were centrifuged and culture media were collected for Proton Nuclear Magnetic Resonance (¹H-NMR) and Ferric Reducing Antioxidant Power Assay (FRAP) analysis. Spermatozoa were washed two times in PBS and a fraction from each condition was collected for viability, motility, mitochondrial membrane potential, or intracellular ROS content analysis.

5.2.7. Spermatozoa viability and motility analysis

The effects of UCPs inhibition in human spermatozoa were assessed using eosin-nigrosin staining, according to an established protocol (Carrageta et al., 2020). The percentage of viable and nonviable spermatozoa was determined by counting a total of 100 spermatozoa per slide in continuous random fields under a bright-field optical microscope (1000 $\times$ magnification). White spermatozoa were considered viable whereas pink-stained spermatozoa, indicating loss of membrane integrity, were considered nonviable. Results are expressed as percentage of viable spermatozoa.

Spermatozoa total and progressive motility were assessed using a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel), as previously described (Carrageta et al., 2020). Spermatozoa total and progressive motility were evaluated using 10 s videos recorded in a bright-field optical microscope (200 $\times$ magnification). The experiment was performed by the same user and the same criteria were applied between samples. New drops were placed at the chamber and the process was repeated until a minimum total of 100 sperm cells per sample were counted. Results are expressed as percentage of total or progressive motile spermatozoa.

5.2.8. Genipin effects on spermatozoa motility

Two approaches were used to assess whether the effects of genipin-induced UCPs inhibition in spermatozoa motility were reversible ($n = 6$ for each procedure). In the first one, genipin-treated spermatozoa were washed twice in PBS and incubated in fresh BWW medium supplemented with BSA (0.4 mM) for 45 min at 37 °C. In the second approach, the

cAMP activator theophylline, was used under the formulation of SpermMobil (GM501, Gynemed, Lensahn, Germany). SpermMobil is clinically used for the in vitro examination of immotile spermatozoa isolated from testicular tissue, as it confers motility to otherwise immotile spermatozoa through the activation of cAMP-dependent pathways. The procedure was conducted according to the manufacturer's instructions. In brief, genipin-treated spermatozoa were washed twice in PBS and incubated in fresh BWW medium supplemented with SpermMobil medium (1:10 dilution, v:v) for 20 min at 37 °C. After each procedure, total and progressive motility were evaluated and reported as aforementioned described.

5.2.9. JC-1 assay for mitochondrial membrane potential analysis

The mitochondrial membrane potential of spermatozoa was analyzed using the fluorogenic dye JC-1 (T3168, Invitrogen™, Carlsbad, CA, USA), according to a protocol previously established (Carrageta et al., 2022). Briefly, 10^6 spermatozoa from each condition were washed and collected after treatments and incubated in a JC-1 working solution (1 µg/mL in PBS) at 37°C for 30 min. Spermatozoa incubated at 20% DMSO solution were used as an assay positive control. Spermatozoa were then transferred into a 96-well clear bottom black plate for the assessment of JC-1 monomers (485/530 nm; excitation/emission) and J-aggregates (535/590 nm; excitation/emission) fluorescence on a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). The ratio between JC-1 J-aggregates/monomers was calculated and used as surrogate of mitochondrial membrane polarization. Results are expressed as mean J-aggregates/monomers ratio.

5.2.10. Detection of intracellular reactive oxygen species (ROS)

The total intracellular ROS production of spermatozoa was quantified using the fluorogenic dye CM-H₂DCFDA (C6827, Invitrogen™, Carlsbad, CA, USA). The protocol was performed as previously described (Carrageta et al., 2020). Briefly, 10^5 spermatozoa were washed and collected after treatments and incubated in a CM-H₂DCFDA working solution (5 µM in PBS) at 37°C for 30 min. Spermatozoa incubated in a 0.1% H₂O₂ solution were used as a positive control. Spermatozoa were then transferred into a 96-well black plate and fluorescence (495/529 nm; excitation/emission) was read on a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). Results are represented in DCF Fluorescence Units (arbitrary units).

5.2.11. Proton nuclear magnetic resonance (^1H -NMR)

The proton nuclear magnetic resonance (^1H -NMR) spectra were acquired using a Bruker Avance III HD 500 MHz spectrometer equipped with a 5-mm TXI probe (Bruker Corporation, Billerica, MA, USA). Solvent-suppressed ^1H -NMR spectra were acquired at 298K with a zgpr pulse with a sweep width of 7 kHz, using a relaxation delay of 7s, a pulse angle of 30° , an acquisition time of 2.3 s, and 32 scans. Before Fourier transform, each free induction decay was multiplied by a 0.2 Hz Lorentzian. A total of 180 μL of the medium of each condition was analyzed using 3-mm NMR tubes and optimizer inserts for 5-mm spinners. Samples were diluted with sodium fumarate (final concentration of 2 mM) in deuterated water solution (final volume of 225 μL), which was used as an internal reference (6.5 ppm) to quantify the following metabolites present at the medium (multiplet, ppm): lactate (doublet, 1.33) and acetate (singlet, 1.90). Spectra were manually phased and baseline corrected. Chosen metabolite peaks were integrated using the NUTS-Pro NMR software (Acorn NMR, Inc, Fremont, CA, USA). Metabolite variation (consumption/production) in each condition was determined and the results are expressed as nmol/ 10^6 spermatozoa.

5.2.12. Ferric-reducing antioxidant power assay (FRAP)

The ferric-reducing antioxidant power assay (FRAP) was performed according to the method described by Benzie and Strain (Benzie & Strain, 1996). Culture media was added to the working FRAP solution, which reduces the Fe^{3+} -TPTZ complex to the colored Fe^{2+} -TPTZ complex. The experiment was performed in duplicates for each sample. After 40 min incubation, the absorbance was read at 593 nm on a SynergyTM H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). Dilutions of ascorbic acid (0-2 μM) were used for the standard curve. FRAP value of the media was calculated as described by Benzie and Strain (Benzie & Strain, 1996). Results are expressed as μmol of antioxidant potential/L.

5.2.13. Statistical analysis

Experimental results are represented as mean $\pm$ standard deviation (SD) or Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). Statistical analysis was performed in GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Data were tested for normal distribution with the Kolmogorov-Smirnov test. Statistical differences were determined by a paired *t*-test or repeated measures one-way ANOVA with *post hoc* Dunnett's multiple comparisons test. $p < 0.05$ was considered significantly different.

5.3. Results

5.3.1. *UCP1-6* mRNAs and *UCP1*, *UCP2*, and *UCP3* proteins are present in human spermatozoa

Few studies focused on studying UCPs expression and function in human spermatozoa. In this study, we screened for mRNA expression of known UCPs homologs (*UCP1-6*) by human spermatozoa. *UCP1*, *UCP2*, *UCP3S* (*UCP3* short isoform), *UCP3L* (*UCP3* long isoform), *UCP4*, *UCP5*, and *UCP6* mRNA were identified in human spermatozoa (Fig. 5.2A). Human peripheral blood leukocytes (PBL) were used as a positive control.

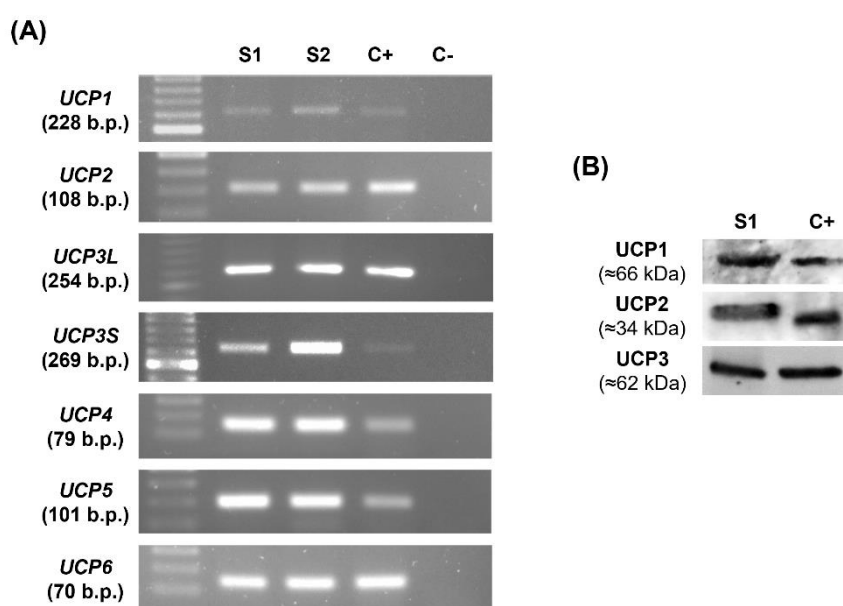


Fig. 5.2 – Identification of mitochondrial uncoupling proteins (UCPs) homologs (*UCP1-6*) mRNA and protein (*UCP1-3*) expression in human spermatozoa. (A) Representative RT-PCR of *UCP1*, *UCP2*, *UCP3L*, *UCP3S*, *UCP4*, *UCP5*, and *UCP6* mRNA expression in human spermatozoa. Samples from two different individuals (S1 and S2) were used and cDNA from human peripheral blood leukocytes (C+) was used as a positive control. **(B)** Representative blots of *UCP1*, *UCP2*, and *UCP3* protein expression in human spermatozoa (S1). Mouse kidney was used as a positive control (C+).

Protein expression of *UCP1*, *UCP2*, and *UCP3* in human spermatozoa was also confirmed by Western Blot (Fig. 5.2B). Protein expression of *UCP4*, *UCP5*, and *UCP6* was not tested as there were no validated antibodies commercially available. Our results show a blot band representative of *UCP2* at the predicted molecular weight (≈34 kDa). However, *UCP1* and *UCP3* blot bands presented a higher molecular weight (≈66 kDa and ≈62 kDa, respectively), which could be suggestive of dimerization, a process that has been previously reported (Ledesma et al., 2002).

5.3.2. UCP1, UCP2, and UCP3 are mainly located at the equatorial and acrosome region of the head of human spermatozoa

UCP1, UCP2, and UCP3 location in human spermatozoa were evaluated by immunofluorescence staining (Fig. 5.3). Notably, all UCPs homologs studied were found to be expressed outside the midpiece, where the mitochondria are mainly present. UCP1 (Fig. 5.3B) and UCP2 (Fig. 5.3C) were found to be expressed at the head, with strong staining in the equatorial region and lighter staining in the acrosome region and midpiece. UCP3 (Fig. 5.3D) was only found to be expressed at the head, specifically at the equatorial and acrosome region. No fluorescent staining was observed in the negative control (Fig. 5.3A).

5.3.3. UCPs inhibition induces motility loss without compromising viability of human spermatozoa

To study the role of UCPs in human spermatozoa physiology we used an UCPs inhibitor, genipin. The percentage of viable spermatozoa following UCPs inhibition by genipin (0, 0.5, 5, and 50 μ M) for 3 h was analyzed through eosin–nigrosin staining (Fig. 5.4A). No differences were observed between all experimental groups ($90 \pm 7\%$, $94 \pm 3\%$, and $90 \pm 5\%$ after treatment with 0.5, 5, or 50 μ M genipin, respectively) when compared to the control group ($90 \pm 6\%$). Interestingly, we observed that UCPs inhibition greatly affected spermatozoa motility (Fig. 5.4B). UCPs inhibition with the highest genipin concentration (50 μ M) greatly decreased spermatozoa total motility ($3 \pm 5\%$) as compared to the control group ($73 \pm 11\%$, $p < 0.001$). A decrease in total motility was not observed in the remaining experimental groups ($75 \pm 8\%$ and $70 \pm 4\%$ after treatment with 0.5 and 5 μ M genipin, respectively) as compared to the control group.

We further evaluated the time of action of UCPs inhibition by 50 μ M genipin in spermatozoa total and progressive motility. For that purpose, spermatozoa were incubated in modified BWW media for 15 min and then genipin was added. Total (Fig. 5.4C) and progressive motility (Fig. 5.4D) were assessed every 15 min for a total of 45 min. A decrease in total motility was observed as early as after 15 min of incubation ($37 \pm 16\%$) when compared to the control group ($69 \pm 16\%$, $p = 0.013$), whereas a tendency for a decrease of progressive motility ($3 \pm 4\%$) was also observed within the same time interval when compared to the control group ($15 \pm 12\%$, $p = 0.07$). A significant motility loss conferred by UCPs inhibition was achieved at 30 min ($3 \pm 4\%$ and $0 \pm 1\%$ for total and progressive motility, respectively) whereas spermatozoa from the control group remained motile ($73 \pm 9\%$, $p < 0.001$; $23 \pm 12\%$, $p = 0.006$ for total and progressive motility, respectively). Virtually full total ($1 \pm 1\%$)

and full progressive motility loss ($0 \pm 0\%$) was observed at 45 min. Spermatozoa from the control group sustained its motility ($63 \pm 15\%$, $p < 0.001$; $17 \pm 10\%$, $p = 0.009$ for total and progressive motility, respectively).

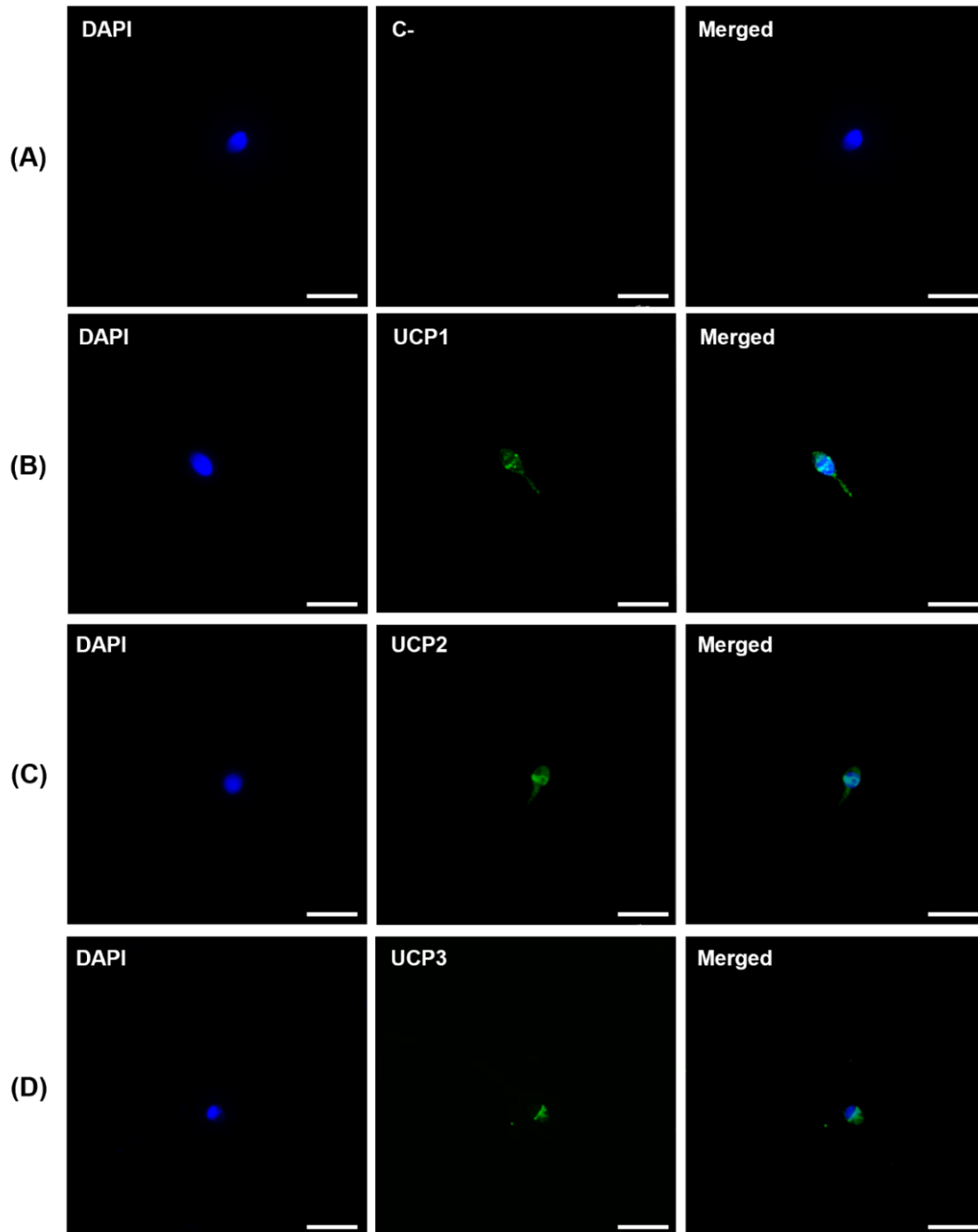


Fig. 5.3 – Immunofluorescence staining for UCP1, UCP2, and UCP3 protein expression in human spermatozoa. (A) Negative control. Immune labeling of UCP1 **(B)**, UCP2 **(C)**, and UCP3 **(D)** was observed in human spermatozoa. Scale bar—10 μm .

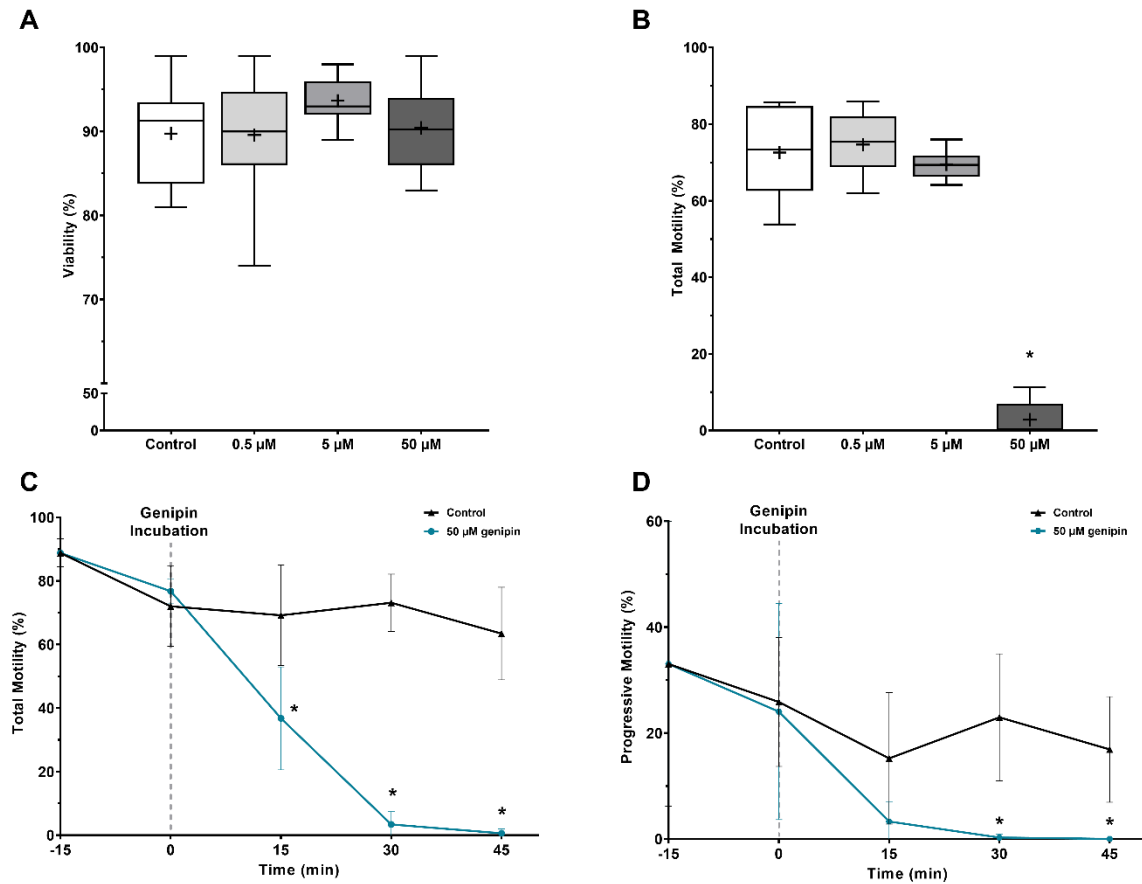


Fig. 5.4 – Evaluation of human spermatozoa viability and motility after UCPs inhibition by increasing concentrations of genipin (0.5, 5, and 50 µM) for 3 h. (A) Human spermatozoa viability was determined by eosin–nigrosin staining ($n = 10$). **(B)** Effect of UCPs inhibition on total motility of human spermatozoa ($n = 10$). **(C)** Evaluation of the time of action of 50 µM genipin on total and **(D)** progressive motility ($n = 6$). Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles ± 1.5 IQR) **(A,B)** or mean $\pm$ SD **(C,D)**. +—Represents group average. Significantly different results are expressed as: *—Relative to the control group.

5.3.4. Human spermatozoa motility loss due to UCPs inhibition is not recovered by incubation with albumin or theophylline

After observing that UCPs inhibition led to complete motility loss, we aimed to study whether this condition was reversible. For that purpose, we washed and incubated genipin-treated spermatozoa in modified BWW media supplemented with albumin. Albumin is reported to form complexes with genipin, thus decreasing its physiological effects (Wang et al., 2021). After 45 min of incubation, total (Fig. 5.5A) and progressive motility (Fig. 5.5B) were analyzed. An increase in total ($84 \pm 5\%$) and progressive motility ($46 \pm 10\%$) was observed in the control group as compared to pre-washing and albumin incubation parameters ($60 \pm$

13%, $p = 0.030$; $17 \pm 10\%$, $p = 0.001$ for total and progressive motility, respectively). Nevertheless, no improvement in total ($1 \pm 1\%$) or progressive motility ($0 \pm 1\%$) was observed as compared to pre-washing and albumin incubation parameters ($1 \pm 1\%$; $0 \pm 0\%$ for total and progressive motility, respectively).

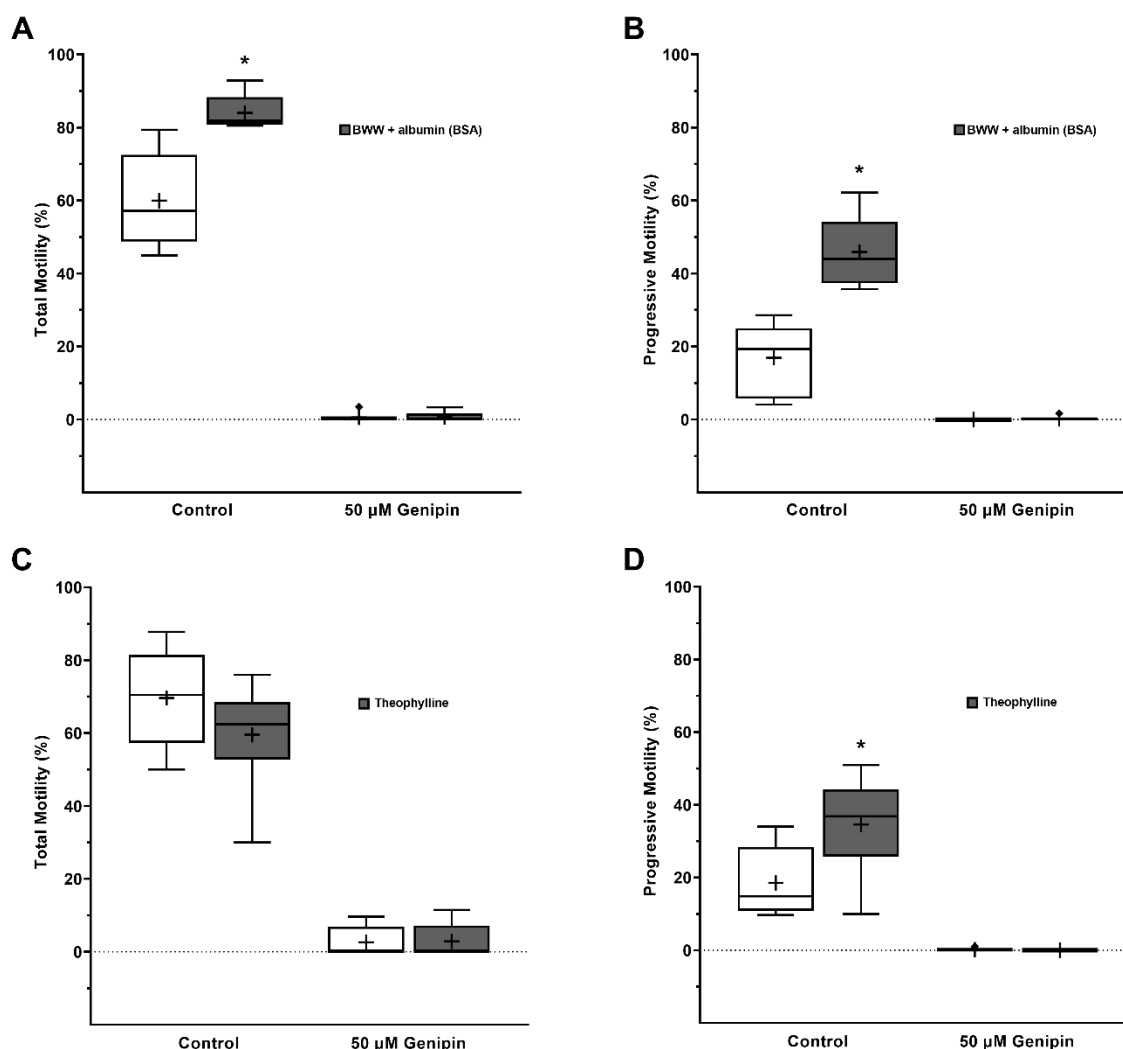


Fig. 5.5 – Evaluation of motility loss reversibility conferred by UCPs inhibition with 50 μ M genipin. (A) Total and (B) progressive motility of genipin-treated spermatozoa upon wash and 45 min incubation in modified BWB media supplemented with albumin ($n = 6$). (C) Total and (D) progressive motility analysis of genipin-treated spermatozoa upon 20 min incubation with theophylline (SpermMobil) ($n = 6$). Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). +–Represents group average. Significantly different results are expressed as: *–Relative to genipin-treated group.

To further evaluate the reversibility of genipin effects, theophylline (SpermMobil), a cAMP activator, was also used. After 20 min incubation, total (Fig. 5.5C) and progressive

motility (Fig. 5.5D) were analyzed. Although no changes in total motility ($60 \pm 16\%$) were observed, progressive motility ($35 \pm 14\%$) increased in the control group, as compared to pre-treatment parameters ($70 \pm 15\%$; $19 \pm 10\%$, $p = 0.036$ for total and progressive motility, respectively). Supporting the previous results, no improvement in total ($3 \pm 5\%$) or progressive motility ($0 \pm 0\%$) was observed when compared to the pre-treatment parameters ($3 \pm 4\%$; $0 \pm 0\%$ for total and progressive motility, respectively). Taken together, these results suggest that UCPs inhibition induced by genipin leads to irreversible motility loss.

5.3.5. UCP3 relative expression in spermatozoa does not differ between normozoospermic and asthenozoospermic men

Taking into consideration that $50 \mu\text{M}$ genipin induced near total motility loss, we hypothesized that these effects could be mainly mediated by UCP3 inhibition (IC_{50} value previously reported as $42 \pm 12 \mu\text{M}$ (Kreiter et al., 2019)). To disclose whether spermatozoa with decreased motility had altered UCP3 expression or dysfunction, we compared UCP3 expression in spermatozoa of asthenozoospermic (low motility, $< 40\%$) and normozoospermic (normal sperm parameters) men by Western Blot (Fig. 5.6A). Although we were not able to detect UCP3 in some samples from asthenozoospermic men, no differences concerning mean UCP3 expression were observed when spermatozoa of asthenozoospermic (0.076 ± 0.084 arbitrary units) were compared to those of normozoospermic (0.057 ± 0.038 arbitrary units) men, suggesting that a potential dysfunction leading to motility loss is unlikely to be mediated by altered UCP3 expression.

5.3.6. UCPs inhibition decreases mitochondrial membrane potential and lactate production in human spermatozoa

To further disclose the molecular mechanisms that lead to motility loss conferred by UCPs inhibition, we sought to evaluate the mitochondrial membrane potential using the fluorescent dye JC-1 (Fig. 5.6B). At the highest concentration of genipin ($50 \mu\text{M}$) there was a decrease in the JC-1 ratio (4.147 ± 2.585 J-aggregates/monomers) when compared to the control group (5.282 ± 2.509 J-aggregates/monomers, $p = 0.020$), indicating mitochondrial depolarization and decreased mitochondrial activity. No differences were observed when the groups incubated with lower genipin concentrations (5.366 ± 2.394 and 5.497 ± 2.699 J-aggregates/monomers for 0.5 and $5 \mu\text{M}$, respectively) were compared to the control group.

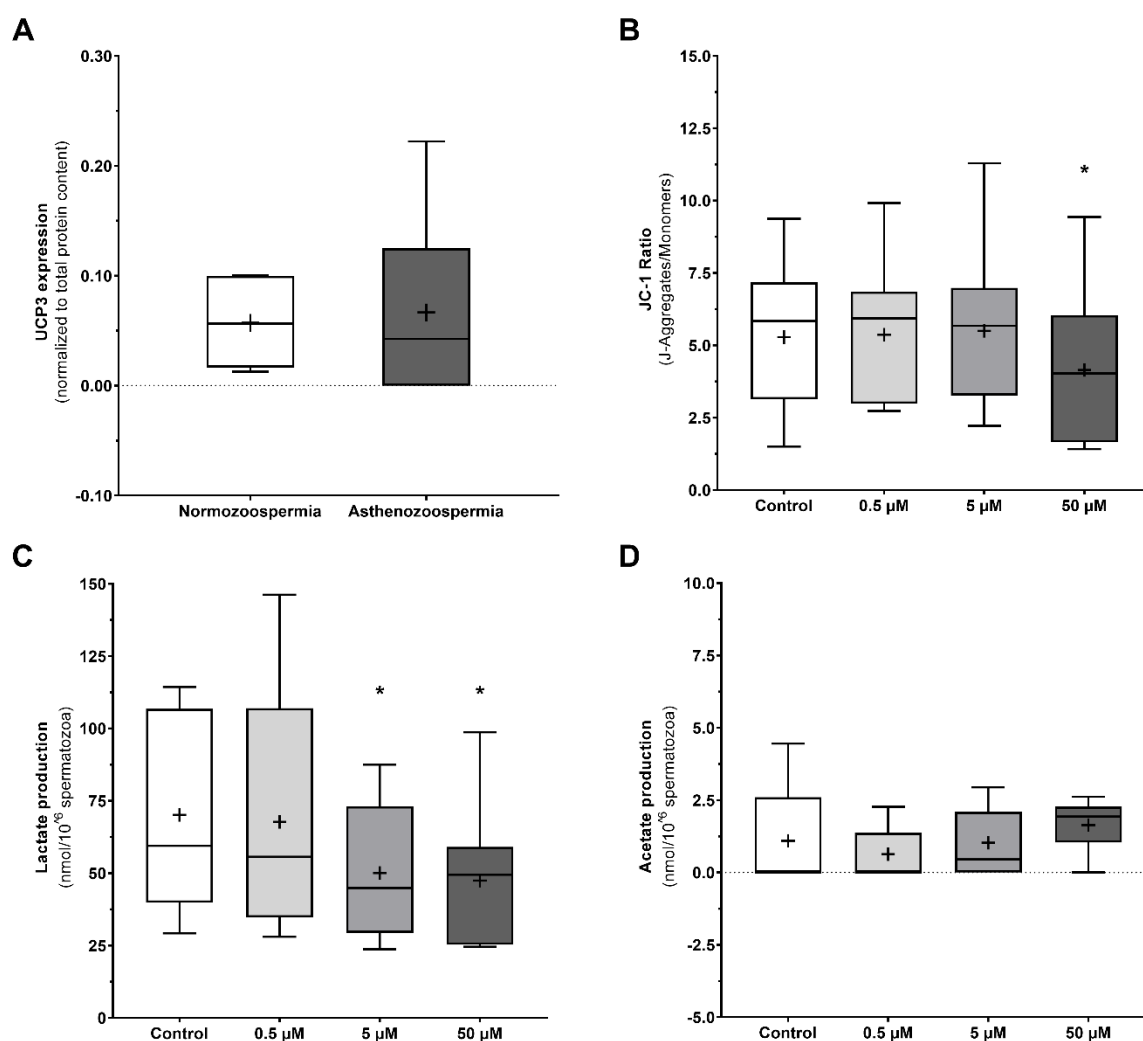


Fig. 5.6 – UCP3 expression, mitochondrial membrane polarization, and metabolic profile of human spermatozoa. (A) UCP3 relative expression in spermatozoa from normozoospermic versus asthenozoospermic men. (B) Evaluation of mitochondrial membrane potential using the JC-1 dye after inhibition of UCPs by increasing concentrations of genipin (0.5, 5, and 50 μ M) for 3 h. (C) Lactate and (D) acetate production by human spermatozoa after UCPs inhibition by increasing concentrations of genipin (0.5, 5, and 50 μ M) for 3 h. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). +-Represents group average. Significantly different results are expressed as: *-Relative to the control group.

We also analyzed the culture media by ^1H -NMR spectra to assess if UCPs inhibition leading motility loss had an impact on the human spermatozoa metabolic signature. Lactate (Fig. 5.6C) and acetate (Fig. 5.6D) were the main metabolites identified and quantified. We observed that both the 5 and 50 μ M of genipin led to lower lactate production (47.43 ± 23.33 and 50.07 ± 23.61 nmol/ 10^6 spermatozoa for 5 and 50 μ M, respectively) as compared to the control group (70.14 ± 34.62 nmol/ 10^6 spermatozoa, $p = 0.025$ and $p = 0.010$ for 5 and 50

μM , respectively). No differences were observed when the group treated with the lower genipin concentration ($67.73 \pm 41.73 \text{ nmol}/10^6 \text{ spermatozoa}$) was compared to the control group. No differences were observed concerning acetate production (0.63 ± 0.89 ; 1.03 ± 1.14 ; $1.64 \pm 0.93 \text{ nmol}/10^6 \text{ spermatozoa}$ for 0.5, 5, and 50 μM , respectively) as compared to the control group ($1.10 \pm 1.63 \text{ nmol}/10^6 \text{ spermatozoa}$).

5.3.7. UCPs inhibition does not increase total ROS production by human spermatozoa

Since UCPs dysfunction is closely linked to redox unbalance, we aimed to evaluate total intracellular ROS production using the fluorogenic dye CM-H₂DCFDA (Fig. 5.7A). Interestingly, there were no significant differences concerning ROS production by spermatozoa after genipin exposure at all studied concentrations (0.304 ± 0.189 ; 0.306 ± 0.172 ; 0.309 ± 0.171 DCF fluorescence units after treatment with 0.5, 5, and 50 μM , respectively) as compared to the control group (0.311 ± 0.162 DCF fluorescence units). In addition, culture media antioxidant potential was tested using the FRAP assay (Fig. 5.7B). We observed that the culture media supplemented with genipin at the highest concentration (50 μM) had an increased antioxidant capacity ($66.63 \pm 4.11 \mu\text{mol antioxidant potential/L}$) as compared to the control group ($22.00 \pm 2.52 \mu\text{mol antioxidant potential/L}$, $p < 0.001$), denoting the genipin antioxidant properties. No differences were observed when the groups treated with lower genipin concentrations (22.77 ± 3.63 and $23.90 \pm 2.06 \mu\text{mol antioxidant potential/L}$ for 0.5 and 5 μM , respectively) were compared to the control group.

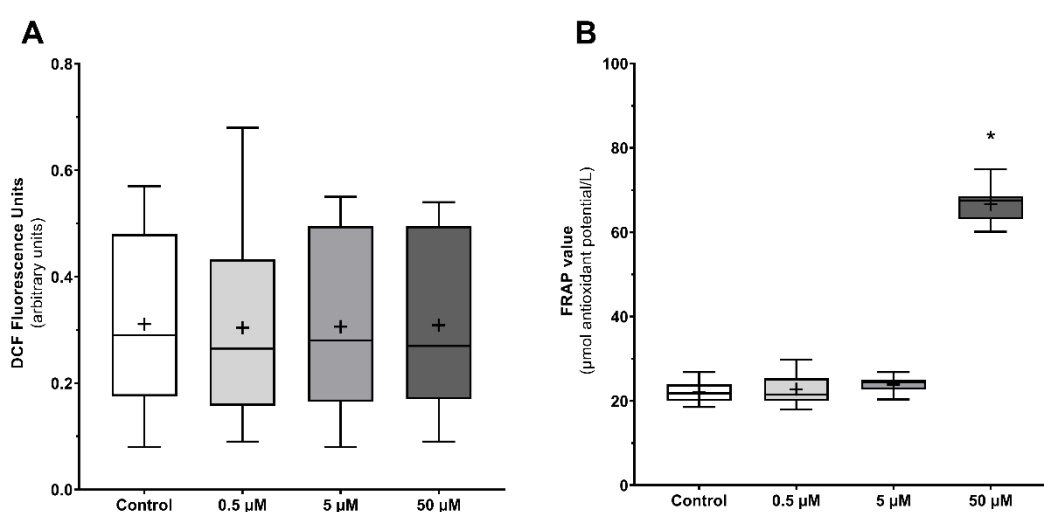


Fig. 5.7 – Culture media total antioxidant capacity and endogenous ROS production by human spermatozoa after UCPs inhibition by increasing concentrations of genipin (0.5, 5, and 50 μM)

for 3 h. (A) Evaluation of endogenous ROS production using the CM-H₂DCFDA dye. **(B)** Analysis of the culture media antioxidant potential through ferric antioxidant power (FRAP) assay. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). +–Represents group average. Significantly different results are expressed as: *–Relative to the control group.

5.4. Discussion

UCPs are mitochondrial proteins known for their activity as regulators of ROS production and oxidative stress. Although initially identified as adipose tissue-specific proteins, it is currently known that UCPs are present almost throughout the human body. However, limited data are available concerning its expression and function in the human male reproductive system, including spermatozoa. For instance, only UCP2 has been reported to be present in human spermatozoa (Wang et al., 2018). Although studies with mRNA microarray screening also predicted the expression of the newly identified UCP5 and UCP6 in the testis, no further studies were conducted since (Karlsson et al., 2021). In this work, we aimed to identify the expression and characterize the function of UCPs in human spermatozoa. For this purpose, we screened mRNA expression of UCP1-6 homologs, and, to the best of our knowledge, we were able to identify for the first time the transcripts of all six UCP homologs — *UCP1*, *UCP2*, *UCP3* (both short and long isoform, *UCP3S* and *UCP3L*), *UCP4*, *UCP5*, and *UCP6*. Due to its function, the expression of all UCP homologs in human spermatozoa suggests its possible involvement in the complex and finely regulated role of ROS production and ROS-dependent signaling pathways, essential for several sperm functions, including capacitation and also acrosome reaction (Du Plessis et al., 2015). We also confirmed the presence of UCP1, UCP2, and UCP3 proteins. Interestingly, the cellular location of these UCPs provided surprising results. We observed that UCP1, UCP2, and UCP3 are also expressed outside its classic location within the mitochondrial inner membrane, found at the midpiece of spermatozoa. While UCP1 and UCP2 were also found in the midpiece, UCP1, UCP2, and UCP3 were found to be mainly expressed in the equatorial region of the head of human spermatozoa. Interestingly, we found that UCP3 was specifically expressed in the head. The expression of UCP2 in the midpiece of human spermatozoa was previously reported by Wang et al. (Wang et al., 2018), but the expression of UCPs outside the mitochondria is herein described for the first time. The equatorial segment is a unique region found at the head of mammalian spermatozoa. It differs from the plasma membrane that covers the anterior and post-acrosomal domains in terms of both structure and function (Jones et al., 2008), as it is reported to express several specific proteins and ionic channels (Anifandis et al., 2014; Wolkowicz et al., 2008). Since it was shown to be the region of fusion

with the oocyte membrane in mammalian species, the equatorial segment function has attracted particular research interest. Spermatozoa only acquire fusion competence after acrosome reaction, the last maturational stage to acquire fertilizing ability, in which the acrosomal proteases are released to activate fusion proteins (Klinovska et al., 2014). In recent work, Ren et al. showed that the acrosomal matrix contains condensed mitochondria, which arise from progressive matrix contraction of orthodox mitochondria during germ cells meiosis (Ren et al., 2022). These condensed mitochondria were reported to exhibit increased respiration and higher levels of respiratory enzymes and supercomplexes. Therefore, we hypothesize that UCPs expression at the head of human spermatozoa could potentially pinpoint the presence of those mitochondria. In addition, mitochondria are the main source of ROS and acrosome reaction is regulated by ROS and ROS-dependent signaling pathways (Du Plessis et al., 2015). Ichikawa et al. observed a strong negative correlation between ROS concentration and acrosome reaction (Ichikawa et al., 1999). In another study, Aitken et al. observed that hydrogen peroxide promoted sperm penetration of zona-free hamster eggs, which would not occur in the presence of catalase (Aitken et al., 1995). Griveau et al. also observed that increased levels of O_2^- radicals induced acrosome reaction (Griveau et al., 1995). Thus, ROS regulation and signaling seem to be essential for acrosome reaction, and due to its location at the acrosomal region, UCPs seem to be potential mediators in the regulation of this mechanism. Nevertheless, future studies are needed to disclose the role of UCPs in the acrosome reaction.

The role and function of UCPs are shrouded in mystery as conflicting results are found in the literature. To further characterize UCPs function in human spermatozoa, we used the UCPs inhibitor genipin. This molecule was firstly described as a selective UCP2 inhibitor but was lately demonstrated to be also able to inhibit UCP1 and UCP3 dose-dependently, without reducing protein expression (Kreiter et al., 2019). The chosen doses of genipin were based on studies previously reported in the literature. The lower doses of genipin (0.5 and 5 μ M) were chosen according to the mitochondrial and metabolic effects observed in pancreatic β -cells by Zhang et al. (Zhang et al., 2006). In this study, these authors observed that small doses of genipin (0.5 and 5 μ M, which are, respectively, approximately 200 and 20 times lower than the reported IC_{50} for UCP2 inhibition— $94 \pm 24 \mu$ M (Kreiter et al., 2019)) increased mitochondrial membrane potential and ATP levels in pancreatic β -cells, thus stimulating insulin secretion. The highest concentration used in our study (50 μ M) was chosen based on the IC_{50} value reported for UCP3 inhibition ($42 \pm 12 \mu$ M) (Kreiter et al., 2019). We performed a pilot study (data not shown) using an even higher concentration (100 μ M, based on the IC_{50} value reported for UCP2 inhibition) but since total loss of motility was observed with 50 μ M of genipin, this concentration was chosen as the highest concentra-

tion. Surprisingly, genipin at 50 μM induced a motility loss without compromising spermatozoa viability. These effects on spermatozoa motility were shown to be relatively fast and enduring, as motility loss was achieved in 30 min and persisted for over 3 h. As motility loss was achieved at 50 μM , we hypothesize that these effects potentially occurred mainly due to UCP3 inhibition, although inhibition of the remaining UCPs could contribute to a smaller extent. Interestingly, UCPs inhibition conferred by genipin seems to be irreversible. At first, we washed and incubated spermatozoa in modified BWB media supplemented with albumin. Albumin is required for spermatozoa capacitation and increased motility (Carrageta et al., 2020), but it was excluded from our experiments since it triggers the formation of complexes with genipin, thus decreasing its effects (Wang et al., 2021). Although total and progressive motility increased when spermatozoa were washed and incubated with albumin, the motility loss conferred by genipin was irreversible. Similar results were observed when spermatozoa were washed and incubated with theophylline (SpermMobil). Theophylline is a methylxanthine, a class of compounds that act as phosphodiesterases (PDEs) inhibitors (Carrageta et al., 2018). Cyclic nucleotide PDEs are responsible for the degradation of cAMP, a molecule that is central in the process of capacitation, and therefore, its inhibition increases cAMP levels which promotes progressive motility (Buffone et al., 2014). Although we observed increased progressive motility upon exposure to theophylline in the control group, genipin-incubated spermatozoa did not recover from the loss of motility. Taken together, these results suggest that the motility loss induced by the inhibition of UCPs by genipin is potentially permanent.

Since the loss of motility induced by genipin is potentially mediated by UCP3 inhibition, we sought to evaluate if UCP3 expression was altered in the spermatozoa of men with asthenozoospermia. Interestingly, no differences in UCP3 expression were observed when spermatozoa from asthenozoospermic and normozoospermic men were compared, which suggests that an altered function rather than altered expression could be associated with decreased motility. The motility of spermatozoa is supported by both glycolysis and mitochondrial function (Guerra-Carvalho et al., 2022); thus, we hypothesized that mitochondrial function could be compromised or at least altered. Indeed, decreased mitochondrial membrane potential was observed at the highest concentration of genipin (50 μM) which led to complete sperm motility loss. Since UCP3 was only found to be expressed at the head, it is unlikely that the observed decrease in mitochondrial membrane potential is due to its inhibition. However, UCP1 and UCP2 were found to be expressed in the midpiece, where mitochondria are located, thus the decreased mitochondrial membrane may potentially occur due to its inhibition. Both UCP1 and UCP2 are well-known modulators of the mitochondrial membrane potential in addition to cellular metabolism (Jo et al., 2019; Li et al., 2018). Thus,

we also studied the metabolic profile of human spermatozoa upon UCPs inhibition and observed decreased lactate production. Lactate is the main metabolite released by human spermatozoa, as a product of glycolysis (Hereng et al., 2011). Notably, the inhibition of UCPs decreased lactate production at both 5 and 50 μ M, even though no motility alterations were observed with the lower concentration. Acetate production, on the other hand, was unaltered under all studied conditions. A decrease in lactate production due to UCPs inhibition has been reported in other cell types. Vozza et al. observed that the silencing of *UCP2* resulted in decreased lactate production by HepG2 cells, leading to the suggestion that UCPs favour glycolytic pathways through the modulation of Krebs cycle intermediates levels (Vozza et al., 2014). Similar results were observed in rat cardiomyocytes, in which *Ucp2* silencing decreased intracellular lactate levels (Kutsche et al., 2020). We hypothesize that the decreased lactate production is linked to decreased ATP production through glycolysis, which in turn would reduce spermatozoa motility. Taken together, our data suggest that UCPs are metabolic modulators of human spermatozoa, although the UCP-mediated molecular mechanisms remain to be elucidated.

Dysfunction of UCPs is positively related to oxidative stress, which is a known major cause of male infertility. While UCP1 is usually related to thermogenesis, UCP2 and UCP3 had been mainly studied as regulators of ROS production. Interestingly, UCP2 and UCP3 do not display a clear uncoupling activity, being activated due to ROS overproduction through a negative feedback mechanism (Anderson et al., 2007). It is reported that UCP2 and UCP3 overexpression reduces ROS production (Brand et al., 2002; Lee et al., 2005; Teshima et al., 2003), whereas its knockout increases oxidative stress (Horimoto et al., 2004; Vidal-Puig et al., 2000). Thus, UCPs are hypothesized to take part in the complex and finely regulated network that maintains cellular ROS balance. The mechanisms, however, are still fairly unknown. In this work, no differences concerning intracellular ROS production upon UCPs inhibition were observed. This finding could be explained by decreased mitochondrial activity. Mitochondria are the main source of ROS in human spermatozoa (Chianese & Pierantoni, 2021). In our previous study, we demonstrated that increased ROS production occurs in spermatozoa submitted to *in vitro* capacitation conditions after mitochondrial activation (Carrageta et al., 2020). As genipin decreases mitochondrial activity, an increased ROS production will not occur, suggesting that UCPs inhibition could, potentially, inhibit capacitation. This hypothesis, however, needs confirmation in future studies. Simultaneously, through the FRAP assay, we observed that genipin has antioxidant properties. The antioxidant properties of genipin have been reported before in the literature (Cho, 2022; Zhao et al., 2019). Interestingly, both the antioxidant properties and decreased mitochondrial activity were observed at 50 μ M, but ROS levels remained unaltered whereas a decrease could be expected. These results suggest that basal ROS levels are maintained,

further suggesting a finely regulated and complex network of ROS and ROS-dependent signaling pathways that regulates spermatozoa function.

Some limitations of this study should be considered. One of the limitations of this study is the small sample size and thus, studies with larger sample sizes should be conducted to confirm our hypotheses. In addition, the use of genipin as an UCPs inhibitor has its own limitations. Although initially reported as a selective UCPs inhibitor, it is currently known that genipin, at 50 μ M, is also able to inhibit the mitochondrial complex III (CIII) by 30% (Kreiter et al., 2019). The inhibition of CIII can be reflected in the decreased mitochondrial membrane potential and can also be related to the decreased motility due to inhibition of oxidative phosphorylation and decreased ATP production. Overall, we hypothesize that it is not the main contributor to the observed motility loss. Spermatozoa are known to have a higher glycolytic activity as compared to the Krebs cycle and oxidative phosphorylation (Guerra-Carvalho et al., 2022). In human spermatozoa, glycolysis is hypothesized to be the main source of ATP, which occurs mainly in the tail to sustain motility (Travis et al., 2001). Additionally, the well-known CIII inhibitor antimycin A was described as being more potent than genipin (Kreiter et al., 2019), and significantly decreased but not conferred loss of motility in spermatozoa (Plaza Davila et al., 2015). Thus, we conclude that UCPs inhibition promoted by genipin is the main cause for the observed motility loss.

In conclusion, our results demonstrate for the first time, to the best of our knowledge, the expression of UCP1-6 homologs in human spermatozoa. Interestingly, UCP1, UCP2, and UCP3 were found to be expressed at the equatorial segment of the head, besides the expression of UCP1 and UCP2 at the midpiece, where the mitochondria are located. UCPs are major regulators of spermatozoa motility, as its inhibition by genipin leads to a relatively rapid and irreversible motility loss. Interestingly, UCPs inhibition in human spermatozoa promotes motility loss without compromising its viability. Taking into consideration the concentration of genipin used, we propose that the motility loss occurs due to UCP3 inhibition, although the inhibition of other homologs could also contribute to a smaller extent. Interestingly, no altered UCP3 expression was found in spermatozoa from asthenozoospermic men, suggesting there are other causes for the dysfunction. UCPs are also modulators of human spermatozoa metabolism, and potentially of capacitation and acrosome reaction, although future studies are needed to address these hypotheses. Since silencing in human spermatozoa cannot be performed, the discovery and characterization of selective UCP inhibitors are required. Taken together, the discovery and characterization of UCPs' role in human spermatozoa physiology can shed some light on spermatozoa bioenergetics and the role of ROS and ROS-related pathways for capacitation and acrosome reaction. The potential role of UCPs (dys)function in male oxidative stress-related infertility and asthenozoospermia should also be highlighted, opening the path for future lines of investigation.

5.5. Authors contribution

David F. Carrageta – conceptualization, methodology, formal analysis, investigation, writing – original draft preparation, writing – review and editing; Laís Freire-Brito – methodology, investigation, writing – original draft preparation, writing – review and editing; Bárbara Guerra-Carvalho – methodology, formal analysis, writing – review and editing; João C. Ribeiro – methodology, writing – review and editing; Bruno S. Monteiro – methodology, writing – review and editing; Alberto Barros – resources, writing – review and editing; Pedro F. Oliveira – conceptualization, formal analysis, investigation, resources, writing – review and editing, funding acquisition; Mariana P. Monteiro – conceptualization, formal analysis, investigation, writing – review and editing; Marco G. Alves – conceptualization, formal analysis, investigation, resources, writing – review and editing, project administration, funding acquisition.

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Chapter 6

Mitochondrial uncoupling proteins regulate the metabolic function of human Sertoli cells



This chapter was adapted from the published work:

Carrageta DF, Freire-Brito L, Guerra-Carvalho B, Bernardino RL, Monteiro BS, Barros A, Oliveira PF, Monteiro MP, Alves MG. (2023). Mitochondrial uncoupling proteins regulate the metabolic function of human Sertoli cells. *Reproduction*, REP-23-0229. <https://doi.org/10.1530/REP-23-0229>

Abstract

Mitochondrial uncoupling proteins (UCPs) are mitochondrial channels responsible for the transport of protons and small molecular substrates across the inner mitochondrial membrane. Altered UCPs expression or function are commonly associated with mitochondrial dysfunction and increased oxidative stress, which are both known causes of male infertility. However, UCPs expression and function in the human testis remains to be characterized. This study aimed to assess the UCP homologs (UCP1-6) expression and function in primary cultures of human Sertoli cells (hSCs). We identified the mRNA expression of all UCP homologs (*UCP1-6*) and protein expression of UCP1, UCP2, and UCP3 in hSCs. UCPs inhibition by genipin for 24 h decreased hSCs proliferation without causing cytotoxicity ($n = 6$). Surprisingly, the prolonged UCPs inhibition for 24 h decreased mitochondrial membrane potential, oxygen consumption rate (OCR), and endogenous reactive oxygen species (ROS) production. The metabolism of hSCs was also affected as UCPs inhibition shifted their metabolism towards an increased pyruvate consumption. Taken together, these findings demonstrate that UCPs play a role as regulators of the mitochondrial function in hSCs, emphasizing their potential as targets in the study of male (in)fertility.

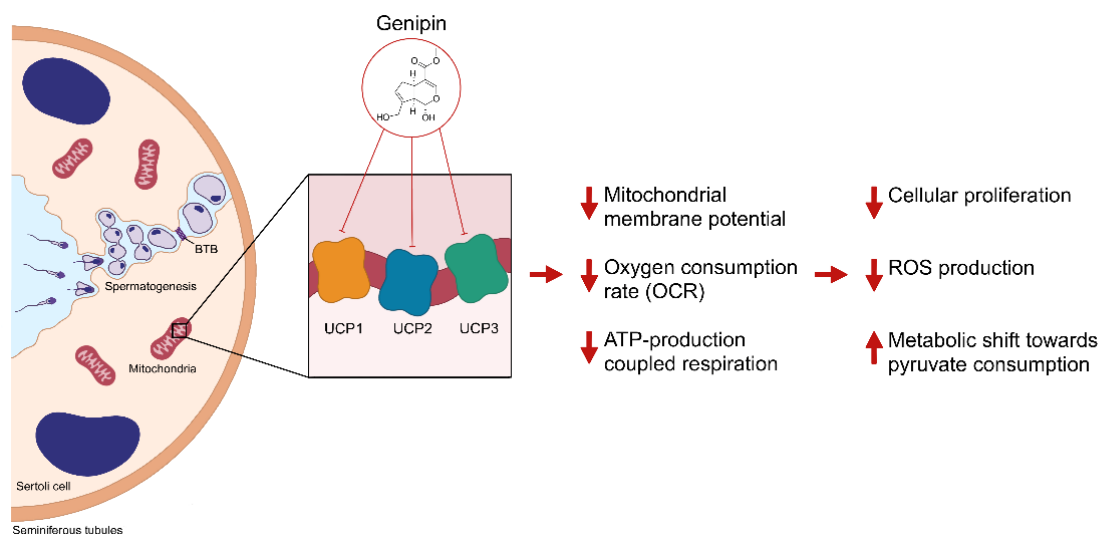


Fig. 6 – Graphical abstract. In this work we provide evidence that UCP1, UCP2, and UCP3 are expressed on the mitochondria of human Sertoli cells. UCPs are major regulators of the mitochondrial activity and its inhibition greatly impairs oxidative phosphorylation, which decreases ATP production and cellular proliferation. Additionally, the inhibition of UCPs decreases reactive oxygen species (ROS) production and promotes a metabolic shift towards pyruvate consumption.

6.1. Introduction

Sertoli cells also take part in the shielding of spermatogenesis against oxidative stress (Aitken & Roman, 2008; Guerriero et al., 2014). Oxidative stress poses a significant threat to germ cells and is a leading cause of male infertility. Studies suggest that approximately 80% of infertile men experience high levels of reactive oxygen species (ROS), leading to defective spermatogenesis and poor sperm quality (Agarwal et al., 2019). Previous studies have demonstrated a positive correlation between high levels of oxidative stress and a reduction in both sperm concentration and motility (Athayde et al., 2007). In addition, DNA fragmentation and genomic instability are also common features of oxidative stress-induced damage in spermatozoa. This damage has been linked to impaired oocyte fertilization, abnormal embryo development, and higher rates of miscarriage (Aitken et al., 2010; Cissen et al., 2016; McQueen et al., 2019; Tarozzi et al., 2007). The aetiology of oxidative stress is multifactorial, where lifestyle factors, including alcohol consumption, smoking, exposure to pollutants or incidence of oxidative stress-related diseases, such as metabolic diseases or chronic inflammation, have been associated with poorer sperm parameters (Martin-Hidalgo et al., 2019). However, the molecular mechanisms responsible for oxidative stress-related Sertoli and germ cell damage are also poorly understood.

Mitochondrial uncoupling proteins (UCPs) are proteins of the anionic transporter family SLC25 expressed in the inner mitochondrial membrane known to regulate mitochondrial function and ROS production. Six homologs of mammalian UCPs (UCP1-6) have already been identified (Monteiro et al., 2021). UCP1 is the classic mitochondrial respiration uncoupler predominantly found in mammals' brown adipose tissue, which facilitates non-shivering thermogenesis through the dissipation of the proton gradient for heat production (Carrageta et al., 2020b; Ikeda & Yamada, 2020). In comparison, evidence highlights that UCP2 to 6 exhibit poorer or even absent uncoupling activity, suggesting that alternative mechanisms are involved in the maintenance of redox homeostasis. Compelling evidence highlights that UCP2 and UCP3 play a protective role in conditions characterized by increased ROS levels, including inflammation (Alves-Guerra et al., 2003; Kizaki et al., 2002), brain and cardiac ischemia (McLeod et al., 2005; Nakase et al., 2007), neurodegenerative (Barnstable et al., 2022; Kumar et al., 2022), and metabolic diseases (Busiello et al., 2015; Monteiro et al., 2021), although the mechanisms are poorly understood. Recent studies demonstrated that the remaining homologs may act as small molecules transporters instead of respiratory uncouplers (Hirschenson et al., 2022). Vozza et al. reported that UCP2 can transport C4 metabolites, promoting the exchange of malate, oxaloacetate, or aspartate in exchange for phosphate and a proton from the mitochondria to the cytosol (Vozza et al., 2014). Thus, UCPs may act as metabolic regulators besides their protective role against OS.

Due to its role in ROS regulation and potential function as metabolic modulators, we hypothesize that UCPs could be relevant targets involved in the metabolic modulation and redox homeostasis of human Sertoli cells (hSCs). In this work, we aimed to characterize the expression and function of UCPs in human Sertoli cells hSCs. For that purpose, we screened the expression of *UCP1* to *6* mRNA in hSCs. Protein expression of UCP1, UCP2, and UCP3 was also analyzed. To evaluate the function of UCPs in hSCs, we used the UCPs chemical inhibitor genipin (Kreiter et al., 2019). The potential cytotoxic effect of UCPs inhibition on hSCs was determined. In addition, the impact of UCPs inhibition on hSCs mitochondrial function, endogenous ROS production, and metabolic profile were also analyzed.

6.2. Materials and methods

6.2.1. Reagents and chemicals

Unless stated otherwise, all reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

6.2.2. Patient recruitment and seminal samples preparation

Participants were recruited among patients attending the Medical Assisted Procreation Center after clinical evaluation performed in compliance with the standards of care. Men with prior history of illicit drug or alcohol abuse, under treatment with drugs known to interfere with fertility, diagnosed with an acute genital inflammatory disease, cancer submitted to any anti-neoplastic treatments, or any systemic disease known to affect fertility were excluded from this study. Testicular biopsies were obtained from six healthy men ($n = 6$) with conserved spermatogenesis (mean age 40.4 ± 4.0 years old and BMI 24.2 ± 3.6 kg/m²) performed for medical procreation treatments in result of anejaculation due to obstructive azoospermia or incidence of varicocele in the non-biopsied testis. Samples were only used after gamete recovery. Patient recruitment and all study procedures were conducted following the ethical guidelines for human sample research, under strict individual anonymity, informed and written consent was obtained from all individual donors, after approval by the Joint Ethics Committee CHUP/ICBAS (2021/CE/P002[P342/CETI/ICBAS]). All experiments were performed in compliance with Good Clinical Practice Guidelines, of the Local, National, and European Ethical Committees, as well as with the Data Protection Regulations.

6.2.3. Human Sertoli cell primary culture

Primary cultures of human Sertoli cells (hSCs) were established according to previously described methods (Bernardino et al., 2018a). In brief, testicular biopsies were washed two times in filtered Hanks' Balanced Salt Solution (HBSS) and centrifuged at 300g for 5 min at room temperature. The resulting pellet was suspended in hSCs culture medium (1:1 mixture of DMEM:F-12 supplemented with 15 mM HEPES, 14 mM NaHCO₃, 50 U/mL penicillin, 50 U/mL streptomycin sulfate, 2.5 mg/mL amphotericin B, 50 µg/mL gentamicin, and 10% fetal bovine serum (FBS)). The pellet was forced through a 20 gauge needle to disaggregate large cell clusters, seeded in culture flasks, and incubated at 37°C with an atmosphere of 95% O₂ and 5% CO₂. Cultures were evaluated under a microscope after 96 hours and purity was then assessed as previously described (Bernardino et al., 2018b). Only cultures with a purity higher than 95% were used. Each "n" used for the subsequent experiments corresponds to a primary cell culture obtained from a distinct testicular biopsy.

6.2.4. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from hSCs using the NZY Total RNA Isolation Kit ((MB13402, NZYTech, Lisbon, Portugal) according to the manufacturer's instructions. Purified RNA concentration was determined using a Take3 microvolume plate on a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). RNA was reversely transcribed using the NZY M-MuLV Reverse Transcriptase Kit (MB083, NZYTech, Lisbon, Portugal). RT-PCR was performed to identify the expression of UCP1, UCP2, UCP3 (UCP3S and UCP3L for short and long isoforms, respectively), UCP4, UCP5, and UCP6 mRNAs in hSCs using the resulting complementary DNA (cDNA) and specific primer sets (Table 6.1). Human peripheral blood leukocytes (PBL) cDNA was used as a positive control. Isolation of PBL was performed using SepMate™ Isolation Tubes (STEMCELL Technologies Inc, Vancouver, Canada) according to the manufacturer's instructions. Total RNA and reverse transcriptase reaction were performed according to the methods herein described.

6.2.5. Western blot

Proteins from hSCs were extracted using radioimmunoprecipitation assay (RIPA) buffer and then quantified using Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA). Western Blot protocol was performed to identify the expression of UCP1, UCP2, and UCP3 using previously described methods (Bernardino et al., 2016). In brief, protein

samples (30 µg) were denatured at 95°C for 5 min (UCP3) or 70°C for 10 min (UCP1 and UCP2). Protein samples were fractionated on a 12.5% polyacrylamide gel from TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad, Hemel Hempstead, UK) and transferred to a low fluorescence polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hemel Hempstead, UK) using a Trans-Blot® Turbo™ System (Bio-Rad, Hemel Hempstead, UK). Membranes were blocked with a solution of 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 solution. Then, membranes were incubated overnight at 4°C separately with primary antibodies rabbit anti-UCP1 (1:500, ab155117, Abcam, Cambridge, UK), rabbit anti-UCP2 (1:1000, PA5-77553, Invitrogen, Waltham, MA, USA), or rabbit anti-UCP3 (1:500, ab180643, Abcam, Cambridge, UK). Proteins were detected upon incubation with goat anti-rabbit IgG-AP (1:5000, A3687, Sigma-Aldrich, St. Louis, MO, USA) and reacted with ECF Substrate (RPN5785, Cytiva, Marlborough, MA, US). Protein bands were detected using a Bio-Rad ChemiDoc XR (Bio-Rad, Hemel Hempstead, UK).

Table 6.1. Oligonucleotides and cycling conditions for RT-PCR amplification of mitochondrial uncoupling proteins (UCPs) homologs.

Target (accession number)	Sequence 5'-3'	Annealing temperature	Number of cycles
UCP1 (NM_021833.5)	FWD: CTAACGACTGGAGGAGTGGC RVS: GTGGAGATGGCTCTGTGCTT	59 °C	35
UCP2 (NM_001381943.1)	FWD: GAAGCCTCTACAATGGGCTGG RVS: CAGAGCCCTTGGTGTAGAACTG	63 °C	30
UCP3S (NM_022803.3)	FWD: CAACCTGGGATGTAGCGGTG RVS: GAGCGTGTGGAGACAGTGAG	60 °C	30
UCP3L (NM_003356.4)	FWD: TGGAACGTGGTGATGTTTCGTA RVS: AGACCAGAATCCCTCCTCCT	60 °C	35
UCP4 (NM_004277.5)	FWD: CGCACAGCTCTAGGGATCAT RVS: CGTGTCTGTAAATGGCGGGT	60 °C	35
UCP5 (NM_001282195.2)	FWD: GACTTTCCCTGTGGACCTTACC RVS: GCGCATGGAACATCCCTCTAT	60 °C	35
UCP6 (NM_001010875.4)	FWD: ACCCTGTTGATGTTGTGAGG RVS: TAGCCAGAACATCTGCCATCTC	63 °C	35

6.2.6. Immunofluorescence staining

An immunofluorescence protocol was performed to observe the expression of UCP1, UCP2, and UCP3, using previously described methods (Pereira et al., 2021). In brief, hSCs were seeded on glass coverslips inside a 12-well culture plate and cultured as described above. When 60-70% confluence was reached, cells were fixed with 10% formalin for 20 min at room temperature. Then, cells were permeabilized using 0.1% Triton X-100 in PBS and blocked in 0.1% gelatin in PBS, both for 15 min at room temperature. Cells were then incubated with primary antibodies rabbit anti-UCP1 (1:50), rabbit anti-UCP2 (1:50), or rabbit anti-UCP3 (1:50) overnight at 4 °C. An anti-ATP synthase β -subunit (1:50, ab14730, Abcam, Cambridge, UK) was used as mitochondrial marker for co-localization analysis. Cells incubated without primary antibodies were used as a negative control. Incubation with the secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (1:1500, A-11001, Thermo Fischer Scientific, Waltham, MA, USA) and Alexa Fluor 555 goat anti-rabbit IgG (1:1500, #4413, Cell Signaling Technology, Danvers, MA, USA) occurred for 1 h at room temperature. Coverslips were mounted and nuclei were stained with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Fluorescence was observed in a Nikon Eclipse E400 microscope equipped with a DS-Fi3 microscope camera, a Y-FL Epi-Fluorescence Attachment, and HB-10103AF Super High-Pressure Mercury Lamp Power Supply (Nikon, Shinagawa, Tokyo, Japan). DAPI filter (395/425 nm; excitation/emission) was used for observing the nuclei, FITC filter (480/510 nm; excitation/emission) was used to observe ATP synthase, and G-2A filter (510/575 nm; excitation/emission) was used to observe UCPs. The obtained pictures were processed using the NIS-Elements Br 4.60.00 software (Nikon, Shinagawa, Tokyo, Japan).

6.2.7. Experimental groups

The function of UCPs for hSCs was studied using genipin (methyl 1-hydroxy-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta[c]pyran-4-carboxylate, Acros Organics™, Thermo Fisher Scientific, Waltham, MA, USA), a chemical inhibitor of UCP1, UCP2, and UCP3 (Kreiter et al., 2019). Cells were cultured in serum-free hSCs medium supplemented with an insulin-transferrin-sodium selenite solution (10 mg/L, 5.5 mg/L, 6.7 μ g/L, respectively) and increasing concentrations of genipin (0.5, 5, 50, and 100 μ M). DMSO was used as vehicle (control group), which was applied in the same concentration in all tested groups. After 24 h incubation, culture media were collected for Proton Nuclear Magnetic Resonance (^{1} H-NMR) analysis. Cells were detached from the culture flask with trypsin-EDTA solution,

centrifuged, collected, and protein extracted and quantified as aforementioned. Culture media and protein samples were stored at -80 °C until further use.

6.2.8. Cellular viability analysis

Cellular viability was examined through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and lactate dehydrogenase (LDH) release assays, according to previously described methods (Carrageta et al., 2018; Martins et al., 2019). Cells were seeded in 24-well plates and cultured as described above. Experiments were performed in triplicates per plate when 70-80% confluence was reached. After 24 h treatment, the culture medium was collected and replaced with 500 µL of fresh serum-free culture medium and 50 µL of MTT solution (5 mg/mL in PBS). Plates were then incubated in a dark environment for 3.5 h at 37 °C. After incubation, the culture medium was replaced with 250 µL of DMSO, followed by agitation to solubilize formazan crystals. Then, 100 µL were transferred to a 96-well plate, and absorbance was measured at 570 nm using a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). DMSO was used as blank. For the LDH release assay, 100 µL of the collected media was transferred to a 96-well plate. LDH released to the extracellular medium was quantified using the LDH Citox™ Assay Kit (Biolegend, San Diego, CA, USA), according to the manufacturer's instructions. Absorbance was read at 490 nm using a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). Results from both assays were normalized and expressed as fold variation to the vehicle group.

6.2.9. Cellular proliferation analysis

Cellular proliferation was determined using the Sulforhodamine B (SRB) dye, according to a previously established method (Martins et al., 2019). In brief, the same number of cells were seeded in 24-well plates and cultured as described above. Experiments were performed with triplicates per plate when 60-70% confluence was reached. After 24 h treatment, cells were washed with PBS and fixed with 1% acetic acid in methanol solution for 1 h at -20 °C. Plates were left to dry and cells were stained with a 0.05% SRB solution for 1 h at 37 °C. Cells were washed with 1% acetic acid and de-stained with a 10 mM Tris (pH = 10) solution. Then, 100 µL were transferred to a 96-well plate and absorbance was read at 490 nm using a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). Results were normalized and expressed as fold variation to the vehicle group.

6.2.10. Mitochondrial membrane potential

Mitochondrial membrane potential was evaluated using the fluorogenic dye JC-1 (T3168, Invitrogen™, Carlsbad, CA, USA) (Carrageta et al., 2022). Cells were seeded in a 96-well black culture plate with a clear bottom (#3904, Corning®, New York, NY, USA) and grown until 80% confluence. Experiments were performed with triplicates per plate, including the assay positive control. After treatment, cells were washed with PBS and incubated in a serum-free culture medium supplemented with JC-1 (1 μ M) at 37°C for 30 min, in a dark environment. Media supplemented with 20% DMSO was used as the assay positive control. Then, cells were washed with PBS, fresh culture media was added, and fluorescence of JC-1 monomers (485/530 nm; excitation/emission) and J-aggregates (535/590 nm; excitation/emission) was evaluated using a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). The ratio between J-aggregates/monomers was calculated and used as an indicator for the mitochondrial membrane potential.

6.2.11. Detection of intracellular reactive oxygen species (ROS)

Total intracellular ROS production of hSCs was quantified using the fluorogenic dye CM-H₂DCFDA (C6827, Invitrogen™, Carlsbad, CA, USA) (Carrageta et al., 2020a). The protocol was performed according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well black culture plate with clear bottom and grown until 80% confluence. Experiments were performed with triplicates per plate, including the assay positive control. After treatment, cells were washed with PBS and incubated in a CM-H₂DCFDA working solution (5 μ M in PBS) at 37°C for 30 min. A working solution supplemented with 0.1% H₂O₂ was used as the positive control. Fluorescence (495/529 nm; excitation/emission) was read on a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). Results are represented in DCF Fluorescence Units (fold variation to the vehicle group).

6.2.12. Mitochondrial oxygen consumption rate (OCR) analysis

Mitochondrial oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of hSCs were evaluated using the Mito Stress Test Kit and the Seahorse XFe24 Analyzer (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. Cells were seeded in 24-well culture plates and grown until 80% confluence. Experiments were performed in duplicates per plate. After treatment, cells were washed with PBS and incubated with Seahorse assay medium (DMEM medium, pH = 7.4, Agilent Technologies)

supplemented with pyruvate (1 mM), glutamine (2 mM), and glucose (10 mM) for 40 min at 37°C in a non-CO₂ incubator. Then, fresh assay medium was added and OCR was measured following a standard protocol: equilibrium phase for basal OCR assessment following injection with oligomycin (1.5 µM), FCCP (1 µM), and antimycin A/rotenone (0.5 µM). Between each injection, three measurements of OCR were recorded. All recordings were 8 min apart, for a total time of 100-120 min per assay. OCR values were normalized for protein concentration using a standard BCA assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, USA), and mean basal respiration, proton leak, maximal respiration, spare respiratory capacity, ATP-production coupled respiration, and non-mitochondrial oxygen consumption was calculated. Results are expressed as mean OCR (pmol/min/µg protein).

6.2.13. Proton nuclear magnetic resonance (¹H-NMR)

Proton nuclear magnetic resonance (¹H-NMR) spectra were acquired using a Bruker Avance III HD 500 MHz spectrometer equipped with a 5-mm TXI probe (Bruker Corporation, Billerica, MA, USA). Solvent-suppressed ¹H-NMR spectra were acquired at 298K with a zgpr pulse with a sweep width of 7 kHz, using a relaxation delay of 7 s, a pulse angle of 30°, an acquisition time of 2.3 s, and 32 scans. Before Fourier transform, each free induction decay was multiplied by a 0.2 Hz Lorentzian. A total of 480 µL of the medium of each condition was analyzed. Samples were diluted with sodium fumarate (final concentration of 2 mM) in deuterated water solution (final volume of 600 µL), which was used as an internal reference (6.50 ppm) to quantify the following metabolites present in the medium (multiplet, chemical shift (ppm)): alanine (doublet, 1.46), formate (singlet, 8.44), glucose (doublet of doublets, 3.52), glutamine (multiplet, 2.43), lactate (doublet, 1.31), phenylalanine (multiplet, 7.41), pyruvate (singlet, 2.35), tyrosine (doublet, 6.88), and valine (doublet, 0.97). Spectra were manually phased and baseline corrected. Chosen metabolite peaks were integrated using the NUTS-Pro NMR software (Acorn NMR, Inc, Fremont, CA, USA). Metabolite variation (consumption/production) in each condition was determined and the results are expressed as nmol/µg protein.

6.2.14. Statistical analysis

Results are represented as Tukey's boxplot (median, 25th to 75th percentiles ± 1.5 IQR). Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Data were tested for normal distribution with the Kolmogorov-Smirnov test.

Statistical differences were determined by a paired *t*-test or repeated measures one-way ANOVA with *post hoc* Dunnett's multiple comparisons test. $p < 0.05$ was considered significantly different.

6.3. Results

6.3.1. Mitochondrial uncoupling proteins (UCPs) are expressed in hSCs

We screened for the mRNA expression of all known UCPs homologs (*UCP1-6*) in hSCs. Human peripheral blood leukocytes (PBL) were used as positive control (Carrageta et al., 2023). We were able to identify the expression of all tested homologs – *UCP1*, *UCP2*, *UCP3S* (*UCP3* short isoform), *UCP3L* (*UCP3* long isoform), *UCP4*, *UCP5*, and *UCP6* mRNA in hSCs (Fig. 6.1A). Then, *UCP1*, *UCP2*, and *UCP3* protein expression were evaluated through Western Blot (Fig. 6.1B) and immunofluorescence staining (Fig. 6.1C). Protein expression of *UCP4*, *UCP5*, and *UCP6* was not tested due to the lack of validated antibodies commercially reliable. We were able to identify a blot band of *UCP2* at the predicted molecular weight (≈ 34 kDa). On the other hand, *UCP1* and *UCP3* presented blot bands at 4 times or 2 times the predicted molecular weight (≈ 132 kDa and ≈ 62 kDa, respectively), which could be indicative of the dimeric or oligomeric assembly, as previously reported in the literature (Ledesma et al., 2002). Through immunofluorescence labeling, we were also able to visualize the expression of *UCP1* (Fig. 6.1Cb), *UCP2* (Fig. 6.1Cc), and *UCP3* (Fig. 6.1Cd) in hSCs. Using ATP synthase as a mitochondrial marker, we observed the same scattered pattern throughout the cells, confirming the expression of *UCP1*, *UCP2*, and *UCP3* in the mitochondria of hSCs.

6.3.2. Inhibition of UCPs for 24 h decreases hSCs cellular proliferation without inducing cytotoxicity

The role of UCPs in hSCs was further studied using genipin, a chemical inhibitor of *UCP1*, *UCP2*, and *UCP3* (Kreiter et al., 2019). Cellular proliferation was assessed after UCPs inhibition by genipin (0.5, 5, 50, and 100 μ M) for 24 h (Fig. 6.2A). We observed that UCPs inhibition for 24 h decreased cellular proliferation at 50 μ M (0.81 ± 0.29 , $p = 0.018$) and 100 μ M (0.80 ± 0.28 , $p = 0.013$), as compared to the vehicle group (1.00 ± 0.30). However, no differences were observed with the lower concentrations of 0.5 μ M (0.88 ± 0.30) and 5 μ M (0.87 ± 0.30).

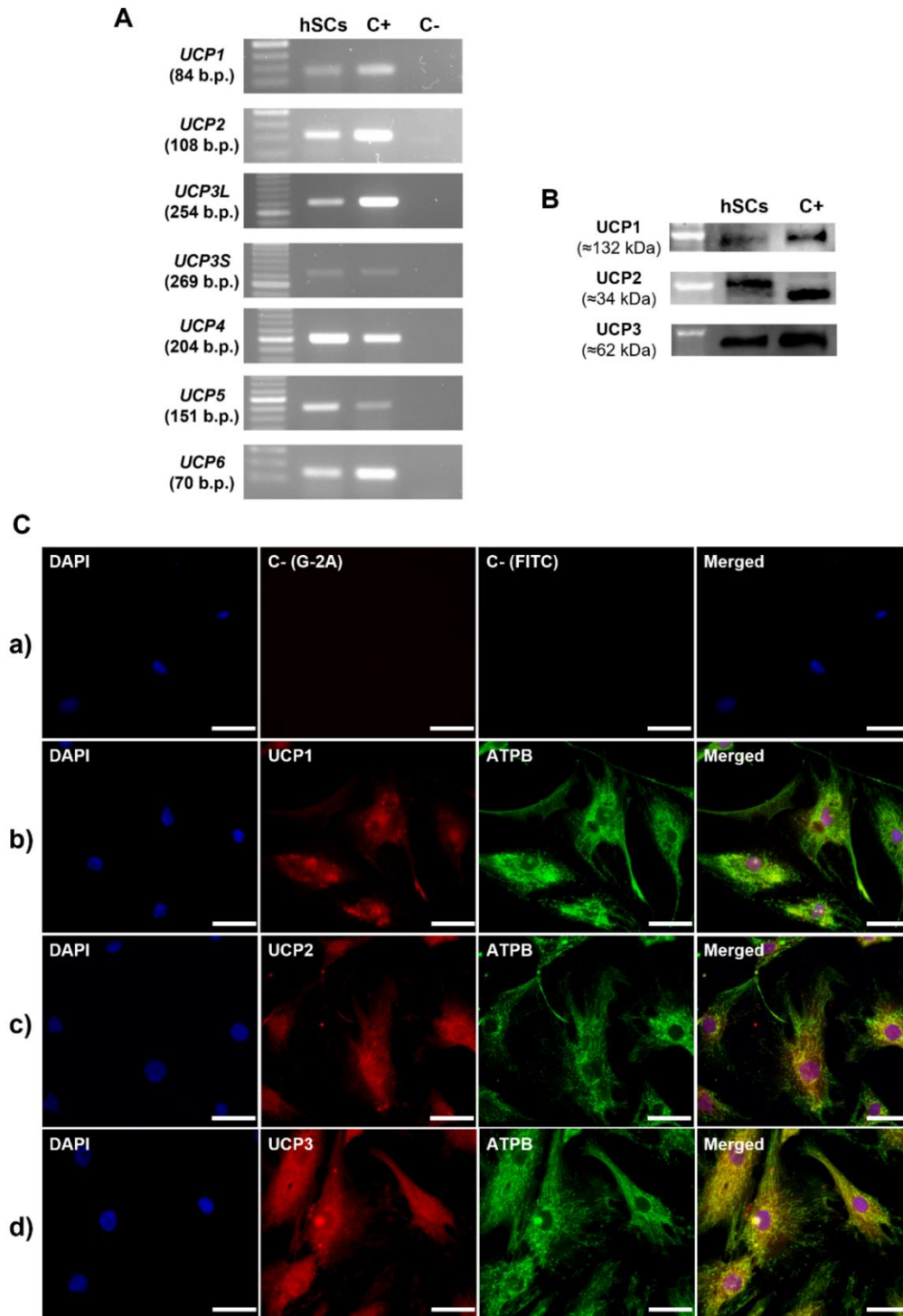


Fig. 6.1 – Identification of mitochondrial uncoupling proteins (UCPs) in human Sertoli cells (hSCs). **(A)** Representative RT-PCR for the evaluation of UCP1, UCP2, UCP3L, UCP3S, UCP4, UCP5, and UCP6 mRNA expression in hSCs. Human peripheral blood leukocytes (C+) were used as a positive control. **(B)** Representative blots of UCP1, UCP2, and UCP3 protein expression in hSCs. Mouse kidney was used as a positive control (C+). **(C)** Immunofluorescence staining for UCP1, UCP2, and UCP3 protein expression at hSCs. ATP synthase β -subunit (ATPB) was used as mitochondrial biomarker for co-localization. **a)** Negative control. Immune labeling of **b)** UCP1, **c)** UCP2, and **d)** UCP3 were observed in hSCs. Scale bar – 50 μ m.

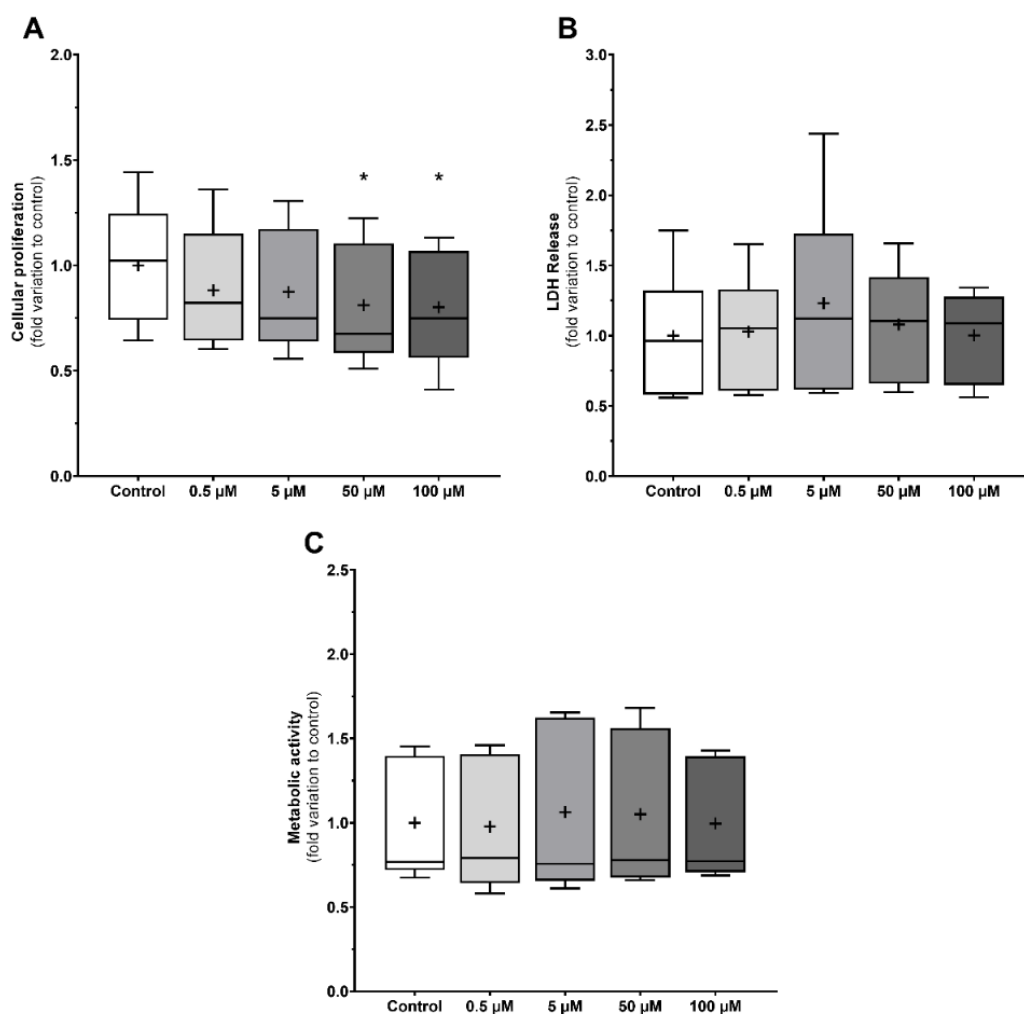


Fig. 6.2 – Cellular proliferation and cytotoxicity in human Sertoli cells (hSCs) upon UCPs inhibition by genipin (0, 0.5, 5, 50, and 100 µM) for 24 h (n = 6, triplicates for each condition). (A) Cellular proliferation as determined by (SRB) assay. **(B)** Quantification of the released lactate dehydrogenase (LDH). **(C)** Evaluation of the metabolic activity as determined by the MTT assay. Results were normalized to the control group and expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). + – Represents group average. Significantly different results are expressed as: * – Relative to the control group.

Although cellular proliferation was affected, no cytotoxicity effects were observed upon UCPs inhibition. No differences in the quantification of extracellular LDH (Fig. 6.2B) were observed between all experimental groups (1.03 ± 0.40 , 1.23 ± 0.68 , 1.08 ± 0.40 , and 1.00 ± 0.32 after treatment with 0.5, 5, 50, or 100 µM genipin, respectively) when compared to the vehicle group (1.00 ± 0.44). Likewise, no differences in the MTT assay (Fig. 6.2C) were observed between all experimental groups (0.98 ± 0.40 , 1.06 ± 0.51 , 1.05 ± 0.48 , and 0.99

± 0.37 after treatment with 0.5, 5, 50, or 100 μM genipin, respectively) when compared to the vehicle group (1.00 ± 0.70), suggesting no alterations in the activity of oxidoreductases.

6.3.3. Inhibition of UCPs for 24 h decreases mitochondrial activity and total ROS production in hSCs

As UCPs are known to modulate mitochondrial activity and ROS production, our study aimed to assess the impact of UCPs inhibition on the physiological activity of mitochondria in hSCs. To evaluate the mitochondrial function, we first assessed the mitochondrial membrane potential by using the fluorogenic dye JC-1 (Fig. 6.3A). Surprisingly, we observed that the inhibition of UCPs for 24 h by genipin (100 μM) decreased the JC-1 ratio (1.22 ± 0.62 J-aggregates/monomers) as compared to the vehicle group (1.83 ± 1.03 J-aggregates/monomers, $p = 0.007$), suggesting mitochondrial depolarization and decreased mitochondrial activity. We also observed a tendency for a dose-dependent decrease of the mitochondrial membrane potential, although no statistical differences were found when the remaining groups (1.71 ± 0.92 ; 1.67 ± 0.92 ; 1.64 ± 0.79 J-aggregates/monomers for 0.5, 5, and 50 μM , respectively) were compared to the vehicle group. Since mitochondria are the paramount source of intracellular ROS and UCPs are directly involved in its regulation, we assessed the total intracellular ROS production (Fig. 6.3B). Likewise, the highest concentration of genipin (100 μM) decreased total ROS production (0.82 ± 0.35) as compared to the vehicle group (1.00 ± 0.43 , $p < 0.001$). No differences were observed when the remaining groups (1.04 ± 0.47 ; 1.05 ± 0.48 ; 0.97 ± 0.43 for 0.5, 5, and 50 μM , respectively) were compared to the vehicle group.

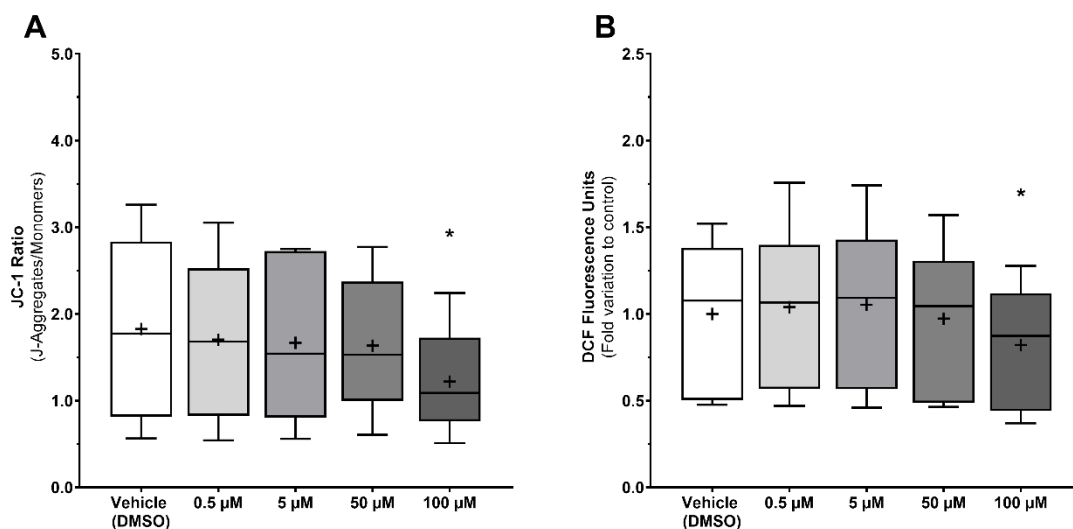


Fig. 6.3 – Mitochondrial membrane polarization and endogenous ROS production in human Sertoli cells (hSCs) after UCPs inhibition by genipin (0, 0.5, 5, 50, and 100 μM) for 24 h ($n = 6$,

triplicates for each condition). (A) Evaluation of mitochondrial membrane potential using the JC-1 dye. **(B)** Evaluation of endogenous ROS production using the CM-H₂DCFDA dye. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). + – Represents group average. Significantly different results are expressed as: * – Relative to the vehicle group.

6.3.4. UCPs inhibition for 24 h decreases hSCs mitochondrial oxygen consumption rate (OCR)

To further investigate the impact of UCPs inhibition on the mitochondrial function in hSCs, we conducted measurements of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) using the Seahorse XF24 Mito Stress Assay (Fig. 6.4). We observed a dose-dependent decrease in both OCR (Fig. 6.4A) and ECAR (Fig. 6.4B) after UCPs inhibition for 24 h. In detail, we detected a dose-dependent decrease in basal respiration (Fig. 6.4C), proton leak (Fig. 6.4D), spare respiratory capacity (Fig. 6.4F), and ATP-production coupled respiration (Fig. 6.4G) as hSCs were incubated with raising concentrations of genipin. In addition, we observed that 100 μ M genipin greatly impaired hSCs mitochondrial function, as it greatly decreased basal respiration (0.062 ± 0.091 pmol/min/ μ g protein) as compared to the vehicle group (0.235 ± 0.229 pmol/min/ μ g protein, $p = 0.016$). We also observe a tendency for a dose-dependent decrease in the remaining groups (0.208 ± 0.135 ; 0.190 ± 0.157 ; 0.157 ± 0.150 pmol/min/ μ g protein for 0.5, 5, and 50 μ M, respectively). Likewise, 100 μ M genipin greatly decreased proton leak (0.014 ± 0.021 pmol/min/ μ g protein) as compared to the vehicle group (0.062 ± 0.058 pmol/min/ μ g protein, $p = 0.014$) but the same effect did not occur in lower concentrations (0.052 ± 0.035 ; 0.047 ± 0.052 ; 0.038 ± 0.044 pmol/min/ μ g protein for 0.5, 5, and 50 μ M, respectively). Maximal respiration (Fig. 6.4E), which reflects the maximal capacity of the electron respiratory chain, was also greatly affected by 100 μ M genipin (0.100 ± 0.056 pmol/min/ μ g protein) as compared to the vehicle group (0.306 ± 0.144 pmol/min/ μ g protein, $p = 0.010$), but the same effect was not observed with lower concentrations (0.402 ± 0.195 ; 0.390 ± 0.225 ; 0.295 ± 0.197 pmol/min/ μ g protein for 0.5, 5, and 50 μ M, respectively). Spare respiratory capacity, which was obtained as the difference between the maximal and basal respiration, was also decreased by 100 μ M genipin (0.052 ± 0.048 pmol/min/ μ g protein) as compared to the vehicle group (0.290 ± 0.350 pmol/min/ μ g protein, $p = 0.035$). Similarly, lower concentrations exhibited a tendency towards a dose-dependent response (0.193 ± 0.126 ; 0.202 ± 0.149 ; 0.140 ± 0.134 pmol/min/ μ g protein for 0.5, 5, and 50 μ M, respectively).

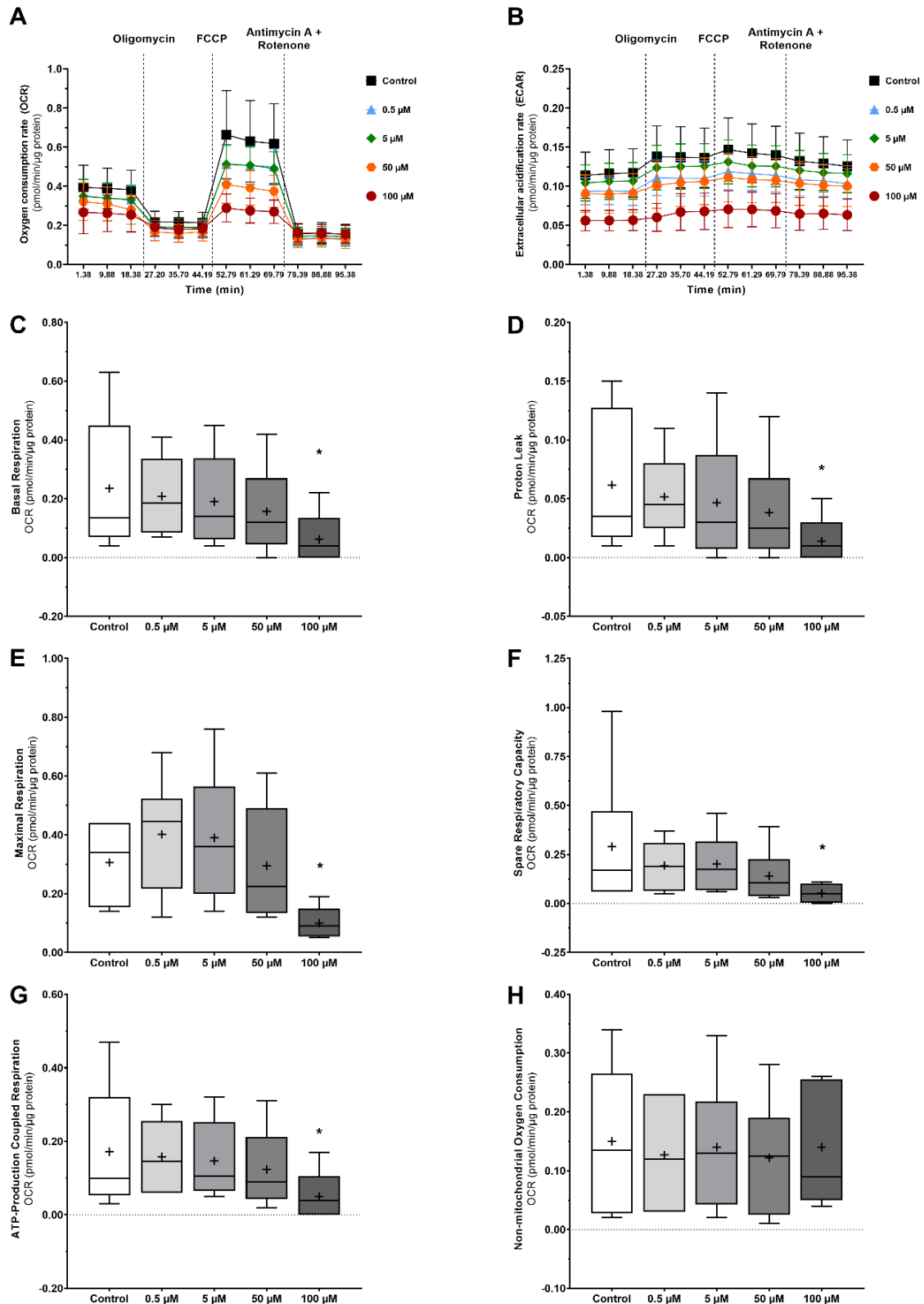


Fig. 6.4 – Mitochondrial respiratory function of human Sertoli cells (hSCs) after UCPs inhibition by genipin (0, 0.5, 5, 50, and 100 μ M) for 24 h (n = 6, triplicates for each condition). (A) Oxygen consumption rate (OCR). (B) Extracellular acidification rate (ECAR). (C) Basal respiration. (D) Proton leak. (E) Maximal respiration. (F) Spare respiratory capacity. (G) ATP-production coupled respiration. (H) Non-mitochondrial oxygen consumption. Results are expressed as Tukey's boxplot

(median, 25th to 75th percentiles $\pm$ 1.5 IQR). + – Represents group average. Significantly different results are expressed as: * – Relative to the control group.

We also observed that 100 μ M genipin had a negative impact on ATP-production coupled respiration (0.050 ± 0.070 pmol/min/ μ g protein) as compared to the vehicle group (0.172 ± 0.168 pmol/min/ μ g protein, $p = 0.018$). Lower concentrations of genipin showed a tendency to a dose-dependent decrease (0.158 ± 0.101 ; 0.147 ± 0.106 ; 0.123 ± 0.107 pmol/min/ μ g protein for 0.5, 5, and 50 μ M, respectively). On the other hand, no differences were observed concerning the non-mitochondrial oxygen consumption (Fig. 6.4H; 0.127 ± 0.090 ; 0.140 ± 0.110 ; 0.122 ± 0.099 ; 0.140 ± 0.107 pmol/min/ μ g protein for 0.5, 5, 50, and 100 μ M, respectively) as compared to the vehicle group (0.150 ± 0.123 pmol/min/ μ g protein), evidencing that the previous results are a consequence of an impaired mitochondrial function.

6.3.5. UCPs inhibition for 24 h promotes pyruvate consumption by hSCs

To examine whether the resulting decrease in mitochondrial activity affected the metabolic signature of hSCs, we analyzed the culture media using ¹H-NMR spectroscopy. For that purpose, we identified and quantified glucose (Fig. 6.5A) and pyruvate (Fig. 6.5B) consumption and lactate (Fig. 6.5C) and alanine (Fig. 6.5D) production. We observe a tendency for a decreased glucose consumption (96 ± 26 ; 98 ± 28 ; 88 ± 44 ; 82 ± 29 nmol/ μ g protein for 0.5, 5, 50, and 100 μ M genipin, respectively), as compared to the vehicle group (107 ± 46 nmol/ μ g protein). Interestingly, we noted an increased pyruvate consumption at 100 μ M genipin (9 ± 6 nmol/ μ g protein, $p = 0.036$) as compared to the vehicle group (6 ± 2 nmol/ μ g protein). No differences concerning pyruvate consumption were observed when lower concentrations (5 ± 3 ; 6 ± 3 ; 7 ± 3 nmol/ μ g protein for 0.5, 5, 50 μ M genipin, respectively) were compared to the vehicle group. No differences were also observed concerning either lactate or alanine production as compared to the vehicle group.

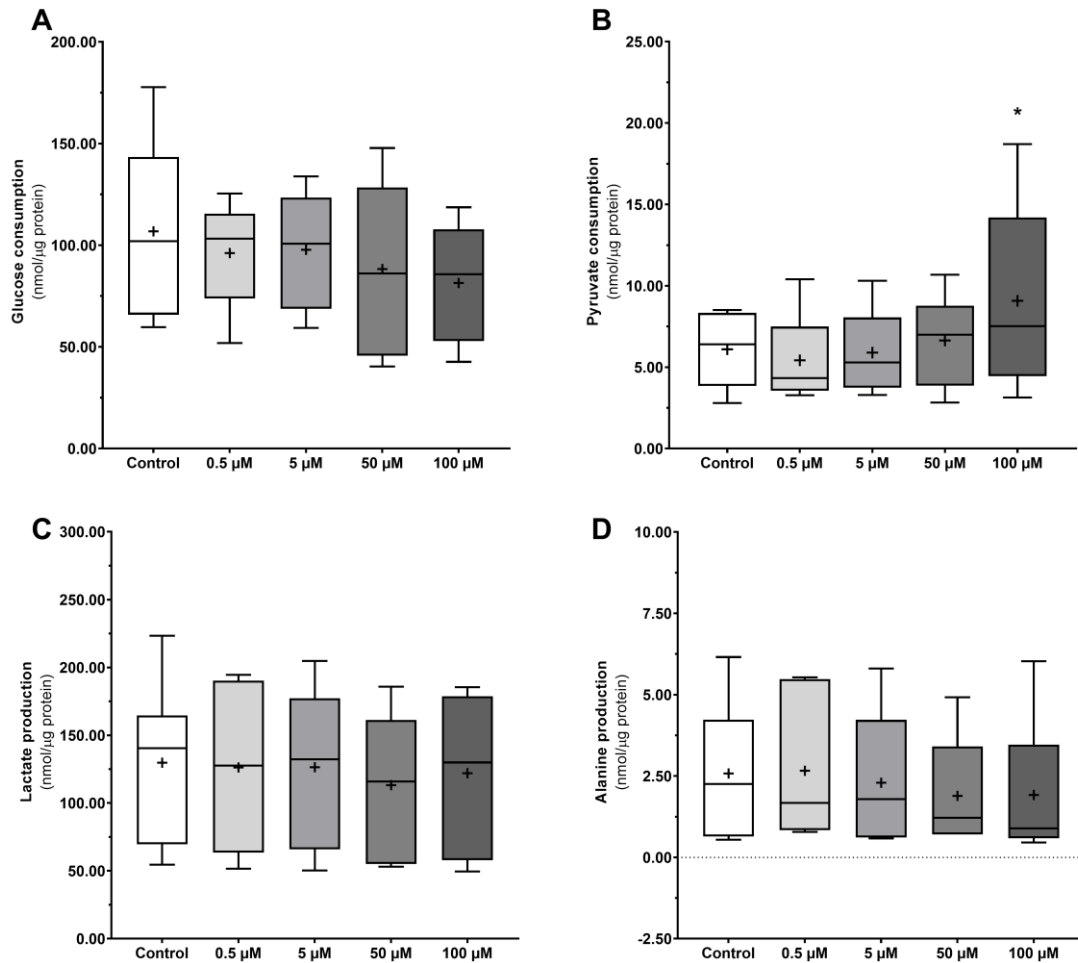


Fig. 6.5 – Metabolic profile of human Sertoli cells (hSCs) after UCPs inhibition by genipin (0, 0.5, 5, 50, and 100 μM) for 24 h ($n = 6$, triplicates for each condition). (A) Glucose consumption. (B) Pyruvate consumption. (C) Lactate production. (D) Alanine production. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). + – Represents group average. Significantly different results are expressed as: * – Relative to the control group.

6.4. Discussion

There is a consensus in the scientific community that UCPs protect cells against exacerbated ROS production and oxidative stress, even though the molecular mechanisms remain elusive. Oxidative stress is one of the main causes of male infertility, however, limited data is available regarding the expression and function of UCPs in the male reproductive system of mammals. UCP2 is the only homolog that was previously identified in the testis of both mice and rats (Amaral et al., 2008; Zhang et al., 2007), whereas studies with mRNA microarray screening predicted the expression of UCP5 and UCP6 in the human and mouse testis (Lizio et al., 2015; Sanchis et al., 1998). In our previous study, we identified UCP1,

UCP2, and UCP3 protein expression in human spermatozoa, whereas *UCP4*, *UCP5*, and *UCP6* mRNA expression were also identified (Carrageta et al., 2023). Furthermore, inhibiting UCPs activity in human spermatozoa resulted in impaired glycolytic and mitochondrial function, ultimately leading to irreversible loss of motility. In this study, we aimed to investigate the expression and function of UCPs in human Sertoli cells (hSCs). We screened the mRNA expression of *UCP1* to 6 homologs and, for the first time, it was detected all six homologs' transcripts in hSCs – *UCP1*, *UCP2*, *UCP3* (*UCP3S* and *UCPL3*, for short and long isoform, respectively), *UCP4*, *UCP5*, and *UCP6*. We also confirmed the expression of UCP1, UCP2, and UCP3 proteins in hSCs through western blot and immunofluorescence. Using ATP synthase as a specific mitochondrial marker, we observed the same scattered pattern throughout the cells, suggesting that UCP1, UCP2, and UCP3 are indeed present in the mitochondria of hSCs. Unfortunately, we were not able to identify the protein expression of UCP4, UCP5, and UCP6 due to the absence of commercially reliable and validated antibodies nor study its role due to the lack of known chemical inhibitors.

Although their name is allusive to mitochondrial uncouplers, it is now known that all homologs, besides UCP1, exhibit poor or absent uncoupling activity (Gaudry & Jastroch, 2019; Hirschenson et al., 2022). As members of the mitochondrial anionic transporter family SLC25 and based on their structure, UCPs were hypothesized to transport small metabolites across the mitochondria membrane, modulating ROS production through the regulation of oxidative phosphorylation and metabolite levels in the mitochondria (Robinson et al., 2008). However, the metabolites being transported remain poorly characterized. Compelling evidence from Vozza et al. demonstrated that UCP2 can exchange C4 metabolites from mitochondria to the cytosol, namely malate, oxaloacetate, and aspartate for phosphate through a proton-assisted mechanism (Vozza et al., 2014). Interestingly, these authors put forth the hypothesis that UCP2 could not only govern ROS production independently of its uncoupling activity but also by orchestrating a glutamate-dependent regulation of the glutathione system. Additionally, a study conducted by Trenker et al. suggested that UCP2 and UCP3 could also play a role on mitochondrial Ca^{2+} uptake (Trenker et al., 2007). These results were later refuted, as neither the incubation with 50 μM genipin or the knockout of *Ucp3* and *Ucp2* did influence Ca^{2+} uptake in isolated mitochondria from rat liver and heart (Brookes et al., 2008). In this work, we aimed to characterize the function of UCPs in hSCs also using genipin, a chemical inhibitor of UCP1, UCP2, and UCP3. Genipin is reported to inhibit the activity of these three homologs physically and dose-dependently without reducing its protein expression (Kreiter et al., 2019). However, the use of genipin, carries some limitations. One of the limitations is the fact that genipin is a general UCP inhibitor, which renders impossible to study the role of each specific homologs. Genipin is also reported to

partially inhibit the mitochondrial complex III (CIII) and could have other unidentified unspecific effects (Kreiter et al., 2019). Despite its limitations, genipin is the only UCP inhibitor commercially available, and as such has been a widely used model for the study of UCPs (Cardoso et al., 2018; Hua et al., 2021). The chosen doses of genipin used in this study were based on the effects previously reported in the literature. The higher doses were chosen based on the reported IC_{50} for UCP3 ($42 \pm 12 \mu\text{M}$) and UCP2 inhibition ($94 \pm 24 \mu\text{M}$) (Kreiter et al., 2019). The lower doses (0.5 and $5 \mu\text{M}$) were chosen based on the beneficial results observed by Zhang et al. on pancreatic β -cells (Zhang et al., 2006). In this study, Zhang et al. reported that a short incubation with genipin (10 min) increased the mitochondrial membrane potential through the inhibition of UCP2, increasing the ATP production by ATP synthase. A high ATP content led to the closure of K_{ATP} channels, which depolarizes the membrane and opens the voltage gated Ca^{2+} channels, stimulating insulin secretion (Zhang et al., 2006). Contrastingly, we observed that a more prolonged inhibition of UCPs (24 h) decreased mitochondrial membrane potential. This result suggests that the effects of the inhibition of UCPs may be dependent on the time of exposure, as low exposure time can stimulate oxidative phosphorylation, but a long exposure greatly impairs its function. The impairment of oxidative phosphorylation was confirmed through the Seahorse Mito Stress Assay analysis, where we observed that the inhibition of UCPs for 24 h significantly decreased OCR of hSCs. Based on previous reports evidencing that UCPs are able to regulate oxidative phosphorylation through the transport of metabolites (Vozza et al., 2014), we hypothesize that the prolonged inhibition of UCPs may impair their exchange into/from the mitochondria, potentially disturbing tricarboxylic acid (TCA) cycle and oxidative phosphorylation. Additionally, basal respiration and ATP-production coupled respiration were highly affected by the prolonged inhibition of UCPs, suggesting that the production of ATP through oxidative phosphorylation was affected, which can explain the observed decreased cellular proliferation. These results are supported by previous studies reported in the literature. For instance, Jo et al. observed that genipin treatment (75 and $150 \mu\text{M}$) for 24 h decreased mitochondrial membrane potential and oxidative phosphorylation in human gastric cancer cell lines, which also resulted in decreased proliferation (Jo et al., 2019). Similar results were reported by Ahani et al., where genipin decreased cellular proliferation in two different human glioblastoma cell lines (Ahani et al., 2019). These authors reported that these effects occur due to UCP2 inhibition and consequent impairment of mitochondrial function. Additionally, it was also observed that the inhibition of UCP2 increased mitochondrial ROS levels, inducing OS and apoptosis (Ahani et al., 2019). In opposition to our initial hypothesis, we observed a decreased intracellular ROS production upon UCPs inhibition for 24 h . These results are unexpected, as several studies report that the inhibition of UCPs by genipin or its knockdown/knockout result in increased ROS production (Blanc et al.,

2003; Horimoto et al., 2004; Lee et al., 2009; Nègre-Salvayre et al., 1997; Pi et al., 2009). We hypothesize that these results are a consequence of the impaired oxidative phosphorylation, which is a major source of intracellular ROS. In addition, antioxidant properties of genipin had been previously reported (Cho, 2022; Zhao et al., 2019), which could further explain the observed decrease in total ROS production. Nonetheless, the inhibition of UCPs could render hSCs more susceptible to exogenous ROS sources, such as in cases of inflammation. For instance, Mailloux et al. observed that the inhibition of UCPs by genipin decreased the oxygen consumption rate without altering ROS production in the chemo-resistant MX2 cell line. However, the inhibition of UCPs sensitized these cells to doxorubicin, as ROS levels only increased in the presence of genipin (Mailloux et al., 2010). Therefore, the potential protective role of UCPs against exogenous ROS sources should be assessed in future studies.

One of the most important functions of hSCs is the nutritional support of spermatogenesis. Sertoli cells exhibit a “Warburg-like” effect where it favours glycolysis and lactate production, as lactate is the preferential substrate for developing germ cells (Rato et al., 2012). Therefore, a consistently high rate of lactate production is one of the most important functions of hSCs. In this study, we did not observe any differences concerning lactate production or glucose consumption after inhibition of UCPs for 24 h, suggesting that the production of germ cells’ preferential energetic substrate is not compromised. It could be hypothesized that the impairment of the oxidative phosphorylation would stimulate glycolysis, thus increasing lactate production, but in highly glycolytic cells it could produce no noticeable effect. For instance, the same results were observed by Jo et al. in gastric cancer cell lines, also highly glycolytic cells, as these authors also reported that glycolysis and lactate production was not affected despite the impaired mitochondrial function (Jo et al., 2019). On the other hand, we found that pyruvate consumption from the media was increased upon UCPs inhibition. These data suggest that the consumption of pyruvate could be compensating for the impaired oxidative phosphorylation through alternative metabolic pathways. We hypothesize that this metabolic shift could help sustaining the cellular energy necessary for the cell to survive despite the compromised mitochondrial function, even if it is inefficient and decreased cellular proliferation occurs. As the oxidoreductases activity through MTT assay shown no alterations, we hypothesize that the TCA cycle may still be active, producing 1 molecule of ATP *per* pyruvate. It is also possible that hSCs are using pyruvate to fuel alternative metabolic pathways, although future studies are required to further explore this hypothesis.

Taken together, our results demonstrate for the first time the expression of *UCP1* to 6 homologs mRNA and UCP1 to 3 protein expression in hSCs. The inhibition of UCPs for 24

h greatly impaired mitochondrial oxidative phosphorylation and cellular proliferation. Despite the inhibition of UCPs, we did not observe an increased intracellular ROS production. In addition, the inhibition of UCPs did not change the lactate production of hSCs but promoted an increased pyruvate consumption, which we hypothesize that could be a compensatory mechanism due to the impairment of oxidative phosphorylation. In sum, our results suggest that UCPs are modulators of the mitochondrial activity in hSCs. Future studies with specific UCP homologs knockdown and/or overexpression will be necessary for disclosure the role of each specific homolog function in Sertoli cells and male reproductive system.

6.5. Authors contribution

David F. Carrageta – conceptualization, methodology, formal analysis, investigation, writing – original draft preparation, writing – review and editing, funding acquisition; Laís Freire-Brito – methodology, investigation, writing – review and editing; Bárbara Guerra-Carvalho – methodology, formal analysis, writing – review and editing; Raquel L. Bernardino – methodology, writing – review and editing; Bruno S. Monteiro – methodology, writing – review and editing; Alberto Barros – resources, writing – review and editing; Pedro F. Oliveira – conceptualization, formal analysis, investigation, resources, writing – review and editing, funding acquisition; Mariana P. Monteiro – conceptualization, formal analysis, investigation, writing – review and editing; Marco G. Alves – conceptualization, formal analysis, investigation, resources, writing – review and editing, project administration, funding acquisition.

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Chapter 7

Insulin promotes capacitation through autocrine/paracrine mechanisms in human spermatozoa



This chapter was adapted from the unpublished work:

Carrageta DF, Guerra-Carvalho B, Pereira SC, Barros A, Oliveira PF, Monteiro MP, Alves MG. (2023). Insulin promotes capacitation through autocrine/paracrine mechanisms in human spermatozoa (unsubmitted journal article).

Abstract

Insulin is an anabolic hormone that has been described as exclusively secreted by pancreatic beta cells. However, recent reports suggest that insulin may be produced by the male gamete. Herein, we aimed to study the presence of insulin and the enzymes responsible for proinsulin cleavage into insulin, PC1 and PC2, in human spermatozoa. In addition, we aimed to disclose if insulin could play a role on capacitation and fertilization. For that purpose, human spermatozoa were submitted to a density gradient centrifugation and two fractions were collected according to its motility (high vs low motility). The presence and levels of insulin, PC1/3 and PC2 mRNA was evaluated by RT-qPCR ($n = 15$). Then, we explored whether there was any association between high motility spermatozoa insulin mRNA content and assisted reproduction techniques (ART) outcomes ($n = 97$). The protein levels of insulin, PC1, PC2, and insulin and IGF-1 receptors were evaluated by Western Blot and immunofluorescence. Insulin content was determined by electrochemiluminescence. The effects of insulin on viability, motility, and mitochondrial and metabolic function were assessed. Insulin signaling pathway was analyzed by Western Blot. Insulin mRNA content was found to be higher in spermatozoa with higher motility, however, we found no significant association between insulin mRNA levels and ART outcomes. Since insulin was found in spermatozoa ($1.25 \pm 0.71 \text{ ng}/10^6 \text{ cells}$), we hypothesized that insulin could regulate motility and capacitation. We were able to demonstrate that insulin and IGF-1 receptors are expressed in human spermatozoa and that the insulin signaling pathway is active during *in vitro* capacitation, even in the absence of exogenous insulin. Supplementation with insulin (1 nM) had no additional effects on spermatozoa viability, motility, and metabolic function, suggesting that the stored insulin is sufficient to stimulate capacitation. Overall, our results show that human spermatozoa have the molecular machinery to produce and secrete insulin and that insulin signaling pathway is active, implying that this hormone is involved in the autocrine/paracrine regulation of capacitation.

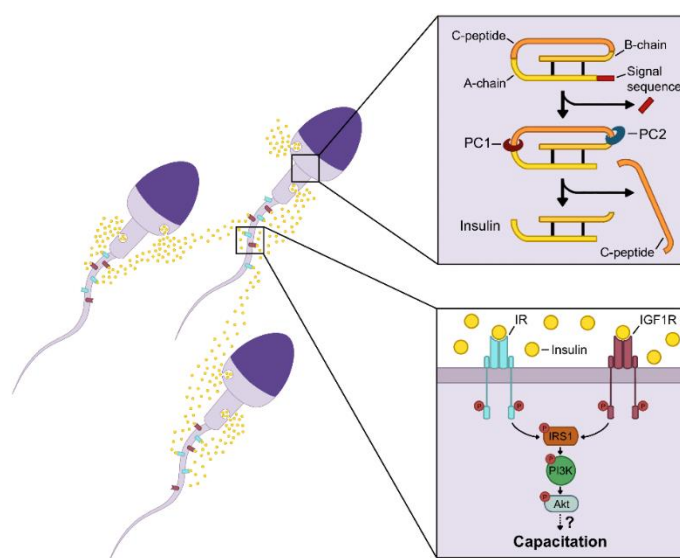


Fig. 7 – Graphical abstract. We were able to identify insulin, as well as PC1 and PC2, enzymes responsible for the cleavage of proinsulin into insulin, in human spermatozoa. Insulin

signaling pathway is active during capacitation, suggesting that insulin is involved in its regulation in an autocrine/paracrine manner.

7.1. Introduction

Sperm capacitation is a process that occurs in mammalian spermatozoa that is essential for the successful fertilization of the oocyte. During capacitation, spermatozoa obtain the ability to undergo acrosome reaction and achieve a state of hyperactivation, characterized by an asymmetric beat with increased flagellar bend amplitude that results in increased motility. These changes improve the sperm's swimming ability, enabling it to penetrate the *zona pellucida* of the oocyte more effectively (Puga Molina et al., 2018). For capacitation to occur, a complex and finely regulated series of structural and functional alterations take place. In the female reproductive tract, human spermatozoa are exposed to high concentrations of bicarbonate, albumin and calcium, in an alkaline pH, which constitute the trigger for capacitation-related modifications of the sperm membrane and activation of specific signaling cascades (Vyklicka & Lishko, 2020). Besides the specific ionic environment, a successful capacitation process requires a fine-tuned energy regulation. Glucose is considered the preferential energy substrate for human sperm capacitation and hyperactivation, as these processes place high demands on ATP production (Williams & Ford, 2001). Human spermatozoa predominantly use two metabolic pathways to generate ATP from glucose – glycolysis and mitochondrial oxidative phosphorylation (Goodson et al., 2012). Although the preferred metabolic pathway of the human male gamete is still debatable, data highlights that both pathways are activated during capacitation (Irigoyen et al., 2022; Piomboni et al., 2012). Additionally, evidence suggests that high glucose concentrations are beneficial for human sperm capacitation (Carrageta et al., 2020). Nevertheless, the regulation of glucose uptake and energy-related pathways during human sperm capacitation remains poorly understood.

Insulin is an anabolic hormone and the main promoter of glucose uptake by the cells. Insulin is synthesized and secreted by pancreatic beta cells in response to glucose levels. Whenever insulin binds to the insulin receptor, it activates a signaling cascade that leads to the facilitation of glucose uptake by cells (Leto & Saltiel, 2012). Insulin signaling pathway also promotes the conversion and storage of glucose into glycogen in the liver and skeletal muscle (Jensen et al., 2011), or into lipids in the liver and adipose tissue (Santoro et al., 2021; Titchenell et al., 2017). Although the effects of insulin in these tissues are well characterized, the role of insulin in the male reproductive system is still poorly understood. In the testis, insulin was demonstrated to have a role in testicular development and regulation

of sex-determination genes (Nef et al., 2003; Pitetti et al., 2013). Insulin was also hypothesized to play a role in the metabolic regulation of spermatogenesis, either directly on the germ cell line, or indirectly through the regulation of Sertoli cells metabolism (Oliveira et al., 2012; Qaid et al., 2016). However, data concerning the effects of insulin on the male germ cell line is conflicting. Some authors reported that insulin signaling is likely involved in human spermatozoa motility, capacitation, and acrosome reaction (Ando & Aquila, 2005; Lampiao & du Plessis, 2008), whereas others stated that insulin produces none of the aforementioned effects (Wang et al., 2015). Interestingly, insulin expression by the human germ cell line was previously suggested, although its function is not clear (Aitken et al., 2021; Aquila et al., 2005).

In this work, we aimed to identify whether insulin was synthesized by human spermatozoa and, if present, to characterize its function. For that purpose, human spermatozoa were screened for the mRNA of insulin, neuroendocrine convertase 1 (NEC1, also known as PC1) and 2 (NEC2, also known as PC2), which are enzymes responsible for the cleavage of proinsulin into insulin. In addition, the potential association between insulin, PC1, and PC2 mRNA levels in human spermatozoa and the individuals age, body mass index (BMI) and assisted reproduction techniques (ART) outcomes, was assessed. Then, the role of insulin during human spermatozoa capacitation was investigated. For that, human spermatozoa total insulin content was quantified; the presence of insulin and insulin-like growth factor 1 (IGF-1) receptors was identified, and the effects of insulin in motility, viability, and mitochondrial function of human spermatozoa was evaluated. Finally, the insulin signaling pathway activation during human spermatozoa capacitation was assessed.

7.2. Materials and methods

7.2.1. Reagents and chemicals

Unless stated otherwise, all reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

7.2.2. Patient recruitment and seminal sample preparation

Human spermatozoa were obtained from patients attending the Medical Assisted Procreation Center, after approval by the Joint Ethics Committee CHUP/ICBAS (2021/CE/P002[P342/CETI/ICBAS]). Patient recruitment, clinical evaluation, and diagnostic

semen analysis were performed at the clinic in compliance with the standards of care. Informed and written consent to participate was obtained from all participants before any study procedure took place. All procedures were conducted according to the ethical guidelines for human research, under strict individual anonymity. Men with a history of alcoholism or drug abuse, under treatment or exposed to drugs known to interfere with fertility, cancer treatment survivors, with any systemic disease known to affect fertility, or diagnosed with any acute genital inflammatory disease before sperm collection, were excluded from this study. Smoking was not an exclusion criterion for the herein study.

This study comprised two different cohorts. The first cohort included men recruited among couples ($n = 97$) undergoing ART treatments. The mean age of the male and female partner was 38.9 ± 5.0 and 38.6 ± 4.4 years old and the mean BMI was 26.1 ± 3.5 and 22.7 ± 3.4 kg/m², respectively. Seminal samples were obtained by masturbation into specific sterile containers after 2-7 days of sexual abstinence, left at 37 °C until complete liquefaction, and then analyzed according to the guidelines of the World Health Organization (WHO, 2021). Experienced board-certified embryologists evaluated all sperm parameters and embryonic development indicators. According to the WHO guidelines, 68% of the men were classified as normozoospermic, 13% as asthenozoospermic, 13% as teratozoospermic, and 6% as oligozoospermic or combination of these conditions. Collected spermatozoa were washed and the high motility fraction was obtained through swim-up technique, before undertaking ART procedures (Pinto et al., 2021). Then, fertilization was achieved either by *in vitro* fertilization (IVF, $n = 15$) or intracytoplasmic sperm injection (ICSI, $n = 82$). Biochemical pregnancy was ascertained through the quantification of serum β -human chorionic gonadotropin (β -hCG) levels on the 12th day following embryo transfer. A serum β -hCG concentration surpassing 20 mIU/mL stood as criterion for biochemical pregnancy (Annan et al., 2013). Clinical pregnancy was confirmed by the detection of fetal heartbeat on ultrasonography scan. The surplus of spermatozoa used in ART procedures was collected, pelleted, and preserved at -80°C until used for total RNA extraction and quantification of insulin (INS), PC1 (*PCSK1*), and PC2 (*PCSK2*) mRNAs.

For the second cohort, seminal samples of male subjects classified as normozoospermic ($n = 14$, mean age 38.3 ± 7.2 years old) were used to study the role of insulin in spermatozoa capacitation. Seminal samples were obtained, handled, and evaluated as aforementioned. A standard discontinuous density gradient centrifugation protocol was performed using the Gems® Sperm Wash Gradient Set (45% and 90%) (SWG-01, Genea, Sydney, Australia) according to the manufacturer's instructions. The subset comprising highly motile spermatozoa and the counterpart encompassing those with low motility were carefully separated, collected, and subjected to two washes with phosphate buffer saline (PBS) before being employed for subsequent experimental procedures.

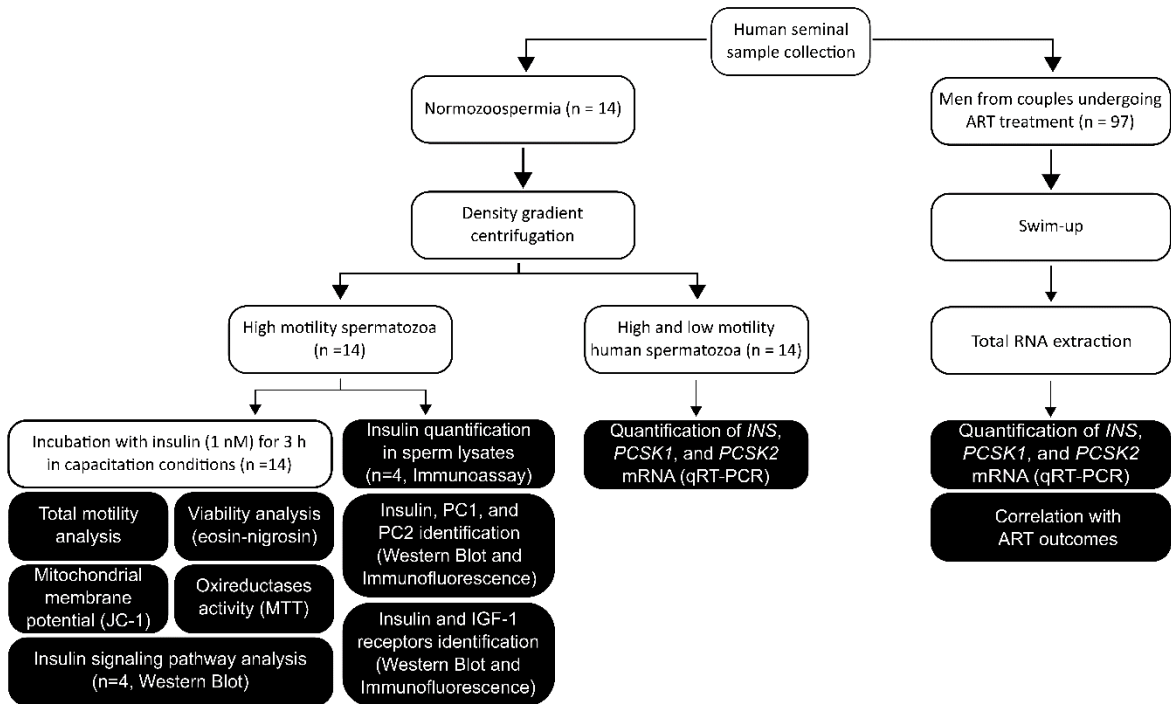


Fig. 7.1 – Schematic workflow of the experimental design applied in this study.

7.2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative RT-PCR

Total RNA was extracted from spermatozoa using the NZY Total RNA Isolation Kit (MB13402, NZYTech, Lisbon, Portugal). Purified RNA concentration was determined using a Take3 microvolume plate on a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). RNA was reversely transcribed using the NZY M-MuLV Reverse Transcriptase Kit (MB083, NZYTech, Lisbon, Portugal). RT-PCR was performed to identify the presence of insulin (*INS*), PC1 (*PCSK1*), and PC2 (*PCSK2*) mRNAs in human spermatozoa using the resulting complementary DNA (cDNA) and specific primer sets (Table 7.1).

A quantitative RT-PCR (qPCR) was performed to assess the *INS*, *PCSK1*, and *PCSK2* mRNA abundance in high motility or low motility human spermatozoa fractions from different donors ($n = 15$) or *INS* mRNA abundance in surplus swim-up spermatozoa selected for ART procedures ($n = 97$), according to a previously described protocol (Bernardino et al., 2018). Briefly, qPCR was performed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hemel Hempstead, UK) and the Speedy NZYtaq 2x Green Master Mix (MB362, NZYTech, Lisbon, Portugal) according to the manufacturer's instructions and with conditions previously optimized: 2 min at 95°C for polymerase activation followed by 40 runs of a 2 steps cycle: 5 s at 95°C for DNA denaturation and 30 s for a combined step of anneal-

ing/extension with a specific temperature for each set of primers (Table 7.1). Primers' efficiency was set to 90-110% and the specificity of the amplicons was determined by melting curves analysis. β 2-microglobulin (*B2M*) or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were used for normalization. *INS*, *PCSK1*, and *PCSK2* mRNA abundance was calculated using the formula $2^{-\Delta Ct}$.

Table 7.1. Oligonucleotides and cycling conditions for RT-PCR and RT-qPCR amplification of insulin (*INS*), PC1 (*PCSK1*), and PC2 (*PCSK2*) mRNA in human spermatozoa.

Target (accession number)	Sequence 5'-3'	Annealing temperature	Number of cycles
<i>Insulin</i> (NM_001185098.1)	FWD: CTGCATCAGAAGAGGCCATCA RVS: CCCCACATGCTTCACGAGC	63 °C	35
<i>PCSK1</i> (NM_000439.5)	FWD: GGGAGTCTGTCTGGCTTTTCTC RVS: TTGCCTTTTCGCTTTTGCAC	63 °C	35
<i>PCSK2</i> (NM_002594.5)	FWD: CACACTGATTGCTGCTTTCC RVS: TGGGAGACACAACCACCCTT	63 °C	35
<i>GAPDH</i> (NM_002046.7)	FWD: CGCCAGCCGAGCCACATC RVS: CGCCCAATACGACCAAATCCG	59 °C	35
<i>B2M</i> (NM_004048.4)	FWD: AGATGAGTATGCCTGCCGTG RVS: TCATCCAATCCAAATGCGGC	59 °C	25

7.2.4. Human insulin immunoassay

Insulin was quantified from spermatozoa lysates using the Elecsys Human Insulin Immunoassay (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. In brief, 100×10^6 spermatozoa were collected before further processing, washed with PBS, and suspended in 100 μ L of Biggers, Whitter, and Whittingham (BWW) medium (in mM: 94.5 NaCl, 4.8 KCl, 1.7 CaCl₂, 1.17 KH₂PO₄, 1.22 MgSO₄, 20 HEPES, 24 NaHCO₃, 0.4 BSA, 5.5 Glucose, pH = 7.4). Then, spermatozoa were sonicated on ice using a pulse of 5 x 5 s. Samples were centrifuged at 14000 $\times$ g for 20 min at 4°C and the supernatant collected for insulin quantification. A total of 20 μ L of sample, standards, and quality control were used in each assay. According to the manufacturer's, there is no cross-reactivity with human proinsulin. A calibration curve was generated within the range of 0.20-1000 μ IU/mL. Total insulin content was calculated and results are expressed as insulin content (ng) *per* 10^6 spermatozoa.

7.2.5. Experimental groups

To assess the effects of insulin on human spermatozoa capacitation, the fraction of highly motile spermatozoa was obtained through density gradient centrifugation and incubated in BWW media supplemented with insulin (1 nM) at 37°C. A group without insulin supplementation in the media was used as control (6×10^6 cells for each condition). After 3 h, cells were collected for viability and motility ($n = 14$), and either mitochondrial membrane potential and oxidoreductases activity ($n = 10$) or protein ($n = 4$) analysis.

7.2.6. Western blot

Proteins were extracted from pelleted human spermatozoa using radioimmunoprecipitation assay (RIPA) buffer and quantified using Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, Waltham, MA, USA). Western blot protocol was performed using previously described methods (Carrageta et al., 2023). Protein samples (30 µg) were denatured at 70°C for 10 min, fractionated on a 10% polyacrylamide gel from TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad, Hemel Hempstead, UK), and transferred to a low fluorescence polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hemel Hempstead, UK) using a Trans-Blot® Turbo™ System (Bio-Rad, Hemel Hempstead, UK). Membranes were blocked with a solution of 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBS-T) solution. Then, membranes were incubated overnight at 4°C separately with the primary antibodies mouse anti-PC1 (1:500, sc-100578, Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-PC2 (1:500, sc-374140, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-insulin receptor β (1:1000, #23413, Cell Signaling, Danvers, MA, USA), or rabbit anti-IGF-1 receptor β (1:1000, #9750, Cell Signaling, Danvers, MA, USA), rabbit anti-phospho-insulin receptor(Y1361) (1:1000, ab303492, Abcam, Cambridge, UK), rabbit anti-phospho-IRS1(Y632) (1:1000, ab109543, Abcam, Cambridge, UK), rabbit anti-phospho-PI3 Kinase P85(Tyr458) (1:1000, #4228, Cell Signaling, Danvers, MA, USA), and rabbit anti-phospho-Akt(Ser473) (1:1000, #9271, Cell Signaling, Danvers, MA, USA). Proteins were detected upon incubation with goat anti-rabbit IgG-HRP (1:5000, AP307P, Sigma-Aldrich, St. Louis, MI, USA), goat anti-rabbit IgG-AP (1:5000, A3687, Sigma-Aldrich St. Louis, MI, USA), or goat anti-mouse IgG-AP (1:5000, A3562, Sigma-Aldrich St. Louis, MI, USA) and reacted with either Clarity™ Western ECL Substrate (Bio-Rad, California, USA) or ECF substrate (RPN5785, Cytiva, Marlborough, MA, USA). Protein bands were detected using a Bio-Rad ChemiDoc XR (Bio-Rad, Hemel Hempstead, UK). Densities from each band were quantified using Image Lab Software (Bio-Rad, California, USA). Results were normalized

to total protein content using the TGX Stain-Free™ technology and according to the manufacturer's instructions.

7.2.7. Immunofluorescence staining

Immunofluorescence staining was performed to observe the cellular location of insulin, PC1, PC2, insulin receptor, and IGF-1 receptor in human spermatozoa, using previously described methods (Carrageta et al., 2023). In brief, spermatozoa were fixed on glass coverslips with 10% formalin for 20 min at room temperature. Then, spermatozoa were rendered permeable with 0.1% Triton X-100 in PBS and blocked in 0.1% gelatin in PBS, both for 15 min at room temperature. An overnight incubation at 4°C was performed using primary antibodies rabbit anti-insulin (1:50, #4590, Cell Signaling, Danvers, MA, USA), mouse anti-PC1 (1:50, sc-100578, Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-PC2 (1:50, sc-374140, Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-insulin receptor β (1:50, #23413, Cell Signaling, Danvers, MA, USA), or rabbit anti-IGF-1 receptor β (1:50, #9750, Cell Signaling, Danvers, MA, USA). Cells incubated without primary antibodies were used as a negative control. Incubation with the secondary antibodies Alexa Fluor 488 goat anti-mouse (1:1500, A-11001, Thermo Fischer Scientific, Waltham, MA, USA) or Alexa Fluor 488 goat anti-rabbit IgG (1:1500, A-11008, Thermo Fischer Scientific, Waltham, MA, USA) occurred for 1 h at room temperature. Coverslips were mounted and nuclei were stained with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Fluorescence was observed in a Nikon Eclipse E400 microscope equipped with a DS-Fi3 microscope camera, a Y-FL Epi-Fluorescence Attachment, and HB-10103AF Super High-Pressure Mercury Lamp Power Supply (Nikon, Shinagawa, Tokyo, Japan). The pictures obtained were processed using the NIS-Elements Br 4.60.00 software (Nikon, Shinagawa, Tokyo, Japan).

7.2.8. Spermatozoa motility and viability analysis

Human spermatozoa viability and motility were assessed according to previously established protocols (Carrageta et al., 2023). The percentage of viable and nonviable spermatozoa was determined using eosin-nigrosin staining by counting a total of 100 spermatozoa per slide in continuous random fields under a bright-field optical microscope (1000× magnification). White spermatozoa were considered viable whereas pink-stained spermatozoa, indicating cytoplasmic staining due to loss of membrane integrity, were considered nonviable. Results are expressed as percentage of viable spermatozoa.

Human spermatozoa motility was evaluated using a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Total and progressive motility was assessed using 10 s videos recorded in a bright-field optical microscope (200× magnification). The experiment was performed by the same user and the same criteria were applied to all samples. New drops were placed at the chamber and the process was repeated until a minimum total of 100 individual spermatozoa were counted for each sample. Results are expressed as percentage of total or progressive motile spermatozoa.

7.2.9. JC-1 assay for mitochondrial membrane potential analysis

Mitochondrial membrane potential of high motility spermatozoa was analyzed using the fluorogenic dye JC-1 (T3168, Invitrogen™, Carlsbad, CA, USA), according to a protocol previously described (Carrageta et al., 2022). Spermatozoa treated with insulin or vehicle (1×10^6 cells for each condition) were washed and incubated in a JC-1 working solution (1 µg/mL in PBS) at 37°C for 30 min in a dark environment. A working solution containing 20% DMSO was used as an assay positive control. Then, spermatozoa were transferred into a 96-well clear bottom black plate for JC-1 monomers quantification (485/530 nm; excitation/emission) and J-aggregates fluorescence (535/590 nm; excitation/emission) on a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). The ratio between JC-1 J-aggregates/monomers was calculated and used as an indicator of mitochondrial membrane potential. Results are expressed as mean J-aggregates/monomers ratio.

7.2.10. MTT assay for oxidoreductase activity analysis

Oxidoreductase activity in human spermatozoa was evaluated using MTT, according to a protocol previously described (Aitken et al., 2020). In brief, 5×10^6 spermatozoa were incubated in 450 µL of BWW medium supplemented with freshly prepared 50 µL of MTT solution (5 mg/mL in PBS). The spermatozoa were then incubated protected from light, at 37°C for 1 h. After incubation, samples were centrifuged at 300×g for 5 min and the culture medium was replaced with 250 µL of DMSO, followed by homogenization to solubilize formazan crystals. Then, 100 µL were transferred into a 96-well plate, and absorbance was measured at 570 nm using a Synergy™ H1 multi-mode microplate reader (BioTek, Winooski, VT, USA). DMSO was used as blank.

7.2.11. Statistical analysis

Experimental results are represented as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR) or Pearson correlation scatterplot. Statistical analysis was performed in GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Data were tested for normal distribution with the Kolmogorov–Smirnov test. Statistical differences were determined by a paired t-test or repeated measures one-way ANOVA with post hoc Dunnett's multiple comparisons test. For correlation analysis, a two-tailed Pearson correlation test was applied. A value of $p < 0.05$ was considered significantly different.

7.3. Results

7.3.1. *INS* and *PCSK1* mRNA content is higher in highly motile human spermatozoa

In this work, we screened human spermatozoa for the mRNA presence of insulin and PC1 and PC2, enzymes responsible for the cleavage of proinsulin into insulin. We were able to identify the presence of insulin (*INS*) and both PC1 (*PCSK1*) and PC2 (*PCSK2*) in human spermatozoa (Fig. 7.2A). We further quantified the relative quantities of *INS*, *PCSK1*, and *PCSK2* mRNA in both high motility (HM) and low motility (LM) human spermatozoa by RT-PCR (Fig. 7.2B).

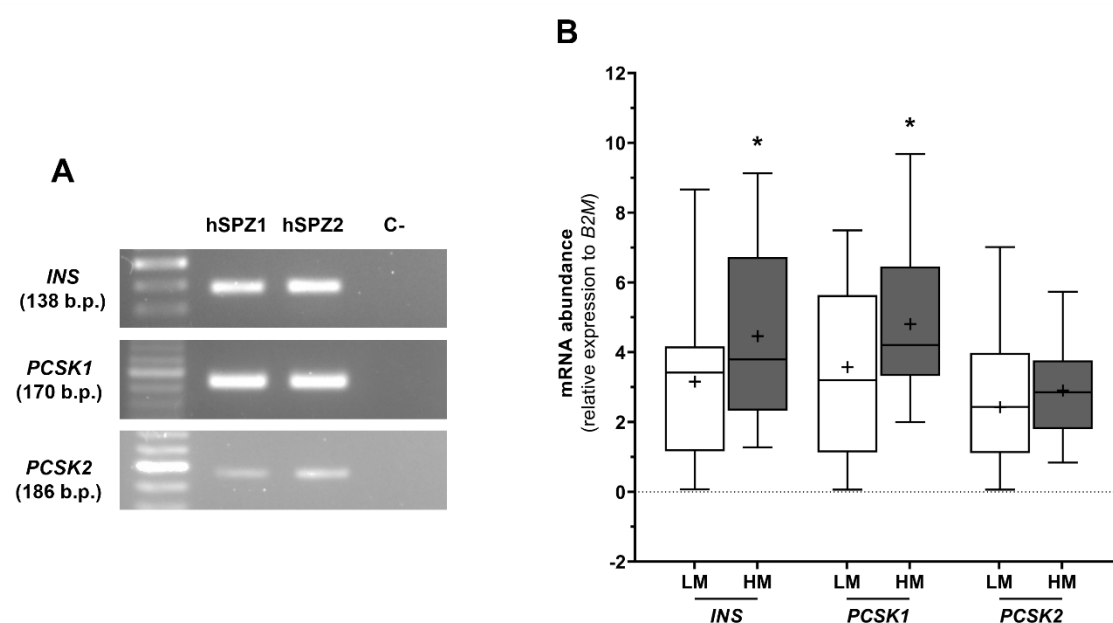


Fig. 7.2 – Identification and quantification of insulin (*INS*), PC1 (*PCSK1*), and PC2 (*PCSK2*) mRNA in human spermatozoa. (A) Representative RT-PCR for the evaluation of *INS*, *PCSK1*, and

PCSK2 mRNA presence in two samples of human spermatozoa (hSPZ1 and hSPZ2) obtained from distinct donors. **(B)** Relative abundance of *INS*, *PCSK1*, and *PCSK2* mRNA in high motility (HM) and low motility (LM) human spermatozoa (n = 15). Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). + – Represents group average. Significantly different results are expressed as: * – Relative to low motility human spermatozoa.

We observed a higher content of *INS* mRNA in HM spermatozoa (4.46 ± 2.35 arbitrary units, $p = 0.035$) and *PCSK1* mRNA (4.81 ± 2.20 arbitrary units, $p = 0.035$) as compared to LM spermatozoa (3.16 ± 2.42 arbitrary units and 3.58 ± 2.30 arbitrary units for *INS* mRNA and *PCSK1* mRNA relative abundance, respectively). However, no differences, concerning *PCSK2* mRNA content, were observed when comparing HM (2.90 ± 1.47 arbitrary units) to LM spermatozoa (2.43 ± 1.92 arbitrary units).

7.3.2. The content of *INS* mRNA in highly motile human spermatozoa is independent of age or the metabolic status of the individual

The production and secretion of insulin by pancreatic beta cells are regulated by a range of factors, including an individual's age, adiposity, and metabolic status (Chang & Halter, 2003; Fu et al., 2013). Hence, our objective was to determine whether the content of *INS* mRNA in spermatozoa could be influenced by the age or body mass index (BMI) (Fig. 7.3). We observed that *INS* mRNA quantities in HM human spermatozoa is neither correlated with the age (Fig. 7.3A) nor BMI (Fig. 7.3C). We further grouped men according to age (Fig. 7.3B), using 35 years old as age threshold (Stone et al., 2013), and according to corpulence classification based on BMI (Fig. 7.3D). No significant differences were observed when comparing younger (0.93 ± 0.37 arbitrary units) to older men (1.03 ± 0.51) or across BMI classes (0.97 ± 0.46 ; 1.11 ± 0.53 ; 0.89 ± 0.48 arbitrary units for normal weight, overweight, and obesity, respectively). Collectively, these results underscore that *INS* mRNA content in highly motile human spermatozoa is conserved, which suggests the homogeneity of this subpopulation of spermatozoa whose intrinsic characteristics appear to be independent of the age or metabolic status of the individual.

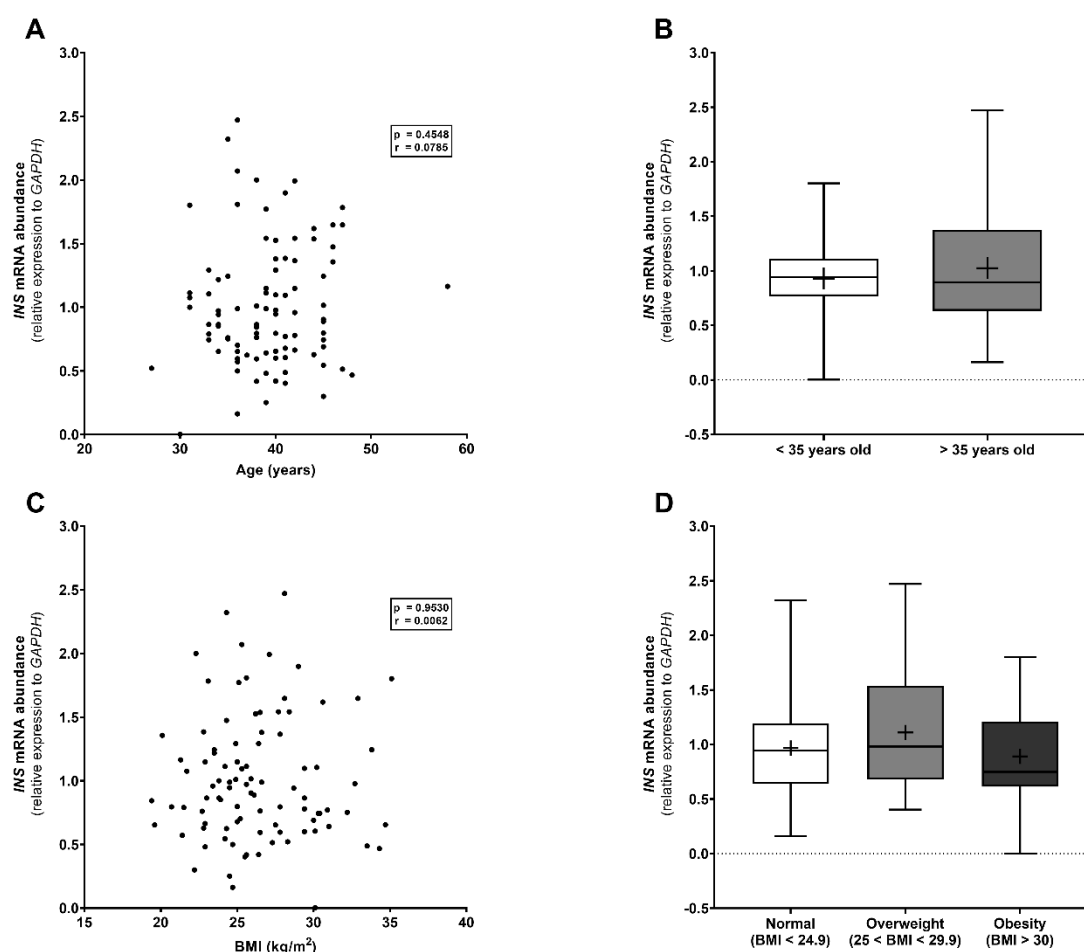


Fig. 7.3 – Correlation between men age, body mass index (BMI), and insulin (*INS*) mRNA content in highly motile human spermatozoa. (A) Pearson correlation between age or (C) the BMI and the *INS* mRNA content in highly motile spermatozoa ($n = 97$). (B) Relative abundance of *INS* mRNA in highly motile spermatozoa in young (< 35 years old) and older men (> 35 years old). (D) Relative abundance of *INS* mRNA in highly motile spermatozoa according to the BMI category (normal weight, overweight, and obesity). Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles ± 1.5 IQR). + – Represents group average.

7.3.3. The *INS* mRNA content in highly motile human spermatozoa displays no correlation with Assisted Reproduction Techniques (ART) outcomes

Given that *INS* mRNA content was demonstrated to be more elevated in HM spermatozoa, regardless of men age or BMI, our next research objective was to explore its potential association with successful outcomes in ART. To address this, we undertook a comprehensive analysis of the correlation between *INS* mRNA content within HM spermatozoa and the efficacy of progressive ART outcomes (Fig. 4). More specifically, focusing on the fertilization

rate (as illustrated in Fig. 7.4A), the rate of embryo cleavage (Fig. 7.4B), high-quality embryo rate (Fig. 7.4C), and blastocyst rate (Fig. 7.4D). This aimed to highlight whether the *INS* mRNA content could serve as a predictive marker for favorable results in ART procedures.

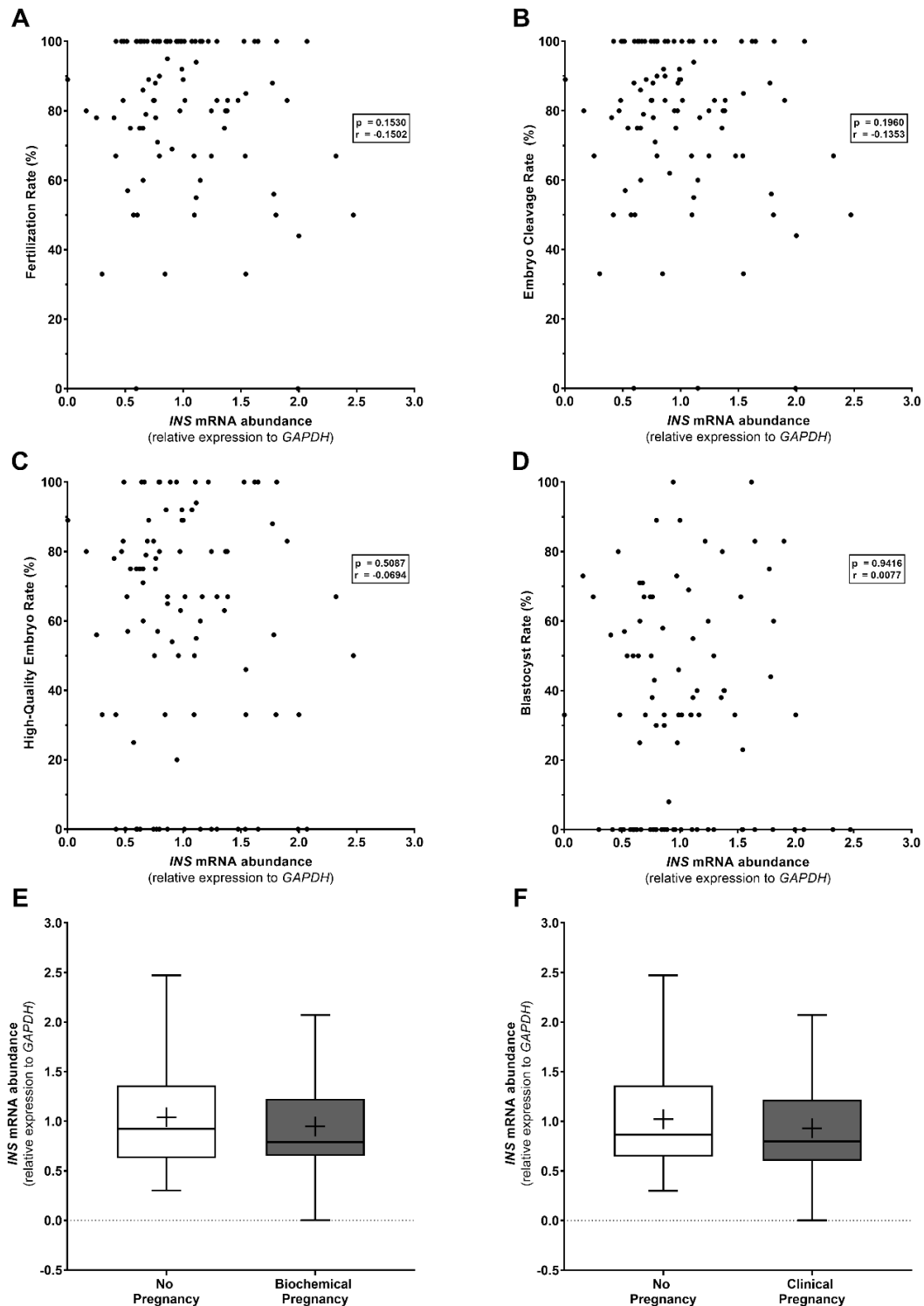


Fig. 7.4 – Correlation between embryonic development or pregnancy with the insulin (*INS*) mRNA content in highly motile (HM) human spermatozoa. (A) Pearson correlation between fertilization rate, **(B)** embryo cleavage, **(C)** high-quality embryo rate, or **(D)** blastocyst rate and the *INS*

mRNA content in HM spermatozoa ($n = 97$). **(E)** Association between the relative abundance of *INS* mRNA in HM spermatozoa and achievement of biochemical or **(F)** clinical pregnancy. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR). + – Represents group average.

However, no significant correlation between *INS* mRNA content in HM human spermatozoa and the outcomes of ART was found. Additionally, we further studied the correlation between *INS* mRNA content in HM spermatozoa with biochemical (Fig. 7.4E) and clinical pregnancy rates (Fig. 7.4F), but no significant association was found between these parameters. These findings indicate a remarkable preservation of *INS* mRNA content in HM human spermatozoa; however, it appears to have no discernible impact on fertilization or embryo development. Subsequently, our hypothesis suggested that the *INS* mRNA content could potentially function as an indicator of insulin production by human spermatozoa. With this hypothesis in mind, we embarked on further investigations to explore its validity.

7.3.4. Insulin, PC1, and PC2 are expressed in the head and midpiece of human spermatozoa

Total insulin content in human spermatozoa lysates was assessed through an electrochemiluminescence immunoassay (Fig. 7.5). In our cohort, the insulin content was 1.25 ± 0.71 ng/ 10^6 spermatozoa.

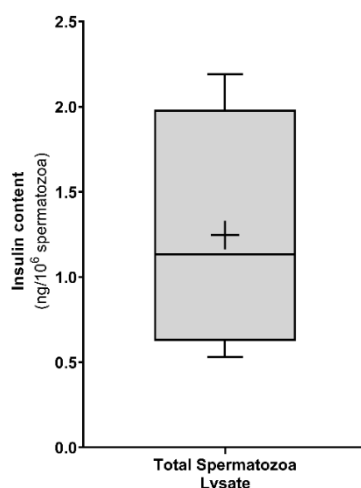


Fig. 7.5 – Quantification of insulin content in human spermatozoa lysates through electrochemiluminescence immunoassay. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles $\pm$ 1.5 IQR; $n = 4$). + – Represents group average.

We further assessed the presence of PC1 and PC2 by Western Blot (Fig. 7.6A). We were able to identify blot bands corresponding to PC1 and PC2 at the predicted molecular

weight (90 kDa). To the best of our knowledge, we were able to identify for the first time the enzymes PC1 and PC2 in human spermatozoa. In addition, we evaluated the cellular localization of insulin, PC1, and PC2 by immunofluorescence staining (Fig. 7.6B).

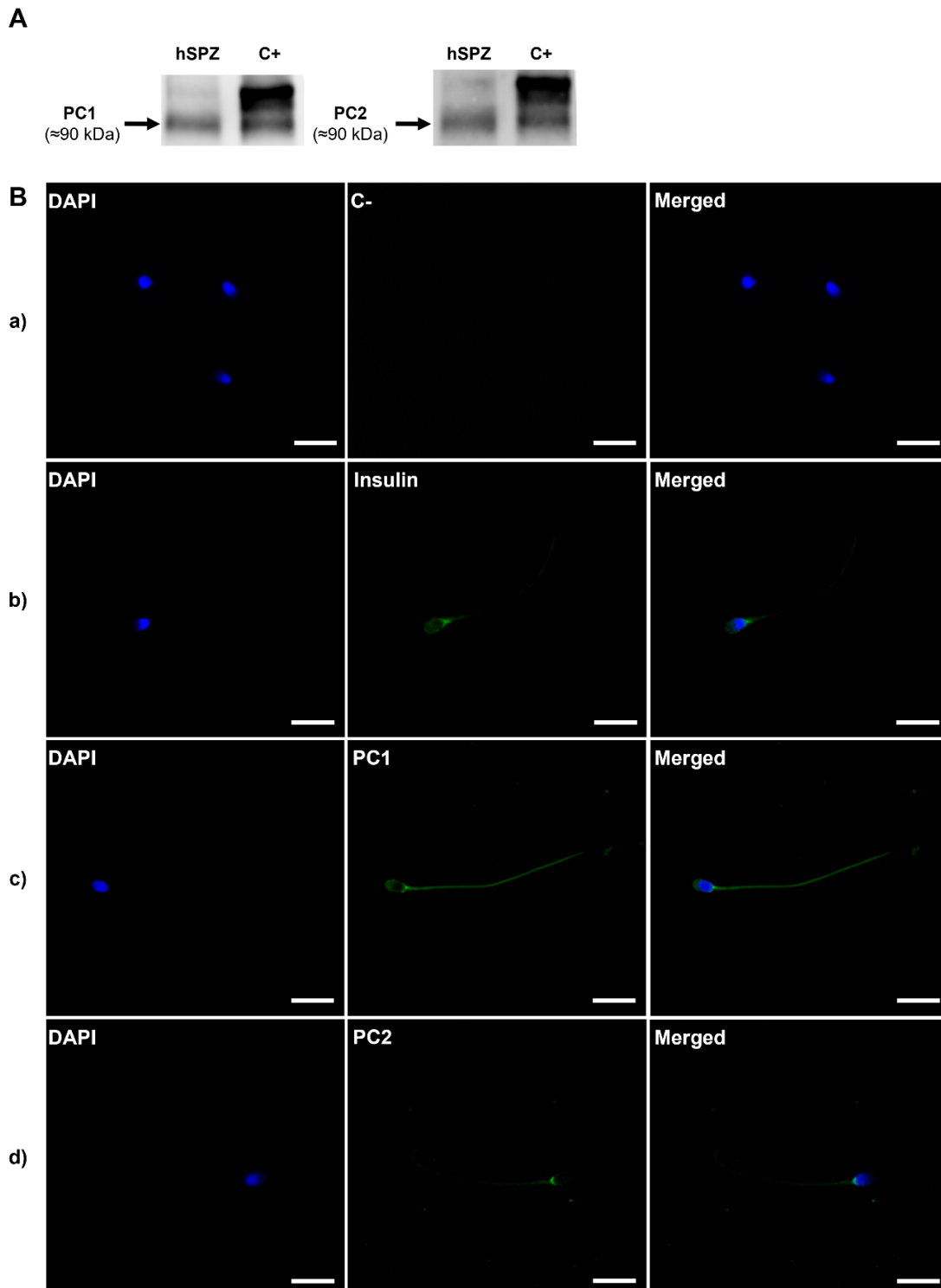


Fig. 7.6 – Identification and immunolocalization of insulin, PC1, and PC2 protein expression in human spermatozoa. (A) Identification of PC1 and PC2 in human spermatozoa (hSPZ) by Western Blot. Mouse kidney lysate was used as positive control (C+). **(B)** Immune labeling of insulin **(b)**, PC1 **(c)**, and PC2 **(d)** was observed in human spermatozoa. **(a)** Negative control. Scale bar—10 μ m.

Interestingly, our observations revealed a shared cellular location for both PC1 and PC2 with insulin, predominantly positioned at the junction of the head and midpiece. While PC2 displayed exclusive localization in this region, insulin was additionally detected in the head and acrosome area. Conversely, PC1 was found in the acrosome region and along the flagellum. Collectively, these findings strongly indicate the presence of the molecular apparatus within human spermatozoa for insulin synthesis.

7.3.5. Insulin receptor and IGF-1 receptor are expressed in the midpiece and tail of human spermatozoa

Following the successful identification and quantification of insulin content, our focus shifted to assessing the presence of its receptors within human spermatozoa. Insulin binds to both the insulin receptor (InsR) and the insulin-like growth factor 1 (IGF-1) receptor (IGF1R), displaying a slightly stronger affinity for the former. To examine this, we evaluated the presence of InsR and IGF1R proteins through Western Blot (Fig. 7.7A) and immunofluorescence staining (Fig. 7.7B). Remarkably, the blot bands corresponding to InsR and IGF1R were successfully identified at the anticipated molecular weight (95 kDa). Equally notable were the identifications of blot bands for Pro-InsR and Pro-IGF1R at the predicted molecular weight (210 kDa). It is intriguing to note that both InsR and IGF1R exhibit analogous cellular location patterns, being present in the midpiece and flagellum of human spermatozoa.

7.3.6. Exogenous insulin does not further improve motility, viability, or metabolic function of human spermatozoa

Human spermatozoa possess both insulin and IGF-1 receptors, implying a potential regulatory role for insulin. To explore this hypothesis, we incubated *in vitro* a subset of HM human spermatozoa in capacitation media supplemented with insulin (1 nM). Following a 3 h incubation period, we proceeded to evaluate spermatozoa viability (Fig. 7.8A) as well as total and progressive motility (Fig. 7.8B). No differences concerning spermatozoa viability upon incubation with insulin ($90 \pm 5\%$) as compared to the control group ($90 \pm 4\%$) were observed. In addition, no differences were observed upon treatment with insulin on total ($79 \pm 11\%$) and progressive motility ($67 \pm 12\%$) as compared to the control group ($76 \pm 13\%$ and $62 \pm 15\%$, respectively).

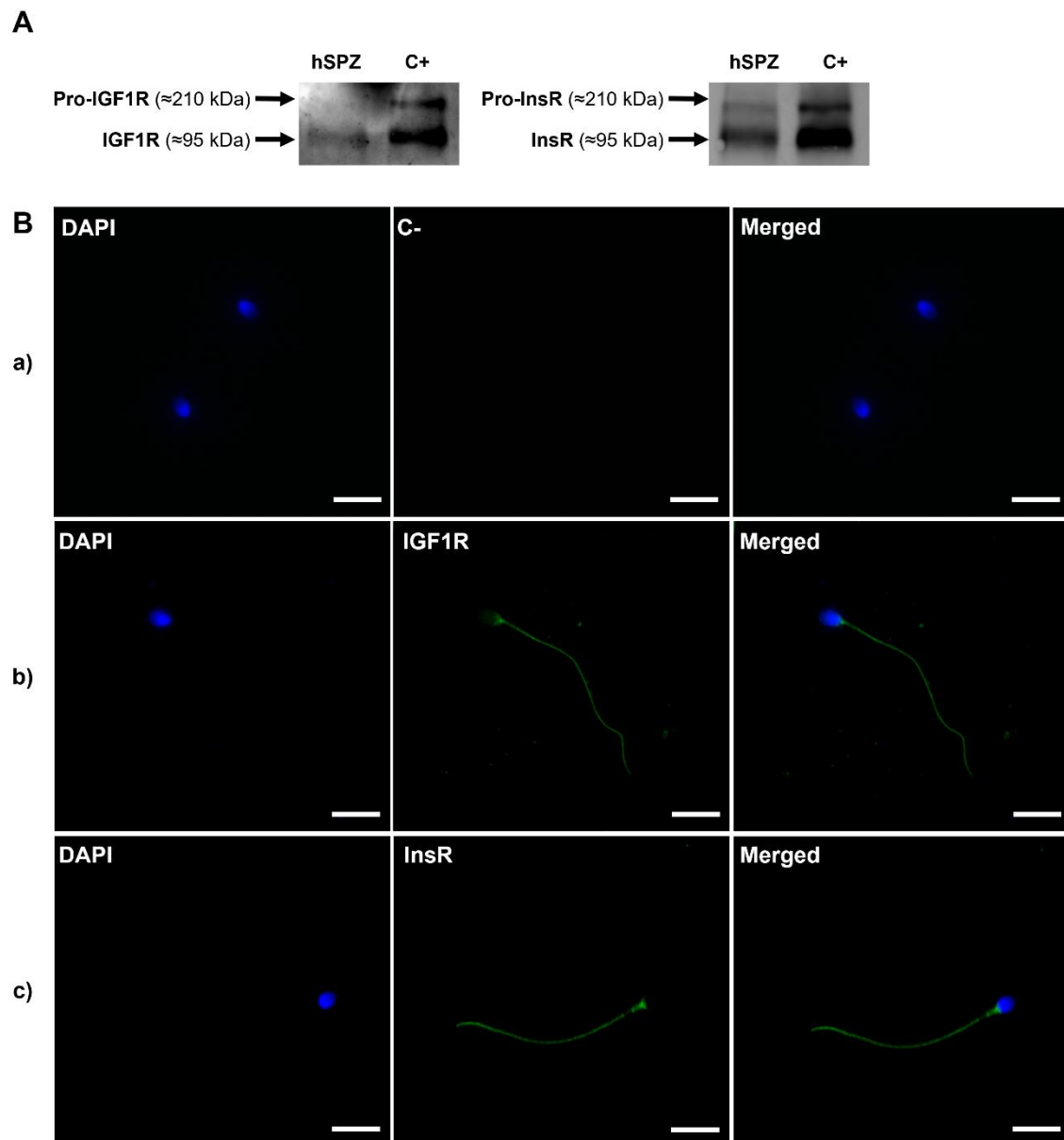


Fig. 7.7 – Identification and immunolocalization of IGF-1 receptor (IGF1R) and insulin receptor (InsR) protein in human spermatozoa. (A) Identification of IGF1R and InsR in human spermatozoa (hSPZ) by Western Blot. Mouse kidney lysate was used as positive control (C+). **(B)** Immune labeling of IGF1R **(b)**, and InsR **(c)** was observed in human spermatozoa. **(a)** Negative control. Scale bar—10 μ m.

To investigate the impact of insulin on human spermatozoa capacitation, we assessed two crucial factors: the mitochondrial membrane potential (Fig. 7.8C) and the overall oxidoreductase activity (Fig. 7.8D). These assessments were conducted using the JC-1 and MTT assays, respectively. However, no differences concerning the JC-1 ratio (3.60 ± 0.63)

J-aggregates/monomers) or oxidoreductases activity (1.03 ± 0.29 arbitrary units) upon insulin treatment were observed when compared to the control group (3.76 ± 0.69 J-aggregates/monomers and 1.00 ± 0.29 arbitrary units, respectively).

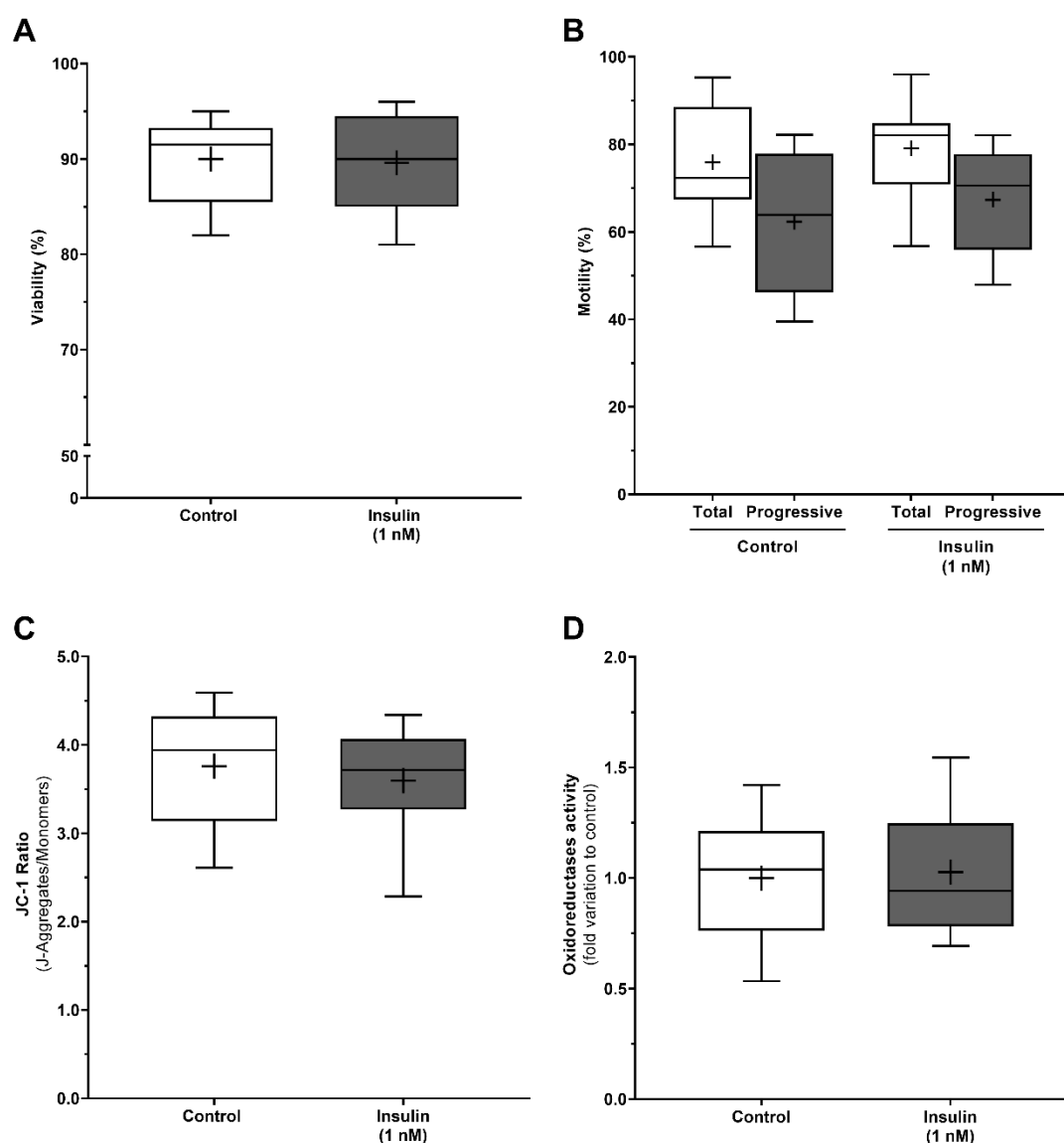


Fig. 7.8 – Effect of insulin (1 nM) incubation for 3 h in *in vitro* capacitation conditions on human spermatozoa viability, motility, mitochondrial membrane potential, and oxidoreductase activity. (A) Spermatozoa viability as determined by eosin-nigrosin staining ($n = 14$ for each condition). (B) Spermatozoa total and progressive motility ($n = 14$ for each condition). (C) Evaluation of mitochondrial membrane potential using the JC-1 assay ($n = 10$ for each condition). (D) Evaluation of oxidoreductases activity as determined by the MTT assay ($n = 10$ for each condition). Results were either normalized to the control group or expressed as Tukey's boxplot (median, 25th to 75th percentiles ± 1.5 IQR). + – Represents group average.

7.3.7. The InsR/IRS-1/PI3K/Akt pathway is active during human spermatozoa capacitation

The addition of exogenous insulin (1 nM) produced no observable effect on human spermatozoa during *in vitro* capacitation. However, we identified both insulin and its receptors in human spermatozoa, suggesting that insulin could already be regulating these cells in an autocrine/paracrine manner. For that purpose, we analyzed the insulin signaling pathway by Western Blot (Fig. 7.9). Interestingly, we observed the same blot bands intensity of phosphorylated insulin receptor and subsequent downstream targets on the control group as compared to the insulin-treated group, suggesting that insulin can be secreted during *in vitro* capacitation, activating the InsR/IRS-1/PI3K/Akt signaling pathway in an auto-crine/paracrine manner.

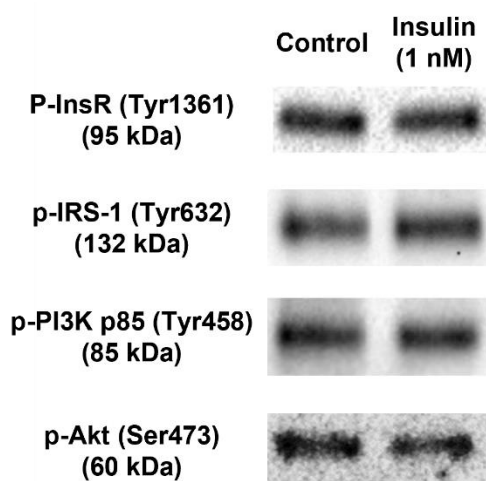


Fig. 7.9 – Assessment of the insulin signaling pathway during *in vitro* capacitation of human spermatozoa with or without exogenous insulin (1 nM) stimulation. Representative blot bands of phosphorylated Insulin Receptor (p-InsR (Tyr1361)), Insulin Receptor Substrate 1 (p-IRS-1 (Tyr632)), PI3 Kinase p85 (p-PI3K p85 (Tyr458)), and Akt (p-Akt (Ser473)). Stain-free TGX total protein content was used as loading control. Experiments were replicated four times ($n = 4$, each sample from a distinct individual).

7.4. Discussion

Insulin is believed to be exclusively produced and secreted by mammalian pancreatic beta cells in response to glucose levels. Insulin is initially synthesized in an inactive form, denominated preproinsulin. Preproinsulin is transported to the endoplasmic reticulum, where it is folded and cleaved into proinsulin, which is then packed into secretory vesicles in the

Golgi apparatus. In these vesicles, proinsulin is cleaved by PC1 and PC2, enzymes that remove the C-peptide, and stored until its secretion (Tokarz et al., 2018). In this work, we were able to demonstrate that human spermatozoa also express insulin. In addition, to the best of your knowledge, we were able to identify for the first time the presence of the mRNA and protein of PC1 and PC2 in human spermatozoa. These results suggest that human spermatozoa have the molecular machinery to produce insulin, whose production can no longer be pinpointed as exclusive of pancreatic beta cells. Interestingly, we observed that mRNA content of insulin and PC1 is higher in highly motile as compared to low motile spermatozoa. In addition, we found that insulin mRNA level is homogeneously expressed in highly motile spermatozoa, independently of age or BMI. Since spermatozoa are mainly translationally silent, except for the mitochondrial ribosomes (Gur & Breitbart, 2008), our hypothesis postulated a potential role for insulin mRNA in fertilization and embryo development. To explore this, we conducted an analysis on the correlation between insulin mRNA levels and the rates of successful fertilization, embryo development, and pregnancy. Surprisingly, our observations revealed no significant correlation between insulin mRNA content and any of these parameters. This suggests that while insulin mRNA remains consistently present in highly motile human spermatozoa, it may not directly impact on fertilization and embryo development. Consequently, we formulated a new hypothesis, speculating that the presence of insulin mRNA might be an indicator of insulin production within the germ cell line during spermatogenesis.

Inside pancreatic beta cells, insulin is stored in a crystalline form in secretory vesicles. It is estimated that insulin accounts for up to 10% of the total protein content on these cells, which corresponds to 8-9 ng/10⁶ cells (Rorsman & Ashcroft, 2018). In this work, we quantified insulin in total spermatozoa lysates and found that the total insulin content is about 10 times lower as compared to pancreatic beta cells (1.25 ± 0.71 ng/10⁶ spermatozoa). We also observed that insulin is mainly located in the junction between the head and midpiece of human spermatozoa, and at smaller extent in the acrosome region and midpiece. These results are supported by a previous study conducted by Aitken et al., who also identified the presence of insulin in human spermatozoa between the head and midpiece (Aitken et al., 2021).

Insulin acts through the insulin receptor, but it may also bind to the IGF-1 receptor, albeit with a lower affinity (Cai et al., 2017). Herein, we were able to identify the presence of both the insulin and IGF-1 receptor in the flagellum of human spermatozoa. The cellular location of both receptors suggests a potential role for the regulation of spermatozoa motility. Spermatozoa are recognized for its elevated glycolytic activity primarily located in the flagellum. This robust glycolysis provides the necessary ATP to sustain and fuel its motility (Travis et al., 2001). In spermatozoa, ATP serves as the energy source utilized by dynein ATPases to

regulate the microtubule movement, thereby facilitating and enhancing flagellar motility (Guerra-Carvalho et al., 2022). However, our results partially contrast with those found in the literature. Aitken et al. observed that the insulin receptor is expressed in the entire surface of the human spermatozoa, whereas we only found it in the flagellum (Aitken et al., 2021). Additionally, we were only able to find one study that reported the location of IGF-1 receptor in human spermatozoa, which was reported to be expressed mainly in the equatorial region of the head and, although weakly, in the acrosome region (Naz & Padman, 1999). The causes for these discrepancies are difficult to pinpoint, as it could be attributed to the use of different antibodies. Both in this study and in the study conducted by Aitken et al., the cellular location was assessed using cells from the high motility fraction acquired using density gradient centrifugation (Aitken et al., 2021). However, putative differences in study population and density gradient centrifugation pelleted cell subpopulation should not be overlooked. Future research is needed to confirm the cellular location of insulin and IGF-1 receptors in highly motile human spermatozoa.

The presence of both insulin and its receptor suggests that insulin may be released for the regulation of human spermatozoa in an autocrine/paracrine manner. In pancreatic beta cells, insulin is secreted in a process denominated glucose-stimulated insulin secretion (GSIS). During GSIS, elevating glucose levels triggers enhanced glucose uptake and glycolysis, subsequently boosting the ATP/ADP ratio. This augmented ratio induces the closure of ATP-sensitive K^+ channels, resulting in plasma membrane depolarization and activation of voltage-gated L-type Ca^{2+} channels. As a result, elevated intracellular Ca^{2+} levels prompt the exocytosis of insulin-containing granules, facilitating the release of insulin into the circulation (Campbell & Newgard, 2021). A potential parallelism can be hypothesized for human spermatozoa. In the female reproductive tract, human spermatozoa are exposed to high concentrations of HCO_3^- , which promote intracellular alkalinization. Spermatozoa intracellular alkalinization is required for the activation of the sperm specific cation channel, CatSper, which promotes the influx of Ca^{2+} (Puga Molina et al., 2018). Therefore, we hypothesize that the release of insulin would only occur when the conditions required for spermatozoa capacitation are met. Nevertheless, the involvement of CatSper and Ca^{2+} influx in insulin release by human spermatozoa remains to be unraveled. Although we hypothesize that insulin is released during capacitation, we were unable to detect insulin in the media with our currently available methods. We hypothesize that either the quantity of released insulin was inferior to the detection limits of our quantification methods ($0.4 \mu U/mL$), and/or insulin is only released in the initial period of capacitation and is no longer detectable in the media 3 h later. Nevertheless, insulin release by human spermatozoa had already been previously reported. The first evidence for insulin release was reported by Aquila et al., who reported that human spermatozoa secrete insulin in a glucose-dependent manner (Aquila

et al., 2005). Contrastingly, Aitken et al. reported that insulin is released in very small quantities to the media and its secretion does not respond to glucose concentrations (Aitken et al., 2021), suggesting that its secretion is only dependent on the ionic requirements necessary to initiate capacitation. Therefore, the potential glucose-dependent release of insulin by human spermatozoa requires confirmation in further studies.

Data concerning the role of insulin in the regulation of spermatozoa are scarce. In 2008, Lampiao and Plessis observed that the addition of insulin (0.35 ng/mL) to the media improved human spermatozoa total and progressive motility and acrosome reaction (Lampiao & du Plessis, 2008). Conversely, Wang et al. reported that higher insulin concentrations (10 and 100 µg/mL) did not produce any effect on human spermatozoa motility (Wang et al., 2015). In this context, our observations revealed that a concentration of 1 nM (equivalent to 5.7 ng/mL) of insulin had no discernible impact on human spermatozoa viability, motility, or metabolic activity throughout the course of *in vitro* capacitation. However, we were able to demonstrate that InsR/IRS-1/PI3K/Akt pathway is active in human spermatozoa during that process, even in the absence of exogenous insulin. This result further suggests that insulin is released during human spermatozoa capacitation and may act in an autocrine/paracrine manner for its regulation. Strong evidence suggests that the PI3K/Akt pathway plays a pivotal role in regulating human spermatozoa motility and acrosome reaction, although the intricate molecular mechanisms underlying this regulation remain incompletely elucidated (Breitbart et al., 2010; Tan & Thomas, 2014). Moreover, delving into the effects of this signaling pathway is complex due to the emerging evidence indicating that its functioning varies based on the spatial location of the involved enzymes. For instance, Reynoso et al. documented that PI3K/Akt pathway activation in the tail region enhances motility and viability (Reynoso et al., 2021). In support of this hypothesis, Koppers et al. demonstrated that the PI3K/Akt pathway is essential for human spermatozoa viability, since its inhibition promotes motility loss and activation of the apoptotic cascade (Koppers et al., 2011). Etkovitz et al. reported that during capacitation PKA and PI3K work together in the spermatozoa head, to promote actin polymerization, a process essential for the initiation of the acrosome reaction (Etkovitz et al., 2007). However, further studies are warranted to fully characterize the potential impact of insulin on the activation of the PI3K/Akt signaling pathway and its broader role in regulating human spermatozoa capacitation.

In summary, our study successfully revealed the presence of insulin and the essential biosynthesis enzymes, PC1 and PC2, within human spermatozoa. Intriguingly, we discovered that the mRNA content of insulin is notably higher in highly motile spermatozoa, maintaining this characteristic consistency regardless of age or BMI. Despite its heightened presence in these spermatozoa, the insulin mRNA content does not appear to correlate with fertilization rates, embryo development, or pregnancy rates. This observation suggests that

the mRNA could be a remnant of germ cell development during spermatogenesis. Furthermore, human spermatozoa express both the insulin and IGF-1 receptors, indicating a potential role for insulin in an autocrine/paracrine manner. Our data also verified the activation of the insulin signaling pathway during human spermatozoa capacitation, leading us to posit that insulin might be involved in regulating this process. However, further research is needed to validate this hypothesis.

7.5. Authors contribution

David F. Carrageta – conceptualization, methodology, formal analysis, investigation, writing – original draft preparation, writing – review and editing, funding acquisition; Bárbara Guerra-Carvalho – methodology, formal analysis, investigation; Sara C. Pereira – methodology; Alberto Barros – resources; Pedro F. Oliveira – conceptualization, formal analysis, investigation, resources, writing – review and editing, funding acquisition; Mariana P. Monteiro – conceptualization, formal analysis, investigation, writing – review and editing; Marco G. Alves – conceptualization, formal analysis, investigation, resources, writing – review and editing, project administration, funding acquisition.

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Chapter 8

Conclusions and future perspectives



8. Conclusions and future perspectives

The discovery and characterization of the physiological phenomena necessary for spermatozoa to fertilize the oocyte were essential for the development of *in vitro* fertilization (IVF) techniques. The process of capacitation was firstly and simultaneously described in the early 1950s by two researchers, Austin and Chang, using the rabbit as a model (Austin, 1951; Chang, 1951). Despite decades of intensive research, the capacitation and bioenergetics of human spermatozoa remain elusive and not fully understood. Besides the technical limitations derived from the lack of standardization, since studies on human spermatozoa range from 3 to 24 h and apply different sperm selection strategies to isolate highly motile cells, the main reason for the lack of knowledge concerns ethical restrictions (Puga Molina et al., 2018). In fact, the role of the human uterus, oviduct, or its secretions on human spermatozoa remains a mystery (De Jonge, 2017). For this reason, our best tool to study spermatozoa resides in *in vitro* experiments.

One of the main aims of this project was to characterize the role of glucose for the metabolic profile and mitochondrial activity of human spermatozoa. For that purpose, in Chapter 4 – “Mitochondrial activation and reactive oxygen species overproduction during sperm capacitation are independent of glucose stimuli” we studied the role of increasing glucose concentrations (5.5, 11, and 22 mM) or the absence of any energetic substrate during the *in vitro* capacitation of human spermatozoa (Carrageta et al., 2020). In this work, we provided further evidence that glucose is essential for human spermatozoa motility and capacitation, stimulating tyrosine residues phosphorylation. However, we also found that the presence of glucose does not seem to be necessary for mitochondrial (hyper)activation. We were able to demonstrate that human spermatozoa mitochondria increase its activity and ROS production in capacitating media, even in the absence of glucose or any other exogenous energy substrates. Considering these results, we hypothesized that the mitochondrial activation is only dependent on the ionic profile of the media, occurring independently of glucose stimuli. We then proposed that human sperm capacitation occurs as a two-step process. On the first step, mitochondria become active and produce ROS as signaling intermediates. As a second step, and when glucose is available, mitochondrial respiration increases to sustain the high ATP demands necessary for mass protein phosphorylation and motility. However, a more comprehensive understanding of the mechanisms triggering mitochondrial activation during capacitation necessitates future research endeavors.

Notably, the mitochondrial activation unfolds even in the absence of external energy substrates, alluding to the presence of an intrinsic energy reservoir. Additionally, the remarkable fact that human spermatozoa maintain their motility and viability for a span of at

least 6 h without external energy sources underscores the existence of a vital, albeit unidentified, internal energy reservoir within these cells. While future studies are necessary to pinpoint these endogenous energy sources, there are several promising contenders, which will be discussed in the subsequent topic.

8.1. Endogenous energy sources in human spermatozoa

In our first study, we observed that human spermatozoa maintain its motility for at least 6 h without the presence of exogenous energetic substrates (Carrageta et al., 2020). Interestingly, previous studies highlighted that human spermatozoa are able to maintain its motility for even longer periods of time (Amaral et al., 2011). Taken together, these results suggest the presence of endogenous sources of energy within human spermatozoa. One potential energy source could be glycogen (Silva et al., 2022). Although to the best of our knowledge no studies have been conducted in human spermatozoa, data from boar and dog spermatozoa provide some evidence that glycogen metabolism plays a role in capacitation. In spermatozoa from these species, glycogen was found to be located in both the tail and the head. In addition, evidence demonstrates its synthesis and catabolism in spermatozoa (Ballester et al., 2000; Marin et al., 2003; Palomo et al., 2003). Albarracín et al. demonstrated that dog spermatozoa are able to synthesize glycogen from lactate, which was found to accumulate after incubation for 2 h in a medium containing U-¹⁴C-lactate (Albarracin et al., 2004). Decreased levels of glycogen were later found after 4 h in capacitation conditions, suggesting that glycogen metabolism occurs during the capacitation of dog spermatozoa. Additionally, these authors also identified the presence of gluconeogenic enzymes, suggesting that dog spermatozoa may be able to use this metabolic pathway. Proteomics studies also suggest the presence of gluconeogenic enzymes in human spermatozoa (Amaral et al., 2013; Sharma et al., 2013), although its characterization and the role of gluconeogenesis for these cells remains to be disclosed.

Another potential endogenous energy source could be related to lipid metabolism through β -oxidation. The ability of spermatozoa to perform mitochondrial β -oxidation have been described in boar spermatozoa. Zhu et al. identified the presence of the mitochondrial β -oxidation enzymes carnitine palmitoyltransferase 1 (CPT1) and long-chain acyl-coenzyme A dehydrogenase (ACADL) in the midpiece of boar spermatozoa (Zhu et al., 2020). In addition, the supplementation of media with oleic acid and palmitic acid for 3 h increased the activity of mitochondrial β -oxidation enzymes, mitochondrial membrane potential, and stimulated ATP production, which resulted in increased motility. These effects were later counteracted through treatment with etomoxir, an irreversible inhibitor of CPT1, enzyme

which catalyzes an essential step in the carnitine shuttle that transports long-chain fatty acids into the mitochondria. Although still poorly understood in humans, proteomics and metabolomics studies have shown that human spermatozoa also express enzymes involved in the mitochondrial β -oxidation (Amaral et al., 2013; Paiva et al., 2015). In the study conducted by Amaral et al., human spermatozoa were incubated with etomoxir (0.01, 0.1, and 1 mM) and observed a dose-dependent decrease of total motility and viability (Amaral et al., 2013). More recently, Li et al. demonstrated the presence of CPT1, acyl-CoA synthetase long-chain family member 1 (ACSL1), ACADL, very long-chain acyl-CoA dehydrogenase (ACADVL) and hydratase subunit A (HADHA) in the midpiece of human spermatozoa (Li et al., 2023). These authors also reported that etomoxir decreases motility, further evidencing the role of mitochondrial β -oxidation in these cells. Additionally, these authors demonstrated that the nuclear factor- κ B (NF- κ B) signaling pathway regulates mitochondrial β -oxidation in human spermatozoa. The NF- κ B inhibitor α (IKBA), which phosphorylates and deactivates NF- κ B, was inhibited by the irreversible inhibitor Bay117082, which stimulated mitochondrial β -oxidation and, as a result, increased spermatozoa motility. These effects were found to be counteracted upon treatment with etomoxir, which supports the regulation of mitochondrial β -oxidation through the NF- κ B pathway. Moreover, these effects were found to be independent of glycolysis, since motility increased upon IKBA inhibition even in the absence of exogenous energy sources in the media and upon glycolysis inhibition through α -chlorohydrin (Li et al., 2023). Taken together, lipid metabolism seems to be one of the most important energy sources for human spermatozoa during capacitation and one of the main hypotheses to explain how motility is sustained without exogenous energy substrates. Additionally, increased mitochondrial β -oxidation would also explain the increased mitochondrial activity in the absence of exogenous energy sources that we observed in our first study. However, several intriguing questions still await answers:

1. Comprehensive lipidomics studies are required to meticulously delineate the lipid composition of human spermatozoa, spotlighting potential disparities in the lipidome between highly motile and less motile spermatozoa;
2. Once the lipidome is characterized, it becomes imperative to unravel the key lipids predominantly metabolized by human spermatozoa and to elucidate the triggering mechanisms behind the activation of mitochondrial β -oxidation during capacitation;
3. Considering lipid metabolism's potential role, its alterations could potentially be related to asthenozoospermia. The potential links between conditions that disrupt lipid metabolism, such as metabolic diseases-associated dyslipidemia or inflammation, and asthenozoospermia merit further in-depth investigation.

8.2. Mitochondria and reactive oxygen species production and signaling pathways in human spermatozoa

Oxidative stress and ROS production is a hot topic in the study of human fertility that has been discussed for decades. It is widely accepted that oxidative stress is a major cause of infertility and it is estimated that up to 80% of infertile men can have high ROS levels (Agarwal et al., 2019). The aetiology of oxidative stress is multifactorial and can be attributed to conditions such as chronic inflammation, metabolic disorders, ageing, and exposure to drugs and pollutants (Forrester et al., 2018; Martin-Hidalgo et al., 2019; Montano et al., 2022; Moreira et al., 2022). Several harmful and irreversible damages on the testes have been associated with oxidative stress, leading to lower sperm counts and high number of spermatozoa morphologically defective and immotile (Aitken et al., 2010; Athayde et al., 2007). Besides impaired spermatogenesis, oxidative stress is also associated with DNA fragmentation and genomic instability, which are positively associated with lower fertilization capacity, abnormal embryo development, and higher miscarriage rates (Cissen et al., 2016; McQueen et al., 2019; Tarozzi et al., 2007). For these reasons, studies targeting oxidative stress and the use of antioxidants to improve male fertility constitute a major fraction of the published literature in the field. Conversely, few studies have focused on the fact that ROS could be a two-edged sword for spermatozoa. While high ROS concentrations are harmful and the cause of oxidative stress-related damage, ROS levels within the physiological range are essential for capacitation and acrosome reaction. Robert John Aitken, a distinguished British reproductive biologist who dedicated his entire career studying the role of ROS in spermatozoa, refers to spermatozoa as “professional generators of ROS because of the fundamental role these molecules play in the induction of sperm capacitation” (Aitken & Drevet, 2020). The main pro-capacitation effects of ROS are hypothesized to occur due to its stimulatory activity on adenylyl cyclase and the simultaneous inhibition of tyrosine phosphatases, enhancing the tyrosine phosphorylation cascade (Aitken et al., 1995; Du Plessis et al., 2015; Rivlin et al., 2004). In addition, ROS increase cholesterol efflux from the membrane, increasing membrane fluidity, and promoting acrosome reaction and the ability to bind to *zona pellucida* (Aitken, 2000; Aitken et al., 1995). Therefore, ROS are regarded as essential to achieve fertilization, whereas high ROS levels are responsible for oxidative damage. The threshold or the mechanisms that regulate ROS levels, however, are still a matter of debate.

Mitochondria are regarded as one of the main sources of ROS in human spermatozoa. Nevertheless, the role of the mitochondria on the capacitation and bioenergetics of human spermatozoa is not fully established, since some authors proposed that motility is mainly fueled by glycolysis (Nascimento et al., 2008; Williams & Ford, 2001), whereas others stated

the Krebs cycle and oxidative phosphorylation to be also important (Ferramosca et al., 2012; Gallon et al., 2006; Marchetti et al., 2002; Piomboni et al., 2012). Besides, spermatozoa mitochondria exhibit unique features. For instance, it is reported that human spermatozoa mitochondria undergo morphological changes during capacitation. Using X-ray microscopy, Vorup-Jensen et al. demonstrated that mitochondria are initially compact and tightly wrapped around the axoneme and distend upon capacitation (Vorup-Jensen et al., 1999). More recently, Ren et al. demonstrated that the acrosome contains condensed mitochondria, which exhibit a higher expression of mitochondrial complexes and respiration rates (Ren et al., 2022). In this work, we further delve into the role of ROS and mitochondria for human spermatozoa. As aforementioned, in Chapter 4 – “Mitochondrial activation and reactive oxygen species overproduction during sperm capacitation are independent of glucose stimuli”, we were able to demonstrate that human spermatozoa mitochondria are active and produce higher amounts of ROS in *in vitro* capacitation media, even in the absence of glucose or any other exogenous energy substrates. These results led us to hypothesized that the mitochondrial activation and subsequent rise in ROS production solely depends on the media ionic profile and occurs independently of glucose stimuli. Based on these results, we decided to further explore the role of mitochondria and ROS in the regulation of human spermatozoa metabolism and function. For that purpose, in Chapter 5 – “Inhibition of mitochondrial uncoupling proteins arrests human spermatozoa motility without compromising viability”, we aimed to study the presence and function of mitochondrial uncoupling proteins (UCPs) in human spermatozoa. In this work, to the best of our knowledge, we were able to identify, for the first time, UCPs in the equatorial segment of the head of human spermatozoa. In addition, UCPs inhibition by the chemical inhibitor genipin irreversible arrests human spermatozoa motility without compromising its viability. We also observed that UCPs inhibition decreased lactate production, suggesting a metabolic shift. This pattern of expression and the effect of its inhibition suggest an active role of UCPs orchestrating the production of ROS and human spermatozoa metabolism, ultimately controlling motility, and potentially capacitation and acrosome reaction. Nevertheless, the mechanisms behind the UCPs’ control of human spermatozoa motility and the full extent of the metabolic modulation remains to be disclosed in future studies. Besides ROS regulation, it is possible that UCPs act as small C4 metabolites transporters in human spermatozoa, regulating the malate-aspartate shuttle as reported by Voza et al. in hepatocytes (Voza et al., 2014). In addition, the hypothesis that UCPs may have a role similar to Hv1 channels, regulating cytoplasm alkalization and Ca^{2+} influx that occurs in the beginning of capacitation, should be considered (Lishko & Kirichok, 2010). However, both hypotheses remain to be investigated in future studies. The fact that genetic manipulation techniques cannot be applied into spermatozoa and the lack of specific UCPs inhibitors increase the difficulty of achieving these goals.

Nevertheless, the discovery of UCPs in human spermatozoa opens the path for novel lines of research. For instance, UCPs are involved in fatty acid oxidation in other tissues (Busiello et al., 2015; Maassen et al., 2007), thus it could be hypothesized that UCPs would also regulate fatty acid oxidation in human spermatozoa. In the previous topic, we highlighted the potential role of mitochondrial β -oxidation as an endogenous energy source for human spermatozoa. Fatty acid oxidation can be a source of ROS in human spermatozoa through peroxisomal β -oxidation. As the name suggests, peroxisomal occurs in peroxisomes and uses very long-chain fatty acids (VLCFA) and branched fatty acids (BFA) as substrates, resulting in the generation of acetyl-CoA and hydrogen peroxide, instead of ATP as occurs in mitochondrial β -oxidation (Demarquoy & Le Borgne, 2015). Interestingly, VLCFA content in human spermatozoa is positively related with human spermatozoa quality and motility (Craig et al., 2019; Gavrizi et al., 2023). Although it is widely accepted that spermatozoa do not possess peroxisomes, proteomics studies identified the presence of several peroxisomal proteins in human spermatozoa (Amaral et al., 2013; Amaral et al., 2014; Chauvin et al., 2012). According to Remedios et al, 71% of all the known peroxisomal proteins have already been identified in human or mice spermatozoa (Horta Remedios et al., 2023). These authors also reported that acyl-CoA oxidase 1 (ACOX1), which regulates the rate-limiting step of peroxisomal fatty acid oxidation and results in the generation of hydrogen peroxide, is among the most abundant proteins expressed in spermatozoa. To the best of our knowledge, up to this date no formal studies on peroxisomal β -oxidation were conducted in human spermatozoa. Taken together, several questions remain to be clarified in future studies:

1. It is necessary to disclosure the mechanisms behind UCPs' regulation of human spermatozoa motility and its potential association with capacitation and acrosome reaction, where a potential role on cytoplasm alkalinization and Ca^{2+} influx should be considered;
2. Although UCPs are classically considered as uncoupling proteins, novel data suggests that its role may be more complex than proton transport. It is essential to clarify the involvement of UCPs in ROS regulation and which signaling pathways are regulated by ROS in human spermatozoa;
3. The metabolic and redox regulation might be orchestrated by UCPs, opening the path for novel lines of investigation. Among those, lipid metabolism pathways merit further research;
4. The development of specific pharmacological inhibitors is imperative to unveil the role of each UCP homolog.

8.3. Relevance of mitochondrial uncoupling proteins for the testis and Sertoli cells

During this work, we highlighted how testicular cells are dependent on the metabolic and oxidative status of the organism. Metabolic alterations and oxidative stress are known to cause testicular damage either directly at germ cells and indirectly through Sertoli and Leydig cells (Guerra-Carvalho et al., 2022). As aforementioned, Sertoli cells play an essential role on the metabolic support of spermatogenesis, besides its mechanical support and immunological protection (Rato et al., 2012). Given the substantial effect of UCPs inhibition on spermatozoa, we sought to evaluate the potential role of UCPs in the metabolic control and function of human Sertoli cells. In the work detailed in Chapter 6 – “Mitochondrial uncoupling proteins regulate the metabolic function of human Sertoli cells” we aimed to study the expression and function of UCPs in human Sertoli cells. In this work, to the best of our knowledge, we were able to demonstrate for the first time the expression of UCP1, UCP2, and UCP3 in human Sertoli cells. Genipin, the chemical inhibitor of UCPs, was also used to study UCPs role on Sertoli cell metabolism. We observed that the prolonged UCPs inhibition greatly impaired oxidative phosphorylation in human Sertoli cells, leading to decreased proliferation. In addition, a decreased ROS production was also observed, suggesting that mitochondria are one of the main source of ROS in human Sertoli cells. These results open the path for novel studies focusing on the role of UCPs in the testis. Given the limitations of genipin as an inhibitor and the lack of selective inhibitors for each homolog, studies with genetic manipulation of UCPs expression could provide evidence on the specific role of each homolog. Besides the potential protective role of UCPs against oxidative stress, an interesting approach would be to study its role on lipid metabolism at the testis. The fact that the testis is an organ able to accumulate fat is often overlooked (Campos-Silva et al., 2015; Li et al., 2020). Adipose tissue accumulation is often associated with chronic inflammation and oxidative stress, which are common causes of poorer spermatogenesis (Dutta et al., 2021). For instance, Crisóstomo et al. demonstrated that mice fed a high-fat diet before adulthood, depict a distinctive testicular lipid content and metabolism, even when the diet is reversed into standard. These authors also observed an over accumulation of proinflammatory n-6 polyunsaturated fatty acids in the testis, which were associated with negative effects on sperm quality (Crisostomo et al., 2020). In a follow-up study, Crisóstomo et al. demonstrated that these alterations persisted in the testis of the offspring for at least two generations (Crisostomo et al., 2022). Sertoli cells are known to accumulate fat in lipid droplets and to use fatty acids as energy source (Gorga et al., 2023; Regueira et al., 2018; Rindone et al., 2023), but palmitic acid accumulation leads to Sertoli cells cytotoxicity and

blood-testis barrier disruption (Ge et al., 2022; Ge et al., 2018). Therefore, several questions can be explored in future studies:

1. Adipose tissue and fat accumulation in the testis are linked to metabolic dysfunction, inflammation, and oxidative stress. Obesity is linked with the dysfunction or altered expression of UCPs in the adipose tissue, which promotes accumulation of fat. Therefore, it is essential to disclosure whether UCPs are involved in the regulation of lipid metabolism in the testis and whether the altered expression or dysfunction of UCPs promote fat accumulation as in the adipose tissue;
2. In case of an active role of UCPs in the testicular lipid metabolism, the potential role of UCPs in the testicular metabolic dysregulation that occurs in men with obesity should be clarified;
3. Fat accumulation and inflammation are known causes of oxidative stress. The testis is greatly affected by oxidative stress, which is one of the major causes for male infertility. It must be determined if UCPs play a role in the protection against oxidative stress in the testis and if altered expression or loss of function of UCPs render Sertoli cells more susceptible to oxidative stress;
4. UCP1 is associated with thermogenesis and temperature regulation. Temperature is critical for the testis and the optimal development and maturation of spermatozoa. We found that human Sertoli cells express UCP1, which suggests that Sertoli cells could also be involved in a potential thermogenic regulation of the testis.

8.4. Human spermatozoa as hormone-producing cells: is fertilization a competitive and solitary swimming marathon or a teamwork effort?

The journey of spermatozoa within the female reproductive system is usually depicted as a colossal swimming marathon where only the healthiest, fittest, and fastest spermatozoon gets to win the prize of fertilizing the oocyte to generate a new life, as if it was a heroic and divine Olympic champion. However, this is not a simple swimming competition, as this journey is full of obstacles that the “chosen one” must overcome. Among the well-known obstacles are the acidic pH of the vaginal epithelium, cervical mucus, and the immune system (Archana et al., 2019; Mahdavinezhad et al., 2022). Additionally, a prolific biochemical and ionic environment is necessary for the capacitation of spermatozoa, a finely regulated and complex biochemical process that only occurs in female reproductive tract and is essential for acquiring fertilizing ability. During capacitation, spermatozoa gain membrane fluidity and

achieve a state of hyperactivation, characterized by a specific type of motility conferred by a high amplitude and asymmetrical beating pattern of the flagellum (Aitken & Nixon, 2013). Therefore, the fittest spermatozoon, which can endure, adapt, and overcome all these difficulties, must go through a long and epic journey until reaching the oocyte. Then, the final challenge arises. The oocyte is covered by the *zona pellucida*, a thick extracellular matrix which must be penetrated to achieve fertilization. Only a low percentage of spermatozoa arrives to the oocyte location, and from those only the few that possess the necessary characteristics will bind and undergo the final step of maturation – the acrosome reaction (Aldana et al., 2021). Both the capacitation and acrosome reaction are necessary for spermatozoa to be able to penetrate the *zona pellucida* and the oocyte membrane, the paternal and maternal gametes fuse, leading to the cortical reaction, a biochemical modification of the oocyte membrane to prevent penetration by further spermatozoa (Cheeseman et al., 2016; Liu, 2011). Finally, the heroic spermatozoon overcame the solitary and competitive swimming marathon, fertilized the oocyte and was able to generate a new lifeform, in all its glory. The story of this epic journey has been told across cultures and generations, propagating the myth that each one of us performed this heroic feat before even being born. But is this epic journey that solitary and competitive or are we, in fact, perpetuating an old-fashioned story? Notwithstanding the sociological implications of this androcentric perspective, where the male gamete is portrayed as the “hero” and the female gamete as the “prize”, current evidence shows that the scientific basis of this popular interpretation is, in fact, outdated.

8.4.1. Interactions between spermatozoa and the female reproductive system

The female reproductive tract is often described as a hostile environment for spermatozoa, and it was hypothesized that the selective environment would ensure that only high-quality gametes would reach the oocyte. It is currently known that the relationship between the female reproductive tract and spermatozoa is much more complex. Several studies demonstrate that there are also positive interactions between the female reproductive tract and spermatozoa (Wolfner et al., 2023). The most obvious positive interaction lies within the fact that it is in the female reproductive tract that spermatozoa encounter the correct ionic and nutritional environment for capacitation (Aitken & Nixon, 2013). Yet, this interaction significance extends far beyond merely inducing capacitation. Research has unveiled that the initial segment of the oviduct offers a protective haven, allowing a subset of spermatozoa to undergo a storage phase, prompting some experts to liken it to a “sperm reservoir” (Rodriguez-Martinez, 2007). The existence of a sperm reservoir was found to occur in some mammalian species almost five decades ago, including pigs (Hunter, 1981), rodents

(Harper, 1973; Suarez, 1987), sheep (Hunter & Nichol, 1983), and cows (Hunter & Wilmut, 1984). Sperm reservoirs are also hypothesized to occur in humans, although there is no clear evidence of its occurrence (Suarez & Pacey, 2006). In these reservoirs, spermatozoa bind to the oviduct epithelial cells and find the correct environment for the maintenance of its viability for longer periods of time and the stimuli to undergo a controlled capacitation (Mirihaigalle et al., 2022). Although in humans we do not have clear *in vivo* evidence of this binding, there is evidence that human spermatozoa can bind to the apical surface of endosalpingeal epithelium *in vitro* (Baillie et al., 1997; Pacey et al., 1995a; Pacey et al., 1995b; Reeve et al., 2003). Then, spermatozoa are quiescent until slowly released towards the site of fertilization, which is thought to limit the number of spermatozoa that reach the oocyte to help reduce the risk of polyspermy (Hunter et al., 1999). Spermatozoa are then guided towards the oocyte by chemoattractants present in the follicular fluid (Suarez & Pacey, 2006). Progesterone, a steroid hormone produced by the *corpus luteum* in the ovary, is regarded as a main chemoattractant (Mirihaigalle et al., 2022). Besides the *corpus luteum*, cumulus cells also produce and secrete progesterone, working as a cellular source of chemoattractants (Guidobaldi et al., 2008). The concentrations of progesterone in the oviduct after ovulation were found to be higher than in the serum (Stefanczyk-Krzymowska et al., 1994). In addition, progesterone is also reported to regulate the release of spermatozoa from the sperm reservoir, further evidencing the interactions between the female reproductive tract and spermatozoa. It was shown that progesterone binds to the Abhydrolase Domain-Containing 2 Acylglycerol Lipase (ABHD2), a serine hydrolase that depletes 2-arachidonylglycerol from the membrane of spermatozoa, activating the Ca^{2+} channel CatSper and inducing a Ca^{2+} influx (Miller et al., 2016). However, the interaction between capacitation and sperm release is a “hen and egg” situation – it still is not clear if capacitation is the reason why spermatozoa are released or if its release is necessary to complete capacitation (Mirihaigalle et al., 2022).

8.4.2. Interactions among spermatozoa: unveiling evidence of collaborative teamwork

In addition to the interaction of spermatozoa with the female reproductive tract environment, highlighting the collaborative nature between the male gamete and the female reproductive tract, spermatozoa also exhibit the ability to engage with one another. At the macro level, the clearest evidence resides on its motility. Although it is known that different species differ concerning the morphology or even metabolic characteristics of its male gametes, it is very curious that different species may have developed similar strategies to achieve fertilization.

One of the first reports concerning the cooperation of mammalian spermatozoa was published two decades ago in *Nature*. Harry Moore and collaborators observed an interesting phenomena in wood mouse spermatozoa (Moore et al., 2002). This rodents' spermatozoa head displays a falciform morphology, resembling a long hook. When spermatozoa were collected from the epididymis these were in a single cell suspension, however, 5 min after incubation in *in vitro* fertilization media, the apical hook of each spermatozoon adhered to other spermatozoa flagellum or hook, forming a motile cluster. The authors described the phenomena as the assemble of motile spermatozoa "trains", constituted by hundreds to several thousand cells. Interestingly, when in media with a viscosity similar to the female reproductive tract secretions, these clusters exhibited a more rapid progressive motility when compared to single spermatozoon. To confirm the occurrence of these spermatozoa "trains" *in situ*, the authors also collected spermatozoa from the uterus of female wood mice 2 h after mating. Not only the occurrence of spermatozoa "trains" was confirmed, but also that it contained approximately 95% of the recovered spermatozoa. The occurrence of spermatozoa clusters was found to be time-limited, as spermatozoa started to disband 90 min after being placed in capacitation media, which was concurrent with a higher percentage of hyperactivated and acrosome-reacted spermatozoa (Moore et al., 2002). This unusual morphology of the spermatozoa head is a characteristic shared by several other rodent species, which some years later were found to exhibit the same cooperative behavior (Fisher & Hoekstra, 2010; Immler et al., 2007). However, rodents are not the only mammals whose spermatozoa exhibit cooperative behavior. Although its morphology is much different and the hook is not present, the same cooperative behavior was found in spermatozoa from bulls. It was observed that bull spermatozoa form dynamic clusters for collective swimming in response to high viscoelastic fluids (Morcillo et al., 2022; Tung et al., 2017). The formation of clusters was found to be advantageous, as spermatozoa swim together and aligned towards the same direction through the synchronization of its flagellar beat (Phuyal et al., 2022; Zhang et al., 2023). Although some authors defend that the formation of clusters occurs due to the presence of adhesion proteins in its head, others report that bulls' spermatozoa do not physically attach to each other and that the composition of the clusters is highly dynamic, as spermatozoa can freely swim into and out of the cluster over time (Tung et al., 2017). Besides the bull, the association of spermatozoa in clusters was also observed in opossums (Moore & Taggart, 1995; Rodger & Bedford, 1982) and sheep (Creppy et al., 2013; David et al., 2015). More recently, evidence suggests that human spermatozoa also cooperate in response to high viscosity fluids. Xiao et al. demonstrated that human spermatozoa attach at the head region of each other and migrate as a cooperative group when swimming from the low viscosity seminal fluid into and through a high viscosity medium (Xiao et al., 2023). The authors also observed that spermatozoa predominantly involved in

cooperative behavior which could be protective against DNA damage and premature capacitation, were those with higher DNA integrity and membrane cholesterol content. Mirroring observations in rodents, this cooperative behavior among human spermatozoa was similarly noted to diminish during capacitation, leading to the dissolution of groups as the surrounding viscosity decreases (Xiao et al., 2023).

Overall, it is becoming clear that spermatozoa do interact with each other, although the underlying molecular mechanisms are still unknown. Besides their potential physical interaction through membrane molecules, human spermatozoa may also interact and regulate its own and nearby spermatozoon's metabolism and capacitation through the production and/or release of certain molecules in an autocrine/paracrine manner. Several pieces of this puzzle can be collected from both this work and previously published studies. For instance, we aforementioned proteomics studies that reported the presence of gluconeogenic enzymes in human spermatozoa (Amaral et al., 2013; Sharma et al., 2013), and although its activity was never demonstrated, it suggests that gluconeogenesis could take part on the metabolism of human spermatozoa. In Chapter 4 – “Mitochondrial activation and reactive oxygen species overproduction during sperm capacitation are independent of glucose stimuli” we observed that human spermatozoa secrete malate during capacitation. Malate is a Krebs cycle intermediate that is reported to improve the metabolism and mitochondrial function of human spermatozoa when added to the capacitation media (Ferramosca et al., 2008; Gerez de Burgos et al., 1994; Storey & Kayne, 1978). This led us to hypothesize that malate release and uptake may be necessary for optimal mitochondrial activity of the surrounding spermatozoa (Carrageta et al., 2020). In addition, malate participates in the malate-pyruvate shuttle for the generation of cytosolic NADPH, which may fuel the generation of hydrogen peroxide through NADPH oxidases (Aitken, 2000). In addition to a potential metabolic cooperation, compelling evidence highlights that human spermatozoa also produce and secrete insulin and leptin, which may act in an autocrine/paracrine manner to support capacitation. In light of these findings, spermatozoa could join a class of hormone-producing cells, which will be detailed in the next section.

8.4.3. Spermatozoa as hormone-producing cells

Several studies provide evidence that human spermatozoa also produce and secrete hormones, namely insulin and leptin, but the designation of spermatozoa as hormone-producing cells has been disregarded by the scientific community. Overall, these findings suggest that insulin and leptin released by spermatozoa may act in an autocrine/paracrine manner to support capacitation (summarized in Fig. 8).

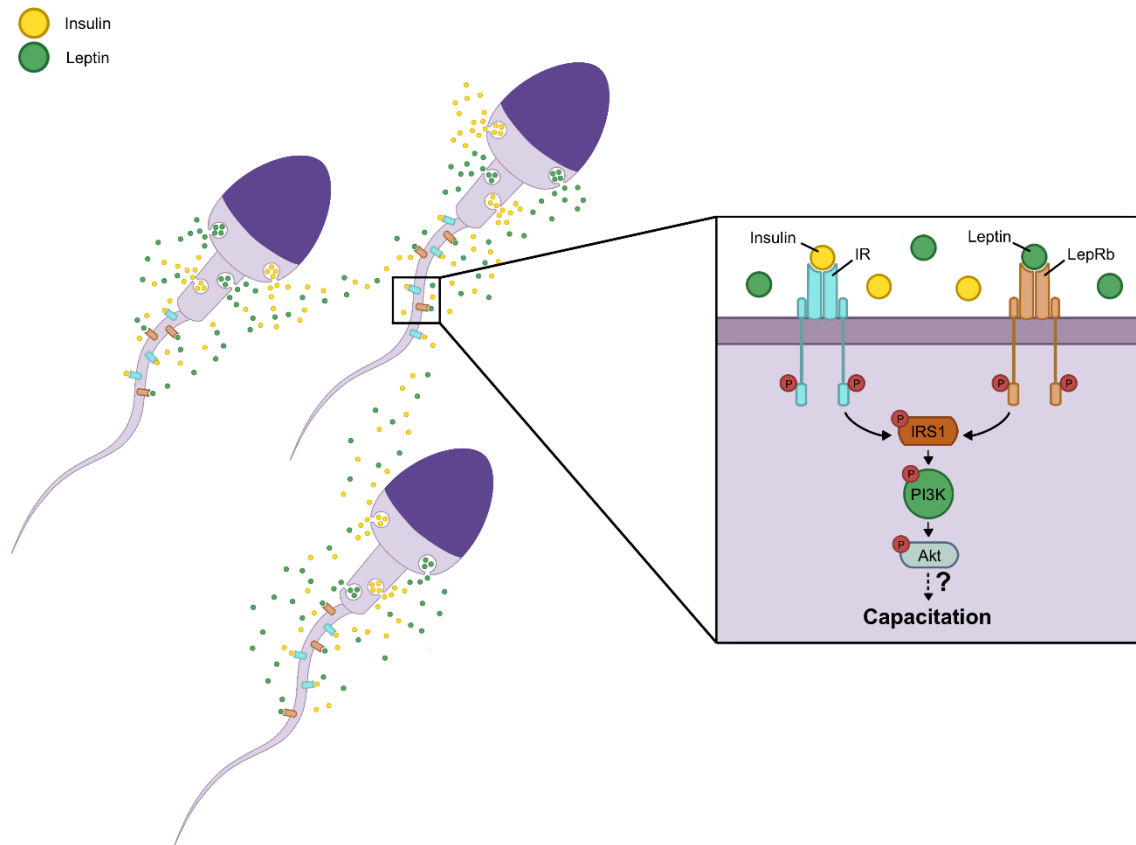


Fig. 8 – Schematic illustration of human spermatozoa hormonal release and paracrine activity.

Human spermatozoa release insulin and leptin, which may act in an autocrine/paracrine manner to regulate capacitation. Insulin and leptin are co-localized in the equatorial region of the head and midpiece of human spermatozoa. It is hypothesized that both hormones are released during capacitation, leading to the activation of the insulin receptor (IR) and leptin receptor (LepRb), present in the principal piece, and induction of capacitation through the IRS1/PI3K/Akt pathway. *Abbreviations:* Akt – protein kinase B; IRS1 – insulin receptor substrate 1; PI3K – phosphoinositide 3 kinase.

In the work described in Chapter 7 – "Insulin promotes capacitation through auto-crine/paracrine mechanisms in human spermatozoa" we demonstrated that human spermatozoa contain insulin, as well as PC1 and PC2, the enzymes that catalyze the conversion of proinsulin into insulin. Additionally, we demonstrated that human spermatozoa also possess both insulin and IGF-1 receptors. Not only this data demonstrates that human spermatozoa possess all the molecular machinery to produce insulin, but also that insulin may play an important role in the physiology of the male gamete. The potential role of insulin on human spermatozoa capacitation had been previously suggested in 2005 by Aquila et al., who reported that human spermatozoa releases insulin in a glucose-dependent manner (Aquila et al., 2005a). However, neither our group nor Aitken et al. could reproduce these results (Aitken et al., 2021). Nonetheless, we observed that the insulin signaling pathway is

active during the capacitation process, suggesting that insulin may indeed have an autocrine/paracrine function in the regulation of human spermatozoa capacitation. While its precise role is yet to be unveiled, the significance of these discoveries is evident — human spermatozoa are capable of synthesizing and releasing hormones under specific conditions. In fact, other studies found in the literature support this hypothesis. For instance, it was reported that human spermatozoa also produce and secrete leptin (Aquila et al., 2005b). Leptin is a hormone that is mainly responsible for the feeling of satiety through its actions in the hypothalamus. However, and depending on the cell type, leptin can also directly modulate its metabolism (Moreira et al., 2018). In 2003, human spermatozoa were found to express the leptin receptor in the tail (Jope et al., 2003). Two years later, Aquila et al. found that leptin is, in fact, secreted during human spermatozoa capacitation (Aquila et al., 2005b). The authors observed that leptin is mainly present in the midpiece and equatorial region of the head of human spermatozoa. Interestingly, if we compare both studies to our results, leptin and leptin receptor seem to be co-localized with insulin and insulin receptor, respectively. These authors also observed that leptin activates the PI3K/Akt pathway, leading to increased membrane cholesterol efflux and protein tyrosine phosphorylation. In a similar fashion to insulin, this data suggests that leptin is secreted by human spermatozoa and may act in an autocrine/paracrine manner to stimulate capacitation. Additionally, Aquila et al. identified the presence of aromatase in the midpiece and tail of human spermatozoa (Aquila et al., 2002). The presence of aromatase in human spermatozoa suggests that these cells may convert testosterone into estradiol. Interestingly, the content of aromatase was found to be higher in highly motile spermatozoa (Lambard et al., 2003). Aromatase activity was also found to be higher in the presence of 2'-O-dibutyryl cAMP, a cell-permeable cAMP analog that activates PKA, suggesting that its activation may occur during capacitation (Aquila et al., 2002). A decade later, the same group also identified the presence of an active form of 5 α -reductase, enzyme that catalyzes the conversion of testosterone in 5 α -dihydrotestosterone (DHT) (Aquila et al., 2015). Overall, these studies demonstrate that human spermatozoa can convert testosterone into both 17 β -estradiol and DHT, although its role on human spermatozoa capacitation is not clear. Human spermatozoa express both active androgen and estrogen receptors (Guido et al., 2011; Zuccarello et al., 2009). Ridley & Blasco reported that the testosterone concentration in seminal fluid of infertile males was higher (750 ± 110 pg/mL) as compared to fertile males (290 ± 30 pg/mL) (Ridley & Blasco, 1981), however, other studies reported higher testosterone levels in normozoospermic men (690 ± 4 pg/mL, (Moreno-Escallon et al., 1982); 710 ± 0.08 pg/mL, (Adamopoulos et al., 1976)). The former authors also observed that testosterone concentrations higher than 1.5 ng/mL severely decreased human spermatozoa motility (Ridley & Blasco, 1981). Con-

versely, Aquila et al. reported that much higher concentrations (1 nmol/mL, which is equivalent to 288 ng/mL) improved human spermatozoa migration and capacitation (Aquila et al., 2003). To the best of our knowledge, no further studies were conducted on the role of testosterone on the *in vitro* human spermatozoa capacitation, and the varied concentration ranges used in these studies pose a challenge to meaningful comparisons. Data on the effects of DHT is even more scarce. It was reported that low DHT levels (1 pmol/mL, which is equivalent to 290 pg/mL) improves cholesterol efflux (Aquila et al., 2003), but higher concentrations were not beneficial for acrosome reaction or tyrosine residues phosphorylation (Guido et al., 2014). In comparison to testosterone, 17 β -estradiol levels were found to be much lower in normozoospermic men (between 30 to 70 pg/mL) (Santemma et al., 1991; Zhang et al., 2010). Adeoya-Osiguwa et al. reported that 17 β -estradiol (1 to 100 nmol/mL, which is equivalent to 0.272 to 27.2 μ g/mL) rapidly improved the capacitation and acrosome reaction of mouse spermatozoa, but had no further effect after 30 min (Adeoya-Osiguwa et al., 2003). In human spermatozoa, Aquila et al. demonstrated that 17 β -estradiol (1 nmol/mL, which is equivalent to 272 ng/mL) improved the migration and capacitation (Aquila et al., 2003). Kotwicka et al. demonstrated that 17 β -estradiol (100 pmol/mL, which is equivalent to 27.2 ng/mL) rapidly improved mitochondrial membrane potential but a higher concentration (1 nmol/mL, which is equivalent to 272 ng/mL) had the opposite effect. Additionally, 17 β -estradiol increased mitochondrial superoxide production in a dose-dependent manner (Kotwicka et al., 2016). Even if there is a lack of standardization on type of media, incubation time, or concentrations used that difficult comparison between studies, the data suggests that steroid hormones may play a role in the initial moments of capacitation.

Overall, the fact that a physical, metabolic, and hormonal cooperation exists among the male gametes challenges the paradigm of the intense competition for fertilization. Our hypothesis postulates that upon entering the female reproductive tract, human spermatozoa encounter the requisite environment crucial for capacitation. In response, these spermatozoa would engage all available mechanisms to accomplish successful capacitation and trigger the indispensable acrosome reaction – both vital attributes for achieving fertilization. However, these mechanisms not only occur within each individual cell, but also result in the production and secretion of factors that influence the action of all spermatozoa. All this evidence suggests that the Olympic swimming marathon of spermatozoa is, in fact, a teamwork effort where all the parts involved contribute for a successful fertilization and win the real prize – the continuation of the species. However, several questions remain to be answered:

1. Our data suggests that insulin may be produced by human spermatozoa, however we cannot pinpoint yet the timing of production. Therefore, it is necessary to disclose if

insulin is produced during spermatogenesis and/or whether human spermatozoa can produce insulin post-ejaculation;

2. Our data suggests that insulin plays a role on human spermatozoa capacitation. It is imperative to study the insulin signaling pathway and underlying mechanisms associated with the capacitation;
3. A potential role of the hormones secreted by human spermatozoa on the female reproductive tract should be considered. Besides the autocrine/paracrine role of insulin or leptin on human spermatozoa capacitation, these hormones may also be involved in the process of fertilization;
4. The acceptance that spermatozoa insulin may have a paracrine action to promote a cooperative behavior suggest that other hormones, metabolites, or signaling molecules may be produced and secreted by human spermatozoa to promote its interaction. It also opens the possibility that an impaired spermatozoon-spermatozoon interaction could be one of the previously unidentified causes for idiopathic infertility;
5. Given the characterization of human spermatozoa's paracrine activity, it is possible to speculate that manipulating spermatozoon-spermatozoon communication could be a target for the development of new therapies or contraceptives.

8.4.4. Final remarks

Human spermatozoa are very specialized and unique cells. Spermatozoa are the only cell type that can leave the organism where it is produced and travel to the female counterpart, where will serve its purpose – fertilize the oocyte and generate a new lifeform. The journey of spermatozoa, however, is not the simple solitary and competitive swimming marathon once thought. Although challenging due to the hostile conditions found in the female reproductive tract, a series of complex cooperative interactions between spermatozoon-spermatozoon and spermatozoa-female reproductive tract do occur. Overall, spermatozoa can be described as paracrine cells capable of producing and secreting hormones such as insulin, and leptin. Besides, evidence points towards a potential cooperation, where each spermatozoon helps each other to achieve a successful capacitation. Taken together, the fact spermatozoa exhibit cooperative behavior constitutes a potential paradigm shift in our understanding of the biology of reproduction. Although several questions remain to be answered, the implications of the study of these interactions pave the way for an exciting new path of research that may revolutionize the field.

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Annexes



Annex A - Integral manuscripts

A1. Mitochondrial uncoupling proteins (UCPs) as key modulators of ROS homeostasis: a crosstalk between diabetes and male infertility?

Abstract

Uncoupling proteins (UCPs) are transmembrane proteins members of the mitochondrial anion transporter family present in the mitochondrial inner membrane. Currently, six homologs have been identified (UCP1-6) in mammals, with ubiquitous tissue distribution and multiple physiological functions. UCPs are regulators of key events for cellular bioenergetic metabolism, such as membrane potential, metabolic efficiency, and energy dissipation also functioning as pivotal modulators of ROS production and general cellular redox state. UCPs can act as proton channels, leading to proton re-entry the mitochondrial matrix from the intermembrane space and thus collapsing the proton gradient and decreasing the membrane potential. Each homolog exhibits its specific functions, from thermogenesis to regulation of ROS production. The expression and function of UCPs are intimately linked to diabetes, with their dysregulation/dysfunction not only associated to diabetes onset, but also by exacerbating oxidative stress-related damage. Male infertility is one of the most overlooked diabetes-related comorbidities, where high oxidative stress takes a major role. In this review, we discuss in detail the expression and function of the different UCP homologs. In addition, the role of UCPs as key regulators of ROS production and redox homeostasis, as well as their influence on the pathophysiology of diabetes and potential role on diabetes-induced male infertility is debated.

A1.1. Introduction

Obesity and diabetes mellitus are perilous health issues that also constitute a huge economic burden worldwide. It is currently estimated that 9.3% of the world population suffers from diabetes mellitus, whereas 39% of adults aged 18 years and over are overweight, and 13% are obese (Nuertey et al., 2017; Saeedi et al., 2019). Obesity and diabetes mellitus are intrinsically related to each other, leading to the term “diabetes”. Both conditions are regarded as metabolic diseases due to the impairment of metabolism and augmented levels of reactive oxygen species (ROS), that result in oxidative stress (Vincent & Taylor, 2006). The biggest source of ROS is the mitochondrial oxidative phosphorylation, which is critical to produce the high quantities of ATP essential for cell survival. However, oxidative phosphorylation is not perfectly coupled to ATP synthesis and some of the energy present in the

electrochemical force is dissipated due to the re-entry of protons in the mitochondrial matrix independently of ATP synthase. Indeed, some electrons escape to oxygen (0.2–2%), essentially in complexes I and III, leading to the appearance of reactive oxygen species (ROS) (Carrageta et al., 2020; Ferramosca & Zara, 2014; Ramsden et al., 2012; Zhao et al., 2019). Although ROS, at physiological levels, are important cell signaling agents, elevated concentrations of these radicals are extremely noxious to cells. Therefore, it is imperative to control their generation (Carrageta et al., 2020; Ferramosca & Zara, 2014; Rhee, 2006; Zhao et al., 2019). The movement of protons to the mitochondrial matrix without passing through the ATP synthase results in an enormous decrease in ROS production while having a minimal effect on ATP synthesis (a reduced attenuation in the potential difference in the inner membrane of the mitochondria can restrain the formation of H_2O_2 by 70%) (Chan et al., 2006; Miwa & Brand, 2003; Ramsden et al., 2012). Proton transport to the mitochondrial matrix independent of ATP synthase occurs through two processes: basal proton leak and inducible proton leak (Fig. A1). Basal proton leak is not finely regulated and depends only on fatty acid composition of the inner mitochondrial membrane and on the abundance of adenine nucleotide translocase (ANT) (Zhao et al., 2019). On the other hand, inducible proton leak is finely regulated, with uncoupling proteins (UCPs) playing a crucial role (Zhao et al., 2019).

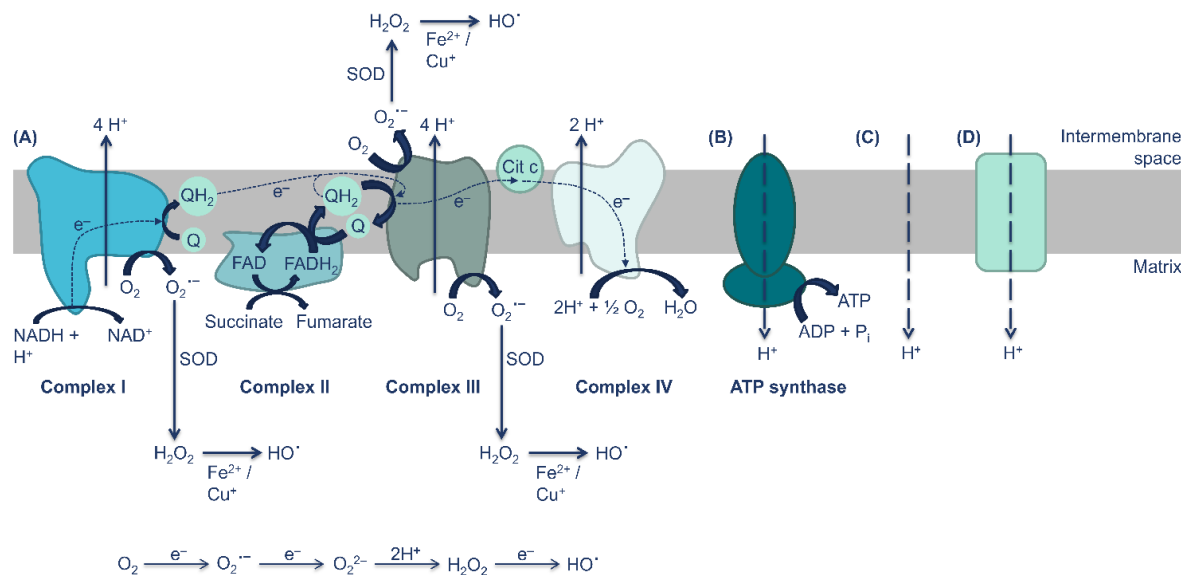


Fig. A1.1 – Proton transport and formation of ROS during oxidative phosphorylation. (A) In complex I, the oxidation of NADH (generated through glycolysis, β -oxidation, and Krebs cycle) to NAD^+ transfers two electrons to the complex. Complex I will then transfer the electrons to the quinone reservoir by reducing ubiquinone to ubiquinol. In complex II occurs the oxidation of succinate to fumarate, a reaction that reduces FAD to FADH_2 . FADH_2 also gives two electrons to ubiquinone, originating FAD and ubiquinol. Ubiquinol is released into the quinone reservoir, joining those from

NADH. Ubiquinol transports electrons through the intermembrane space to complex III, where they are again oxidized to quinones. Complex III transfers electrons to cytochrome c, which moves to complex IV. Complex IV receives the electrons from cytochrome c and transfers them to molecular oxygen, reducing it to water. Some electrons escape to oxygen, which lead to the formation of superoxide anions. Superoxide leads to a cascade of redox reactions, which leads to the formation of other ROS, such as the hydroxyl radical ($\cdot\text{OH}$) and hydrogen peroxide (H_2O_2). During the transport of electrons through the respiratory chain, protons are pumped from the matrix into the intermembrane space by complexes I, II, and IV. This action creates an electrochemical gradient that is used to **(B)** convert ADP into ATP, by ATP synthase. The proton transport to the mitochondrial matrix can be also made through **(C)** basal proton leak or **(D)** inducible proton leak.

Herein we discuss in detail the expression and function of the different UCP homologs in human, mouse, and rat. In addition, the role of UCPs as key regulators of ROS production and redox homeostasis and their potential role on the pathophysiology of diabetes and on diabetes-induced male infertility will be discussed.

A1.2. Mitochondrial uncoupling proteins (UCPs) expression and function

UCPs are integral transmembrane proteins of the inner mitochondrial membrane and members of the mitochondrial anionic transporter family SLC25 (Chan et al., 2006; Hinz et al., 1999; Pecqueur et al., 2001; Ramsden et al., 2012). UCPs are constituted by three repetition domains, each composed of two α -helix regions (Arsenijevic et al., 2000; Hinz et al., 1999; Mao et al., 1999; Ramsden et al., 2012; Sanchis et al., 1998). The carboxy and amino-terminal regions are on the intermembrane space while the α -helix regions are linked by long loops that reside on the matrix side of mitochondria (Fig. A1.2A) (Eckerskorn & Klingenberg, 1987; Miroux et al., 1993; Ramsden et al., 2012). There are two main hypotheses concerning the proton transport mechanism by UCPs: the flip-flop model and the cofactor model (Fig. A1.2B) (Hoang et al., 2012; Ricquier & Bouillaud, 2000). The flip-flop model occurs in the presence of high concentrations of fatty acids, in which the protonated form of the fatty acid freely crosses the membrane towards the matrix and dissociates the proton in the matrix due to the pH difference (higher in the matrix). The anionic fatty acid returns to the intermembrane space with the help of the UCP, completing the cycle. In the cofactor model, protons can be transported independently or, at a higher rate, linked to the carboxyl group of fatty acids, which operate as prosthetic groups.

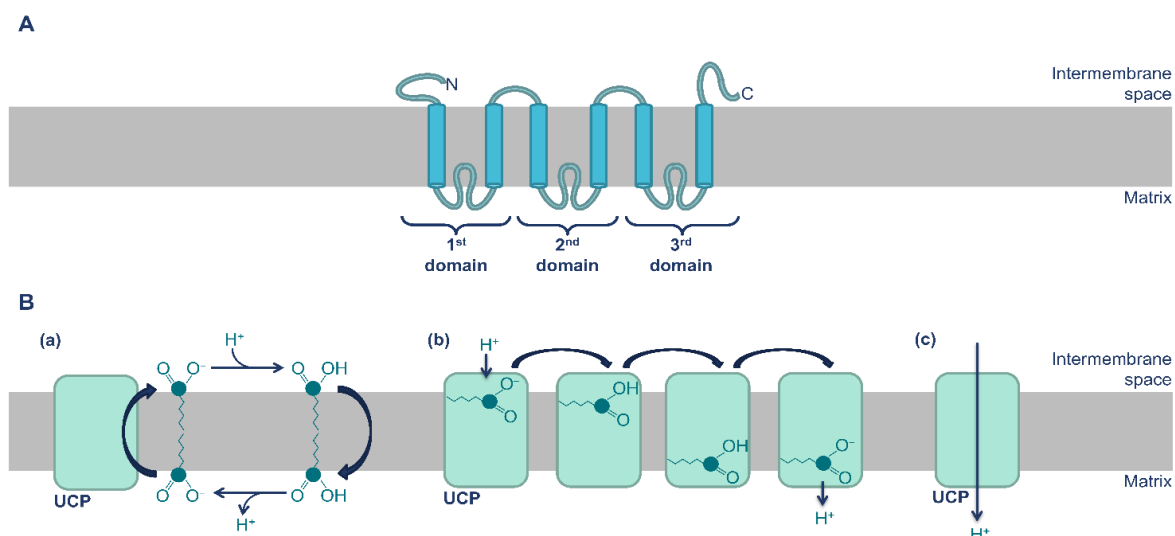


Fig. A1.2 – Molecular structure and hypothesized models for proton transport by UCPs. (A) UCPs are constituted by three repetition domains, each composed of two α -helix regions. (B) The proposed models for proton transport by UCPs are (a) flip-flop model; (b) cofactor model (transport linked to fatty acids); (c) cofactor model (independent transport).

UCPs play a central role in regulating the potential of mitochondrial membrane, exhibiting several distinct functions from thermogenesis (dissipating energy in the form of heat) to oxidative phosphorylation or ROS levels regulation (Arsenijevic et al., 2000; Liu et al., 2006; Ricquier & Bouillaud, 2000). Currently, six homologs of mammalian UCPs (UCP1-6) have been identified (Table A1), which will be discussed in detail on the following topics.

A1.2.1. UCP1

UCP1 is predominantly expressed in brown adipose tissue (BAT), where it constitutes up to 10% of the mitochondrial proteins in this tissue (Arsenijevic et al., 2000). Humans present UCP1 expression almost exclusively in brown adipocytes (Virtanen et al., 2009), however, it has also been found to be expressed in white adipose tissue (WAT), on brite or beige adipocytes (Okamatsu-Ogura et al., 2013; Tiraby et al., 2003) and skin (Mori et al., 2008). This expression pattern is shared by mice and rats, where UCP1 expression is almost confined to BAT (Jakus et al., 2002; Matthias et al., 2000), with some studies describing its presence in WAT (including epididymal WAT) (Sepa-Kishi et al., 2019; Shabalina et al., 2013), and mice adrenal gland (Fujita et al., 2021). Moreover, UCP1 has been identified in smooth muscle cells from mice (including those from the female and male reproductive tracts) (Nibbelink et al., 2001), and mouse and rat thymocytes (Carroll et al., 2005). Furthermore, the existence of *UCP1* mRNA in human ductus deferens, testis, epididymis,

Table A1. Expression and hypothesized functions of all mitochondrial uncoupling proteins (UCPs) homologs.

Isoforms	Localization	Putative Function	References
UCP1	Human brown adipose tissue, white adipose tissue, keratinocytes, sweat glands, sebaceous glands, hair follicles, and granular layer of the epidermis	Non-shivering thermogenesis, metabolic and bioenergetic regulation, modulation of ROS production	(Mori et al., 2008; Tiraby et al., 2003; Virtanen et al., 2009)
	Mouse brown adipose tissue, white adipose tissue, and adrenal gland		(Fujita et al., 2021; Matthias et al., 2000; Shabalina et al., 2013)
	Rat brown adipose tissue and white adipose tissue		(Jakus et al., 2002; Sepa-Kishi et al., 2019)
UCP2	Human proximal tubular cells, cytotrophoblasts, syncytiotrophoblast, lungs*, keratinocytes*, skin fibroblasts*, spermatozoa, white adipose tissue*, skeletal muscle*, brown adipose tissue*, and pancreatic β -cells*, brain	Modulation of ROS production, insulin sensitivity and secretion, lipid and glucose metabolism, control of mitochondrial Ca^{2+} -uptake, inflammation, immunomodulation	(Boss et al., 1997; Chan et al., 2001; Fleury et al., 1997; Haguenauer et al., 2005; Mori et al., 2008; Nigro et al., 2018; Stark et al., 2012; Wang et al., 2018)
	Mouse thymocytes, tubular epithelial cells, granulosa cells*, theca cells*, endometrium glandular epithelium cells*, uterine glands*, oviduct mucosa epithelial cells*, mammary gland, stomach, macrophages, splenocytes, B lymphocytes, T lymphocytes, dendritic cells, neutrophils, microglial cells*, neurons*, cardiomyocytes, lungs, testicular germ cells, testicular interstitial cells, white adipose tissue*, skeletal muscle*, brown adipose tissue*, pancreatic β -cells*, pancreatic α -cells, and hepatocytes*		(Arsenijevic et al., 2007; Arsenijevic et al., 2000; Cao et al., 2013; Chan et al., 2001; Chen et al., 2009; Diao et al., 2008; Fleury et al., 1997; Jiang et al., 2013; Krauss et al., 2002; Moloch et al., 2016; Rousset et al., 2003; Rousset et al., 2006; Yu et al., 2000a; Zhang et al., 2007)
	Rat proximal tubular cells, Kupffer cells*, microglial cells, neurons, cardiomyocytes, skeletal muscle*, testis, brown adipose tissue*, white adipose tissue*, pancreatic β -cells, lungs*, spleen*, and thymus*		(Alan et al., 2009; Amara et al., 2008a; Boss et al., 1997; Cadenas et al., 1999; Chan et al., 1999; De Simone et al., 2015; Friedreich et al., 2008; Hovath et al., 1995; Larrouy et al., 1997; Negre-Salvayre et al., 1997; Teshima et al., 2003)
UCP3	Human skeletal muscle, heart*, spleen*, thymus*, keratinocytes*, skin fibroblasts, sweat glands, hair follicles, stratum basale of the epidermis, pancreatic β -cells, thyroid*, and bone marrow*	Modulation of ROS production, insulin secretion, lipid metabolism, protection against lipotoxicity, control of mitochondrial Ca^{2+} -uptake, immunomodulation	(Boss et al., 1997; Carroll & Porter, 2004; Hesselink et al., 2003; Li et al., 2008; Mori et al., 2008; Vidal-Puig et al., 1997)
	Mouse skeletal muscle, brown adipose tissue, brain*, kidney*, colon*, liver*, heart, white adipose tissue, thymocytes, splenic lymphocytes, and peripheral naive CD4+ T cells*		(Hise et al., 2016; Kelly & Porter, 2011; O'Connor et al., 2020; Vidal-Puig et al., 1997)
	Rat skeletal muscle, brown adipose tissue*, white adipose tissue*, kidney*, spleen reticulocytes, spleen monocytes, spleen lymphocytes, thymocytes, and heart		(Boss et al., 1997; Bordinina et al., 2012; Cadenas et al., 1999; Carroll & Porter, 2004)

Table A1. Expression and hypothesized functions of all mitochondrial uncoupling proteins (UCPs) homologs (cont.).

Isoforms	Localization	Putative Function	References
UCP4	Human Purkinje cells, most of the brain tissues*, and cartilage*		(Chan et al., 2006; Huang et al., 2016; Mao et al., 1999; Ramsden et al., 2012)
	Mouse brain (neurons, astrocytes, cortex, brainstem, cerebellum), spinal cord, mast cells, spiral ganglion*, vestibular ganglion*, kidney*, heart*, white adipose tissue*, brown adipose tissue*, skeletal muscle*, and liver*	Regulation of oxidative stress, thermoregulation, protection against mitochondrial Ca ²⁺ overload	(Alan et al., 2009; Kempuraj et al., 2016; Kishihara et al., 2005; Lengacher et al., 2004; Smorodchenko et al., 2009)
	Rat Merkel cells, modiolus ear (fibrocyte, satellite cells), organ of Corti (spiral ganglion neurons, supporting cells, hair cells), brain (pyramidal cells of hippocampus and cortex, Purkinje cells of cerebellum, neurons of hippocampus, substantia nigra, striatum, neocortex*), chondrocytes, vestibular ganglion*, heart*, lungs*, skeletal muscle*, kidney*, and liver*		(Alan et al., 2009; Donhoff et al., 1999; Huang et al., 2016; Kishihara et al., 2004; Ramsden et al., 2012; Smorodchenko et al., 2011; Smorodchenko et al., 2009)
	Human kidney*, heart*, lungs*, stomach*, liver*, spleen*, skeletal muscle*, brain (cerebellum, cortex, medulla, occipital pole, frontal lobe, putamen, amygdala, caudate nucleus, hippocampus, substantia nigra, thalamus, corpus callosum)*, spinal cord*, pituitary*, uterus*, and testis*		(Kim-Han et al., 2001; Sanchis et al., 1998; Yu et al., 2006b)
UCP5	Mouse heart, kidney, skeletal muscle, white adipose tissue, spinal cord, brain (neurons, astrocytes, cortex, thalamus, hippocampus, substantia nigra, cerebellum, basal ganglia, hypothalamus*, amygdala*, neocortex*, caudate putamen*), spiral ganglion*, vestibular ganglion*, hepatocytes*, brown adipose tissue*, spleen*, intestine*, lungs*, testis*, uterus*, and periovarian fat*	Regulation of oxidative stress, thermoregulation, transport of metabolites, regulation of mitochondrial metabolism	(Gorgoglione et al., 2019; Haguenauer et al., 2005; Huang et al., 2011; Kim-Han et al., 2001; Kishihara et al., 2005; Lengacher et al., 2004; Sanchis et al., 1998; Vidal-Puig et al., 2000; Yu et al., 2006a)
	Rat heart*, lungs*, adrenals*, kidney*, gonadal fat*, ovary*, brain (striatum, cortex, hippocampus, cerebellum)*, skeletal muscle*, and liver*		(Alan et al., 2009; Eriden et al., 2016; Sanchis et al., 1998)
UCP6	Mouse kidney (proximal tubules, distal tubules, surrounding nephron segments, glomeruli, medullary part of the loop of Henle, collecting duct)*, white adipose tissue*, brown adipose tissue*, brain*, heart*, muscle*, liver*, lungs*, spleen*, and testis*	Regulation of oxidative stress, transport of metabolites, regulation of mitochondrial metabolism	(Haguenauer et al., 2005; Kim-Han et al., 2001)

* Only mRNA has been reported or predicted based on mRNA microarrays.

seminal vesicle, and prostate (Lizio et al., 2015) has been predicted. The main function attributed to UCP1 is the production of heat through thermogenesis (Darley-Usmar, 2004). BAT mainly consists of brown adipocytes, cells specialized in thermogenesis that have high expression of thermogenic genes, including UCP1. Exposure to cold leads to adrenergic activation of brown adipocytes and a higher expression of *Ucp1* (Ricquier et al., 1986). As a result, there is an increase in the number and size of mitochondria and the rate of lipolysis is also increased, leading to a higher content of free fatty acids (Busiello et al., 2015). These free fatty acids activate UCP1, instigating the uncoupling of the respiratory chain and the production of heat (Liu et al., 2006). In addition to the cold exposure and adrenergic regulation, including triiodothyronine (T3) and norepinephrine (Rabelo et al., 1995), UCP1 activity and expression is also stimulated by superoxide (Echtay et al., 2002b), and compounds with a 2-alkenal functional group, including trans-retinoic acid, trans-retinal, trans-2-nonenal, and retinoic acid through a non-adrenergic pathway (Alvarez et al., 1995). In addition, UCP1 also responds to the nutritional status of the organism. Gong et al. reported that *Ucp1* mRNA levels drop with starvation and recover after feeding (Gong et al., 1997). UCP1 is also regulated by nucleotides. Interestingly, the binding site of fatty acids to the protein is different from that of nucleotides. Through a FTIR spectra analysis, Chan et al. observed that the addition of nucleotides causes considerable changes in the secondary, and probably tertiary, structure of UCP1 of hamster BAT (Rial et al., 1990). In contrast, no secondary or tertiary structure changes were detected upon fatty acid binding.

A1.2.2. UCP2

UCP2, which is 59% identical to UCP1, is probably the most widely expressed UCP homolog (Fleury et al., 1997; Gimeno et al., 1997). Unlike UCP1, UCP2 is presumably ubiquitously expressed throughout the body. In humans, UCP2 expression has been detected in kidneys (Nigro et al., 2018), placenta (Stark et al., 2012), spermatozoa (Wang et al., 2018), and brain (Fleury et al., 1997). In skin (Mori et al., 2008), lungs (Fleury et al., 1997), WAT, BAT, skeletal muscle (Boss et al., 1997), and pancreatic β -cells (Chan et al., 2001), only the mRNA expression has been reported in humans. Widespread UCP2 expression is also observed in rodents, where in mice expression has been identified in thymus (Krauss et al., 2002), kidney (Jiang et al., 2013), mammary gland, stomach (Rousset et al., 2003), macrophages (Arsenijevic et al., 2000), B lymphocytes, T lymphocytes, dendritic cells, neutrophils (Rousset et al., 2006), spleen (Cao et al., 2013), heart (Motloch et al., 2016), lungs (Chen et al., 2009), testis (Zhang et al., 2007), and pancreatic α -cells (Diao et al., 2008), while *Ucp2* mRNA was reported in pancreatic β -cells (Chan et al., 2001), skin (Jiang et al., 2013),

female reproductive tract (Rousset et al., 2003), brain (Arsenijevic et al., 2007), WAT, BAT, skeletal muscle (Fleury et al., 1997), and liver (Yu et al., 2000a). In rats, UCP2 presence has been described in kidneys (Friederich et al., 2008), neurons (Horvath et al., 1999), microglia (De Simone et al., 2015), heart (Teshima et al., 2003), testis (Amaral et al., 2008a), and pancreatic β -cells (Chan et al., 1999), and its mRNA in liver (Larrouy et al., 1997), skeletal muscle (Cadenas et al., 1999), WAT, BAT (Boss et al., 1997), lungs (Alan et al., 2009), spleen (Alan et al., 2009), and thymus (Negre-Salvayre et al., 1997). Additionally, the existence of *UCP2* mRNA has been predicted in human ductus deferens, testis, epididymis, seminal vesicle, and prostate (Lizio et al., 2015).

The main function attributed to UCP2 comprises the regulation of ROS production and protection against oxidative damage (Horimoto et al., 2004; Krauss et al., 2003). Thus, UCP2 has been studied for its protective effects on several diseases associated with increased oxidative stress, including Alzheimer's disease, Parkinson's disease, arteriosclerosis, diabetes mellitus (Alves-Guerra et al., 2003; Brand & Esteves, 2005; Lee et al., 2005; Nowak et al., 2017). In addition, UCP2 is reported to have a protective role against stroke and trauma (Mattiasson et al., 2003), ethanol intoxication (Horvath et al., 2002), brain and cardiac ischemia (McLeod et al., 2005; Nakase et al., 2007), and a regulatory role on inflammation (Alves-Guerra et al., 2003; Kizaki et al., 2002) and glucose-stimulated insulin secretion (Zhang et al., 2001). Some studies also report that UCP2 may transport inorganic anions such as aspartate, malate, phosphate, or oxaloacetate (Vozza et al., 2014). However, it is not known whether the anion transport can be part of the uncoupling cycle or if it is just a side reaction (Brand & Esteves, 2005). Interestingly, UCP2 appears to mediate the transport of protons only when specifically activated (Brand & Esteves, 2005). UCP2 is activated by high ROS levels, fatty acids, and molecules containing the 2-alkenal functional group (Azzu & Brand, 2010; Brand & Esteves, 2005; Echtay et al., 2002b). *Ucp2* mRNA expression is reported to be regulated by glutamine and leptin (Hurtaud et al., 2007; Zhou et al., 1997). On the other hand, UCP2 is inhibited by purine nucleotides (Azzu & Brand, 2010; Echtay et al., 2002b).

A1.2.3. UCP3

UCP3 shares an 73% identity with UCP2 and 57% with UCP1 (Boss et al., 1997; Vidal-Puig et al., 1997). In humans, UCP3 is predominantly expressed in skeletal muscle (Hesslink et al., 2003) and, to a lesser extent, skin (Mori et al., 2008), and pancreatic β -cells (Li et al., 2008). An equivalent UCP3 expression pattern is observed in mice which is preferentially expressed in skeletal muscle and BAT (Vidal-Puig et al., 1997), and, in a smaller degree, in

heart, WAT (Hilse et al., 2016), spleen, and thymus (Kelly & Porter, 2011). Likewise, rats have predominant UCP3 expression in skeletal muscle (Cadenas et al., 1999), and smaller amounts in heart (Boudina et al., 2012), spleen, and thymus (Carroll & Porter, 2004). mRNA has been detected in human heart (Boss et al., 1997), spleen (Carroll & Porter, 2004), thymus (Carroll & Porter, 2004), keratinocytes (Mori et al., 2008), thyroid, and bone marrow (Vidal-Puig et al., 1997), in mice brain, kidney, colon, liver (Vidal-Puig et al., 1997), and circulating leucocytes (O'Connor et al., 2020), and in rat BAT (Cadenas et al., 1999), WAT and kidney (Boss et al., 1997). The existence of *UCP3* mRNA was predicted in human ductus deferens, testis, epididymis, seminal vesicle, and prostate (Lizio et al., 2015).

Similar to UCP2, UCP3 also plays a role on the regulation of ROS production and protection against oxidative damage (Brand et al., 2002; Talbot et al., 2004; Vincent et al., 2004). In addition, UCP3 is hypothesized to play a protective role against cardiac ischemia (McLeod et al., 2005). As UCP2, UCP3 is positively regulated by ROS levels (Cadenas et al., 2002), fatty acids, molecules containing 2-alkenal functional group (Brand & Esteves, 2005), and leptin (Liu et al., 1998), while it is inhibited by purine nucleotides (Azzu & Brand, 2010). However, UCP3 also has several resemblances with UCP1. Although it is not normally thermogenic or involved in the adaptive response to cold, UCP3 may be significantly thermogenic under specific conditions, including glucocorticoids or T3 stimulation, and cold exposure (Brand & Esteves, 2005; Gong et al., 1997; Larkin et al., 1997). In addition, *UCP3* mRNA is also regulated by fasting in both human and rat (Boss et al., 1998; Millet et al., 1997).

A1.2.4. UCP4

UCP4, along with UCP5 and UCP6, are less identical to the other three homologs (Mao et al., 1999). UCP4 is predominantly expressed in the central nervous system either from human (Ramsden et al., 2012), rat (Huang et al., 2016) or mouse (Smorodchenko et al., 2009), but its expression has also been detected in other tissues and cells such as Merkel cells, modiolus ear, organ of Corti (Smorodchenko et al., 2011), and chondrocytes (Huang et al., 2016) of rat, and spinal cord (Smorodchenko et al., 2009), and mast cells (Kempuraj et al., 2016) of mouse. In human cartilage (Huang et al., 2016), rat vestibular ganglion (Kitahara et al., 2004), heart, lungs, skeletal muscle, kidney, and liver (Alan et al., 2009) and mouse spiral ganglion, vestibular ganglion, kidney (Kitahara et al., 2005), heart, liver, skeletal muscle, WAT (Alan et al., 2009), and BAT (Lengacher et al., 2004), only the mRNA expression was reported. Furthermore, the existence of *UCP4* mRNA in human ductus deferens, testis, epididymis, seminal vesicle, and prostate (Lizio et al., 2015) has been predicted.

The activation of UCP4 attenuates mitochondrial ROS levels (Chan et al., 2006). Since the apoptosis of neurons is related to the action of ROS, UCP4 is hypothesized to play neuroprotective role in Alzheimer's and Parkinson's disease (Huang et al., 2017; Xu et al., 2018). This uncoupling protein may also be responsible for the observed mitochondrial proton leakage in the brain (Xu et al., 2021), acting as a thermoregulator in response to cold (Donhoffer et al., 1959). Another suggested role for UCP4 in neurons is the regulation of mitochondrial Ca^{2+} signalling (Chan et al., 2006). Analogously to the other homologs, UCP4 is inhibited by purine nucleotides and activated by ROS and fatty acids (Hoang et al., 2012). Calorie restriction and cold induce *Ucp4* mRNA expression in the rat brain as well (Liu et al., 2006).

A1.2.5. UCP5

UCP5 is mainly present in human (Yu et al., 2000b), rat (Erden et al., 2016) and mouse (Kim-Han et al., 2001) central nervous system. It is also expressed in other tissues, organs and cells as kidney, heart, lungs, stomach, liver, spleen, skeletal muscle, spinal cord, uterus (Yu et al., 2000b), pituitary, and testis (Sanchis et al., 1998) in humans, heart, lungs, adrenals, kidney, gonadal WAT, ovary (Sanchis et al., 1998), skeletal muscle, and liver (Alan et al., 2009) in rat, and spiral ganglion, vestibular ganglion (Kitahara et al., 2005), hepatocytes (Yu et al., 2000a), BAT, spleen, intestine, lungs, testis, uterus, and periovarian (Sanchis et al., 1998) fat in mouse. Its protein has been detected in mouse brain, heart, kidney, skeletal muscle, WAT, and spinal cord (Kim-Han et al., 2001). Additionally, the existence of *UCP5* mRNA has been predicted in human ductus deferens, epididymis, seminal vesicle, and prostate (Lizio et al., 2015).

As UCP4, UCP5 may have a neuroprotective effect against damage related to oxidative stress (Kim-Han et al., 2001). This can have implications in several pathological conditions, including Alzheimer's disease (Huang et al., 2017), and cortical spreading depression (Viggiano et al., 2016). It is also hypothesized that UCP5 is involved in metabolic rate changes associated with starvation and a high-fat diet (Yu et al., 2000b). In addition, *Ucp5* mRNA levels decrease during brain hypoxia and increase under hyperoxic conditions (Pichiule et al., 2003). UCP5 is also hypothesized to play a role in thermoregulation induced by cold exposure (Yu et al., 2000b). Interestingly, UCP5 transports mainly sulphate and thiosulfate (Gorgoglione et al., 2019). These compounds are products of the degradation of H_2S , so UCP5 is a potential regulator of the mitochondrial levels of this important signalling

molecule. UCP5 also efficiently transports other inorganic anions, including sulphite, phosphate and, to a lesser extent, maleate, oxalate, malonate, malate, citramalate, aspartate, and glutamate (Gorgoglione et al., 2019).

A1.2.6. UCP6

Ucp6 mRNA was found to be mainly expressed in mouse kidneys (Haguenauer et al., 2005). It is also considerably expressed in the testis of mice. Its expression has also been detected in other mouse tissues and organs such as WAT, BAT, brain, heart, muscle, liver, lungs, and spleen. The existence of *UCP6* mRNA was predicted in human ductus deferens, testis, epididymis, seminal vesicle, and prostate (Lizio et al., 2015).

UCP6 is upregulated in response to oxidative stress (Haguenauer et al., 2005). Although UCP6 seems to have no uncoupling capacity (Haguenauer et al., 2005), its antioxidant role is hypothesized to be due to the regulation of H₂S concentration, which at low concentrations inhibits ROS production in mitochondria (Xie et al., 2016). In fact, *Ucp6* was found to be upregulated during cancer (Nohara et al., 2012) or the regenerative phase after renal tubular damage (Haguenauer et al., 2005), which are conditions characterized by high oxidative damage. Moreover, it is assumed that increased *Ucp6* expression is associated with increased mitochondrial activity (Haguenauer et al., 2005). Similar to UCP5, UCP6 primarily transports sulphate and thiosulfate, which supports its role as regulator of H₂S levels (Gorgoglione et al., 2019). UCP6 is also able to transport the same inorganic anions as UCP5, except for glutamate (Gorgoglione et al., 2019).

A1.3. UCPs are key regulators of ROS production and redox homeostasis

ROS are an essential part of the cell function, participating in numerous processes such as immune response, cellular proliferation and differentiation, angiogenesis, aging, and several signaling pathways. They are a natural byproduct of the mitochondria's oxidative phosphorylation, arising when a single unpaired electron reduces O₂ at the oxidative chain level, which results in formation of the superoxide anion. However, an unregulated increase in the production of these free oxygen radicals renders the cellular antioxidant system incapable of mitigating their harmful effects, causing a shift in the homeostasis of the redox balance towards oxidative stress, a damaging state that participates on the pathophysiology of several diseases and capable of inducing macromolecular damage towards apoptosis/necrosis

(Papa & Skulachev, 1997). It has been proposed that mild uncoupling of the mitochondrial oxidative phosphorylation system might prevent the oversupply of electrons to the various protein complexes of the electron transport chain, diminishing the possibility of electron leaks and interaction with oxygen. Hence, controlled proton conductance via the mitochondrial inner membrane has been suggested as a key regulator of ROS production, through membrane potential modulation (Azzu et al., 2010; Cadenas, 2018; Mailloux & Harper, 2011; Papa & Skulachev, 1997).

All UCPs homologs are well known for regulating ROS homeostasis, although UCP1 role as an antioxidant remains debatable. Given the extensively described uncoupling activity of UCP1, it would be expected that this protein has an active role in the control of mitochondrial ROS production. However, there are some conflicting data. For instance, Dlasková et al. observed that BAT mitochondria from both wild-type C57BL/6J mice treated with guanosine diphosphate (GDP), an UCP1 inhibitor, and *Ucp1* knockout mice exhibited greater H₂O₂ production and higher mitochondrial membrane potential (Dlaskova et al., 2010). In accordance with these results, ablation of *Ucp1* in the BAT mitochondria of mice exposed to cold stress leads to higher superoxide production, and subsequently oxidative stress, when compared to wild-type mice (Oelkrug et al., 2010; Stier et al., 2014). Besides, ROS have been shown to be capable of modulating UCP1 activity via sulfonylation of the Cys253 residue, increasing thermogenesis (Chouchani et al., 2016). Conversely, Shabalina et al. reported that UCP1-dependent proton leak is not affected by the oxidative stress by-product 4-hydroxy-2-nonenal (HNE), and *Ucp1* deletion in mice does not predispose them to higher oxidative damage (Shabalina et al., 2006). In a later study, also using mitochondria from BAT of wild-type and *Ucp1* knockout mice, they determined that UCP1 activity bears no weight in the reduction of ROS emission, except when succinate was endogenously added. Interestingly, the authors also inferred that low membrane potential may not necessarily correlate with lower oxidative stress, challenging the claim that mild uncoupling protects mitochondria against oxidative damage (Shabalina et al., 2014).

UCP2 and UCP3 role as modulators of ROS production is extensively described. UCP2 and UCP3 do not display a clear uncoupling activity, so it is unlikely that they regulate superoxide production in a mitochondrial uncoupling-dependent manner. The redox balance mediated by UCP2 was first demonstrated by Nègre-Salvayre et al., where it was shown that mitochondria fractions containing high levels of UCP2 treated with the inhibitor GDP presented a significant increase in H₂O₂ generation, whereas the treatment in mitochondria with low expression of UCP2 led to no changes in the oxidative state (Negre-Salvayre et al., 1997). Other studies focusing on the overexpression of UCP2, using an adenoviral vector demonstrated that UCP2 plays a protective role against oxidative stress, shielding both cardiomyocytes and endothelial cells from ROS-induced cell death (Lee et al., 2005;

Teshima et al., 2003). UCP2 neuroprotective role against oxidative stress has also been reported (Chan et al., 2009; Jun et al., 2015; Liu et al., 2009). Compelling evidence highlights that ablation of *Ucp2* in multiple mice strains induces chronic high levels of ROS in pancreatic cells (Lee et al., 2009; Pi et al., 2009; Robson-Doucette et al., 2011). Elevated ROS levels in *Ucp2*-deficient mice also results in higher levels of oxidative stress markers in the liver and delayed hepatic regeneration (Horimoto et al., 2004), as well as aggravated atherosclerosis injury (Blanc et al., 2003). On another note, Arsenijevic et al. observed that *Ucp2* knockout mice infected with *Toxoplasma gondii* presented a stronger infection fighting capacity, when compared to wild type, due to higher ROS emission in macrophages (Arsenijevic et al., 2000). Due to its ROS suppressing activity, targeted UCP2 inhibition has been proposed as a potential novel treatment for cancer (Baffy, 2010; Derdak et al., 2008; Mailloux et al., 2010).

Akin to UCP2, it was hypothesized that the overproduction of ROS activates UCP3 through a negative feedback mechanism, diminishing oxidative damage (Anderson et al., 2007). The presence of superoxide from both endogenous (Anderson et al., 2007; Talbot & Brand, 2005; Talbot et al., 2004) and exogenous sources (Echtay et al., 2002a; Echtay et al., 2002b), along with byproducts of lipid peroxidation (Echtay et al., 2005; Murphy et al., 2003) have been shown to induce UCP3-mediated proton leak. In addition, *Ucp3* knockout mice display higher rates of ROS production and are more vulnerable to oxidative damage, including increased vulnerability to ischemia-reperfusion injury (Brand et al., 2002; Ozcan et al., 2013; Toime & Brand, 2010; Vidal-Puig et al., 2000). Toime and Brand studied isolated energized skeletal muscle mitochondria from wild-type and *Ucp3* knockout mice and reported that UCP3 decreases the rate of ROS production (Toime & Brand, 2010). Interestingly, these authors treated the isolated mitochondria with FCCP, a well-known chemical uncoupler, which mirrored the UCP3 antioxidant activity. However, inhibition with GDP was ineffective at reducing UCP3-dependent ROS suppression and no differences in the mitochondrial membrane potential were observed, which suggests that UCP3 can modulate ROS production through a membrane potential-independent mechanism. In support to these results, MacLellan et al. demonstrated that moderate overexpression of UCP3 in L6 myocytes does not interfere with basal cellular oxygen consumption or membrane potential despite potentiating its antioxidant activity (MacLellan et al., 2005). UCP3 is also reported to be upregulated in myocytes upon muscle/fiber contraction, suggesting a role on protection against physical exercise-related ROS production (Anderson et al., 2007; Jiang et al., 2009). Overexpression of UCP3 has also been associated with a protective effect against age-related rise in mitochondrial oxidative damage (Nabben et al., 2008). On a final note, UCP3-mediated translocation of lipid peroxides to the extramitochondrial medium has also

been reported to be a competent strategy in minimizing oxidative stress (Goglia & Skulachev, 2003).

Although fewer studies focus on UCP4-6, compelling evidence highlights their role on the regulation of ROS production. Increased levels of *Ucp4* and *Ucp5* mRNA and respective proteins were found to protect neurons against oxidative stress damage (Huang et al., 2017). In addition, other studies shown that UCP5 expression decreased the production of superoxide radicals in mouse neurons (Kim-Han et al., 2001), whereas it protected cells against oxidative stress due to hyperoxic conditions (Pichiule et al., 2003). UCP6 represents an intriguing case. Although UCP6 does not display an uncoupling function, this homolog can be upregulated in response to oxidative stress (Haguenauer et al., 2005). It was hypothesized that its antioxidant effect must be due to the regulation of H₂S levels. Although H₂S exerts cytotoxic effects when in high concentrations, at low concentrations it exhibits beneficial and protective effects (Xie et al., 2016). One of the beneficial effects of low H₂S concentrations is the inhibition of ROS production in mitochondria through the stimulation of cellular antioxidants (Gorgoglione et al., 2019; Xie et al., 2016). Hence, UCP6 seems to contribute to the maintenance of low levels of ROS by keeping the concentration of H₂S low.

A1.4. UCPs dysregulation leads to diabetes

UCPs have a significant influence in mitochondrial bioenergetics, thermogenesis, and control of ROS production, which are all key processes known to progressively become impaired with the onset of metabolic disorders (including obesity and diabetes mellitus). Obesity and diabetes mellitus are closely linked, where a prolonged and persistent positive intake of energy lead to chronic hyperglycemia and subsequent insulin resistance (Carrageta et al., 2019). Even though physical activity accounts for a large percentage of total energy expenditure, resting metabolic rate (RMR) and adaptive thermogenesis are fundamental mechanisms (Betz & Enerback, 2018; Hosseini et al., 2016). For instance, it is estimated that proton leak through the inner membrane of mitochondria is responsible for 20–30% of the RMR in rats (Rolfe & Brand, 1996). In fact, alterations in proton conductance and UCPs expression have been implicated in the development of obesity and are considered potential targets for its treatment (Harper et al., 2008).

UCP1-mediated thermogenesis can increase 12–20% the total daily energy expenditure (Abumrad, 2017). However, early studies in *Ucp1* knockout mice were incapable of establish a clear connection between UCP1 deficiency and obesity. Enerbäck et al. were the first to report the absence of an obesogenic effect, as *Ucp1*-deficient mice fed a standard or

high-fat diet did not become obese (Enerback et al., 1997). These findings were later supported by other studies (Kontani et al., 2005; Liu et al., 2003). Notably, Feldmann et al. suggested that the housing temperature at which the mice were kept might have confounded some of the results from these studies (Feldmann et al., 2009). Mice housed at a standard temperature (18–22 °C) are under chronic cold stress, which requires an increase in their metabolic rate of 50% to 60% just to sustain body temperature. This increase in energy expenditure might have masked any possible effect of UCP1 in the energy metabolism. In this same study, the authors demonstrated that when *Ucp1* knockout mice were housed at thermoneutral temperature (i.e., 30 °C), they do indeed develop obesity even when fed standard diet. In accordance with these results, more recent studies have also described the obesogenic role of *Ucp1* ablation in mice kept at ~30 °C (Rowland et al., 2016; von Essen et al., 2017), even for notoriously obesity resistant 129 mice strain (Luijten et al., 2019). Interestingly, it was observed that this strain had markedly higher metabolic rate with increased energy expenditure when compared to other obesity prone strain, the C57BL/6 mice. Raised RMR was explained by higher expression of UCP1 and uncoupling in mitochondria from muscle. After histological analysis it was possible to determine that higher expression of UCP1 correlates with the presence of intramuscular depots of BAT (Almind et al., 2007). In addition, Winn et al. reported that *Ucp1* knockout mice fed Western diet and kept at 25 °C developed insulin tolerance, although no significant differences in body weight, visceral adiposity, and energy expenditure in comparison with wild-type mice were observed (Winn et al., 2017). Loss of *Ucp1* also causes whitening of BAT (Winn et al., 2017), decreasing its protective role against the development of insulin resistance, type 2 diabetes, and obesity (Chondronikola et al., 2014; Cypess et al., 2009). In fact, an UCP1-dependent role for BAT in glucose homeostasis has been previously described. *Ucp1*-deficient mice lack BAT glucose uptake after norepinephrine administration, suggesting that sympathetic stimulation leads to UCP1 mediated glucose disposal by BAT (Inokuma et al., 2005). Notably, BAT transplant to *Ucp1* knockout mice was able to eliminate glucose intolerance (Thoonen et al., 2015). Furthermore, stimulation of UCP1 dependent BAT thermogenesis, either through cold or diet, has been shown to increase energy expenditure via catabolic acceleration. When active, BAT oxidizes significant amounts of metabolic substrates such as stored fatty acids, as well as plasma glucose and triglycerides. Thus, through reduction of the circulating glucose and lipids in order to supply the energy costly thermogenesis, BAT is a tissue of great interest to treat metabolic disorders such as obesity and type 2 diabetes mellitus (Busiello et al., 2015; Rowland et al., 2016; Saito et al., 2020; von Essen et al., 2017; Winn et al., 2017). In accordance with these results, Vázquez et al. reported that Zucker diabetic fatty rats, an experimental model for obesity and type 2 diabetes mellitus, exhibited significantly lower levels of UCP1 protein and mRNA expression

in BAT when compared to their lean littermates. Interestingly, treatment with melatonin, which has been described as having an anti-obesity effect, was able to restore not only UCP1 expression but also its functionality in obese rats. This increase in UCP1 expression and function resulted in higher thermogenic activity, which was linked with enhanced energy expenditure and overall improved metabolic state of treated rats (Fernandez Vazquez et al., 2018). Further evidence for the UCP1 antidiabetic and anti-obesity effect comes from studies in transgenic mice models overexpressing UCP1. Transgenic mice with upregulation of UCP1 in WAT showed resistance to diet-induced obesity even when fed a high-fat diet (Klaus et al., 2012; Kopecky et al., 1996). Moreover, several studies have linked ectopic overexpression of UCP1 in the skeletal muscle with higher energy expenditure, lean phenotype, lower levels of glucose, insulin, cholesterol, and adiposity, enhanced lipid metabolism and glucose transport, and improved responsiveness to insulin (Adjeitey et al., 2013; Keipert et al., 2013; Li et al., 2000; Neschen et al., 2008; Ost et al., 2014).

UCP2 has also been implicated in the pathogenesis of diabetes, almost since its discovery. A study by Zhang et al. observed that *Ucp2* knockout mice had improved glucose-stimulated insulin secretion (GSIS), indicating that UCP2 activity negatively regulates insulin secretion (Zhang et al., 2001). The authors also reported that UCP2 was significantly upregulated in isolated pancreatic islets of a model of obesity-induced diabetic mice (*ob/ob* mice), when compared to control. Interestingly, *ob/ob* mice lacking *Ucp2* had enhanced β -cell responsiveness to glucose, decreased levels of glycemia and restored first phase insulin secretion. Overall, these results seem to indicate that obesity-dependent induction of UCP2 in pancreatic β -cells has a pathogenic effect towards the development of insulin resistance and in turn diabetes mellitus. In accordance with this hypothesis, overexpression of UCP2 in isolated β -cells was reported to impair GSIS (Chan et al., 2001; Chan et al., 1999; Hong et al., 2001), while inhibition with genipin, an UCP2 inhibitor, improved glucose secretion (Zhang et al., 2006). Desouza et al. observed that partial inhibition of UCP2 with an antisense oligonucleotide in two mice models of type 2 diabetes mellitus, *ob/ob* mice and diet-induced obese and diabetic mice, led not only to improvements in insulin secretion in β -cell but also enhanced insulin action in peripheral tissues (eg. adipose tissue) (De Souza et al., 2007). Saleh et al. shown that acute knockdown of UCP2 in isolated pancreatic islets from lean mice increased in vitro insulin secretion but failed to improve GSIS in isolated pancreatic islets from *ob/ob* mice (Saleh et al., 2006). Nevertheless, dissenting results have highlighted the possibility of the confounding effects of genetic background and systemic absence of protein activity in studies with *Ucp2*-ablated mice (detailedly reviewed in (Pi & Collins, 2010)). In addition, early reports negatively correlated UCP2 induction with β -cell ATP levels (Chan et al., 2001; Chan et al., 1999; Saleh et al., 2006; Zhang et al., 2001; Zhang et al., 2006). A study by Affourtit and Brand using acute knockdown of *Ucp2* in clonal

β -cells, INS-1E cells, reported that UCP2 is responsible for the high mitochondrial proton conductance observed in this model (Affourtit & Brand, 2008). ATP is a powerful stimulus for insulin secretion, which led the authors to speculate that UCP2 modulates GSIS in pancreatic islets through the coupling efficiency of oxidative phosphorylation. In fact, this hypothesis is shared by several other authors (Chan et al., 1999; De Souza et al., 2007; Zhang et al., 2001). Growing evidence also has revealed the pivotal role of ROS production in β -cell function. Glucose-dependent ROS production and overall changes in the redox status of pancreatic islets have been shown to stimulate insulin production and secretion, which in turn can be suppressed through antioxidative activity. Therefore, a role for ROS in controlling β -cell function under pathological conditions, such as obesity and diabetes mellitus, has been proposed (Chen et al., 2018; Diano & Horvath, 2012; Pi et al., 2007). In support, several studies focused on the potential role of UCP2 in GSIS through the control of mitochondrial ROS production and protection against oxidative stress. For instance, *Ucp2* ablation in INS-1E insulinoma cells resulted in improved GSIS, which can be mimicked using the cell-permeative antioxidant MnTMPyP (Affourtit et al., 2011). A study using a refined model of β -cell specific *Ucp2* knockout reported that *Ucp2*-ablated pancreatic islets had increased ROS levels correlating with increased GSIS (Robson-Doucette et al., 2011). Interestingly, while no changes in the uncoupling respiration rates and ATP/ADP ratio were observed, *Ucp2*-deficient islets exhibit mildly increased mitochondrial membrane potential (Robson-Doucette et al., 2011). Taken together, these findings suggest that *Ucp2* knockout increases intracellular ROS and thus stimulate insulin secretion. Thus, UCP2 might be seen as a double-edged sword: decreasing its activity seems to improve GSIS, however it leaves insulin-secretion β -cell susceptible to oxidative damage, which suggests a finely regulated activity.

UCP3 plays an active role in ROS production and fatty acid oxidation, which makes it a candidate for better understanding the pathophysiology of metabolic disorders such as obesity and type 2 diabetes mellitus. Experimental evidence from multiple studies have proposed UCP3 as a modulator of energy metabolism and a possible protective role in obesity. For instance, transgenic mice overexpressing UCP3 fed a fatty diet exhibit lower body weight, decreased adipose tissue, and triglyceride accumulation (Clapham et al., 2000; Costford et al., 2006; Son et al., 2004). Furthermore, moderate upregulation of UCP3 can mimic the beneficial consequences of physical exercise, promoting fatty acid oxidation, increasing energy expenditure and consequentially increase weight loss (Aguer et al., 2013). In contrast, studies using *Ucp3*^{-/-} mice failed to demonstrate an obesogenic effect for *Ucp3* ablation (Gong et al., 2000; Vidal-Puig et al., 2000). More surprisingly, a recent study by Lomax et al. linked *Ucp3* deficiency with protection from diet-induced obesity (Lomax et al., 2020). However, it is important to highlight that housing of animals in conditions of chronic thermal stress may have conflicting results. In regard to diabetes mellitus, overexpression

of UCP3 improves glucose tolerance and protected the animals against fat-induced insulin resistance (Choi et al., 2007; Costford et al., 2008). Studies focusing in *Ucp3* ablation have conflicting evidence as *Ucp3*^{-/-} animals fed a fatty diet exhibited either enhanced (Lomax et al., 2020), decrease (Senese et al., 2011) or unaltered (Vidal-Puig et al., 2000) insulin resistance. In humans, high glucose exposure decreased UCP3 protein expression in isolated islets, whereas overexpression of UCP3, contrary to UCP2, improves GSIS (Li et al., 2008). Concurrently, clinical studies have reported lower expression of *UCP3* mRNA and protein in prediabetic and diabetic individuals, when compared to healthy individuals (Mensink et al., 2007; Schrauwen et al., 2001; Schrauwen et al., 2006). Interestingly, both 8-week treatment with rosiglitazone and physical exercise training were able to restore UCP3 levels in diabetic patients (Schrauwen et al., 2006).

A1.5. The potential role of UCPs on diabetes-induced male infertility

The link between oxidative stress and male factor infertility is well documented since it was first described by MacLeod in 1943, where a connection between H₂O₂ production and loss of spermatozoa mobility was observed (MacLeod, 1943). In fact, clinical studies have reported that 30% to 80% of infertile men exhibit supraphysiological levels of seminal ROS (Agarwal et al., 2006; Iwasaki & Gagnon, 1992; Ochsendorf et al., 1994; Shekarriz et al., 1995; Tremellen, 2008; Zini et al., 1993). Furthermore, a study comparing ROS levels in semen from proven donor (those who resulted in a pregnancy) and infertile men, using chemiluminescence assay, reported that fertile men possessed reduced ROS levels (Agarwal et al., 2014). Several studies have already established a correlation between oxidative stress and poor sperm parameters, including reduced sperm concentrations and motility, aberrant morphology, as well as increased DNA fragmentation (Agarwal et al., 2003; Aziz et al., 2004; Moustafa et al., 2004; Said et al., 2004; Smith et al., 2006).

Interestingly, ROS have been described as a double-edged sword for spermatozoa. ROS have a crucial influence in sperm physiology and spermatogenesis, as well as in the fertilization process. Seminal ROS participate as a secondary messenger in several pathways necessary for spermatozoa hyperactivation, capacitation, sperm–oocyte fusion, chemotaxis, acrosome reaction, and sperm head decondensation (Aitken et al., 1991; de Lamirande & Gagnon, 1993; de Lamirande et al., 1997; Du Plessis et al., 2015). In addition to endogenous sources, ROS may also arise from exposure to pollutants, radiation, and drugs (Dias et al., 2020). An imbalance in the redox homeostasis, as a result of increased ROS production or a deficient antioxidant system (or both), result in pathological oxidative

damage to spermatozoa. Interestingly, it has been recently shown that antioxidant mechanisms, as is the case of Sperm Nuclear Basic Proteins (SNBP), when in contact with certain pollutants such as heavy metals can overturn their protective role and contribute to oxidative damage, which highlights how vulnerable spermatozoa are to oxidative stress (Lettieri et al., 2020a; Lettieri et al., 2020b). Even though infertile men present lower seminal plasma antioxidant capacity (Ko et al., 2014; Martin-Hidalgo et al., 2019), oxidative damage in spermatozoa is mostly associated with exacerbated levels of ROS as opposed to impaired superoxide scavenging activity (Zini et al., 1993). Loss of motility is one of the major consequences of oxidative stress in spermatozoa (Athayde et al., 2007), arising from a combination of reduced membrane fluidity, due to dysregulated lipid peroxidation (Hosseinzadeh Colagar et al., 2013), and defective axonemal phosphorylation (de Lamirande & Gagnon, 1992a, 1992b; Kurkowska et al., 2020). Furthermore, oxidative stress has been associated with spermatozoa DNA damage. Disruption in sperm genomic integrity clinically relates to augmented teratozoospermia, impaired oocyte fertilization, abnormal embryonic development, and increased miscarriage rates (Cissen et al., 2016; Lewis & Simon, 2010; Tarozzi et al., 2007).

As previously mentioned, individuals who suffer from obesity or diabetes mellitus present augmented levels of ROS, which results in oxidative stress and ultimately subfertility or even infertility. In fact, the deleterious effects of diabetes mellitus and obesity in male reproductive function are well established. Patients suffering from diabetes and/or obesity exhibit poor sperm parameter, decreased motility, abnormal morphology, reduced density, and higher damage to both nuclear and mitochondrial sperm DNA (La Vignera et al., 2012; Oliveira et al., 2017). It was also reported that obese men have a higher mean scrotal temperature than healthy individuals, which can be related to male infertility (Garolla et al., 2015; Mieusset et al., 1987). Besides, hormonal dysfunctions that decrease steroidogenesis in Leydig cells, resulting in hypogonadism, and alterations in the body energy metabolism disturb the normal function of testis (Carrageta et al., 2019; Oliveira et al., 2017). On the other hand, weight loss and improved lifestyle has been associated with better sperm parameters (Hakonsen et al., 2011), although a series of studies by Crisóstomo et al. demonstrated that the consumption of a high fat diet during childhood permanently alters testicular metabolism which led to an irreversible decline in sperm quality (Crisostomo et al., 2019; Crisostomo et al., 2020). Glucose metabolism is of great importance in the testis as sperm cells are highly sensitive to glucose concentrations. Accordingly, glucose transport through the blood-germ barrier is a highly controlled process. Hyperglycemia is particularly harmful to spermatogenesis being correlated with poor reproductive outcomes (Maresch et al., 2018). Sertoli and Leydig cells as well as spermatozoa have been shown to be sensitive to insulin, therefore, glucose homeostasis is necessary for male reproductive

function (Alves et al., 2013). Furthermore, both obese and diabetic patients have been reported to exhibit elevated ROS in testis and seminal fluid, which is, as described above, extremely detrimental (Amaral et al., 2008b; Leisegang et al., 2021).

Further understanding of the nefarious impact of ROS production and antioxidant mechanism in male related infertility will surely lead to more focused treatment and accurate diagnostic, as well as better outcomes for subfertile and infertile men. Hence, UCPs by virtue of their antioxidant activity may represent a clinically relevant target to improve reproductive outcomes. UCPs seem to play an important part in mediating the deleterious effects of diabetes in male infertility and subfertility, however little is known about their function in the male reproductive tract. Given the extensively described role of UCPs as key regulators of the mitochondria redox state, it should not come as a surprise that most of the experimental data comes from studies on the activity of UCPs in the testis/sperm ROS production, more precisely UCP2. Zhang et al. reported that UCP2 protects germ cells from hyperthermia-derived ROS production. Furthermore, *Ucp2* mRNA expression was increased in the mouse testis of experimental cryptorchidism. Hyperthermia in the testis arises from multiple pathological conditions such as varicocele, cryptorchidism, or obesity, and correlates with exacerbated spermatozoa apoptosis (Zhang et al., 2007). In another study, it was found that ROS production is the main mechanism behind hyperthermia-induced sperm cell apoptosis. Acute exposure of mouse testicles to heat (43°C for 5 min) was sufficient to induce a 6-fold increase in UCP2 expression. Furthermore, overexpression of UCP2 in mouse GC-2 cell line, a model for germ cell differentiating, improved the survival rate after exposure to the ROS-inducing agent menadione (Zhang et al., 2007). Moreover, older mice exhibit increased UCP2 activity and proton leak in the testis. Testicular age involves higher rates of mitochondrial dysfunction which correlates with increased oxidative stress, therefore, UCP2 upregulation in older testis might be a protective mechanism (Amaral et al., 2008a). In humans, UCP2 expression was found to be significantly increased in spermatozoa from donors with normal sperm motility when compared to asthenospermic men (Wang et al., 2018). The link between UCP2 and ROS production was further highlighted through treatment of spermatozoa with genipin, which resulted in elevated levels of mROS and decreased sperm motility. It should be noted that it was recently reported by Kreiter et al. that genipin is incapable of specifically inhibit UCP2, but rather is a nonspecific inhibitor of UCPs activity (Kreiter et al., 2019). Additionally, treatment of spermatozoa with H₂O₂ was shown to induce UCP2 expression (Wang et al., 2018). Although the experimental data on the role of UCPs in the male reproductive function is scarce, given their presence in testis and their established function in other tissues it should be expected that they are important modula-

tors of testicular bioenergetics and redox state. Therefore, they are enticing targets for further research in the crosstalk between diabetes mellitus and obesity and their secondary effects in male reproduction.

A1.6. Conclusions and future perspectives

UCPs are widely expressed throughout the organism and play an important role on ROS production and homeostasis. UCP1 is responsible for thermogenesis, but the other homologs display broader functions, including insulin release and regulation of oxidative phosphorylation. Due to their functions, UCPs take part in the pathophysiology of diabetes, where its altered expression and/or dysfunction is associated with the onset of the disease. Studying UCPs expression and function will lead to a better understanding of the pathophysiology of metabolic diseases and potentially to novel therapeutic targets. As mentioned above, diabetes is positively associated with high oxidative stress and, consequently, to male subfertility or even infertility, which is one of the most overlooked comorbidities. Working towards a better understanding of the relationship of UCPs activity with male reproductive function could lead to the development of novel fertility therapies and better reproductive outcomes. However, not much is known concerning the expression and function of UCPs in the male reproductive tract. Further studies are needed to disclose the role of UCPs in male fertility and redox signaling and homeostasis during spermatogenesis and spermatozoa physiology.

A1.7. References

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A2. Molecular mechanisms regulating spermatogenesis in vertebrates: environmental, metabolic, and epigenetic factor effects

Abstract

The renewal of the natural resources is one of the most concerning aspects of modern farming. In animal production, there are many barriers that breeders and researchers have to overcome to develop new practices to improve reproductive potential and hasten sexual maturation of the commercially viable species, while maintaining meat quality and sustainability. With the utilization of molecular biology techniques, there have been relevant advances in the knowledge of spermatogenesis, especially in mammals, resulting in new possibilities to control male fertility and the selection of desirable characteristics. Most of these discoveries have not been implemented in animal production. In this review, recent studies are highlighted on the molecular pathways involved in spermatogenesis in the context of animal production. There is also exploration of the interaction between environmental factors and spermatogenesis and how this knowledge may revolutionize animal production techniques. Furthermore, new insights are described about the inheritance of desired characteristics in mammals and there is a review of nefarious actions of pollutants, nutrition, and metabolism on reproductive potential in subsequent generations. Even though there are these advances in knowledge base, results from recent studies indicate there are previously unrecognized environmental effects on spermatogenesis. The molecular mechanisms underlying this interaction are not well understood. Research in spermatogenesis, therefore, remains pivotal as a pillar of animal production sustainability.

A2.1. Introduction

Spermatogenesis is the process where male gametes (i.e., spermatozoa) are produced in the testes. Spermatogenesis comprises several phases, such as the mitotic increase of germ cell numbers, (the spermatogonia), meiotic divisions, spermiogenesis and subsequently spermiation. This process is supported by the testicular somatic cells, where Sertoli cells are highlighted as the primary cells responsible for the mechanical and nutritional support of the germ cell line (Rato et al., 2012). Even though the ultimate goal is similar – producing the spermatozoa – structural differences of male gonads are present among vertebrate species, which can be divided into two distinct groups, amniotes and anamniotes. Amniotes (reptiles, birds, and mammals) can produce eggs and embryonic membranes that

cannot be penetrated by water, whereas in anamniotes (fish and amphibians), spermatogenesis occurs inside a cyst of Sertoli cells and spermatogonia, leading to cystic spermatogenesis (Uribe et al., 2014). Cystic spermatogenesis is similar to a certain extent to oogenesis because a cyst of Sertoli cells surrounds the germ cell and nourishes the gamete until there is maturation, meiosis and subsequently spermiation. During this process, there is a change in gene expression in Sertoli cells and, therefore, protein secretion patterns to support the respective stages of spermatogenesis. When the cycle ends, Sertoli cells are degraded. Interestingly, this replenishment of Sertoli cells indicated there is a Sertoli-related stem cell niche, although it has not been ascertained as to whether there are actually such cells (Yoshida, 2016). Because results from studies focused on anamniote spermatogenesis are limited, this review will mainly focus on the advances of molecular mechanisms regulating spermatogenesis of amniote species.

In amniotes, spermatogenesis occurs inside the seminiferous tubules, the functional unit of the testes, characterized by a long and convoluted tubular structure that culminate in the rete testis. The seminiferous tubules contain Sertoli cells and germ cells, which are in physical contact with the basement membrane. Notably, Sertoli cells comprise 17–20% of the mass of the seminiferous tubules (Russell et al., 1990). Sertoli cells form a highly polarized epithelium due to its prominent basal membrane and tight junctions between adjacent cells that is the basis of the blood-testis barrier (BTB) (Mruk & Cheng, 2015). The BTB serves as a functional barrier, controlling the exchange of molecules that enter into or are removed from the testis milieu due to the complex and precisely regulated network of transporters and channels in the basal membrane. In addition, the BTB functions as an immunological barrier resulting in there being few immune cells and inflammatory responses in the seminiferous tubules (Su et al., 2011; Wong & Cheng, 2005). Sertoli cells, therefore, are the primary tissues responsible for maintaining the homeostasis of the milieu that is contained in seminiferous tubules, providing not only protection but also mechanical and nutritional support for germ cells. If germ cells do not receive the appropriate quantity of substrates that are sources for energy and if there is not an adequate ionic milieu for gamete development, these cells will degenerate as a result of apoptosis (Boussouar & Benahmed, 2004; Jutte et al., 1981). Each Sertoli cell supports as many as 30–50 germ cells at all stages of development which is facilitated by the numerous cytoplasmic prolongations of these cells (Cheng et al., 2010; Weber et al., 1983). At each stage, Sertoli cells are closely associated with the germ cells and provide all the nutrients and metabolic intermediates needed for gamete homeostasis, including amino acids, carbohydrates, lipids, vitamins, and ions (Mruk & Cheng, 2004; Robinson & Fritz, 1981). Spermatogenesis occurs in a precise germ cell developmental sequence from the basement membrane to the lumen of seminiferous tubules (Fig. A2.1). In brief, spermatogonia, which are located near the basement membrane,

undergo mitosis to replicate and maintain the germ cell line (spermatogonia A), while there is generation of a new population (spermatogonia B) that differentiate into spermatozoa. Spermatogonia B subsequently differentiate into primary spermatocytes, which cross the BTB and as a consequence of the first meiotic division there is development into secondary spermatocytes. With the second meiotic division, there is development of round spermatids from secondary spermatocytes and subsequently elongated spermatids develop from round spermatids during the spermiogenic process. Spermiation occurs as the last of the sperm maturation processes and elongated spermatids are released into the seminiferous tubule lumen as immature spermatozoa (Hess & de Franca, 2008). Immature spermatozoa are non-motile and are transported to the epididymis in testicular fluid through peristaltic contractions. In the epididymis, spermatozoa further mature and gain the capacity for motility and fertilization.

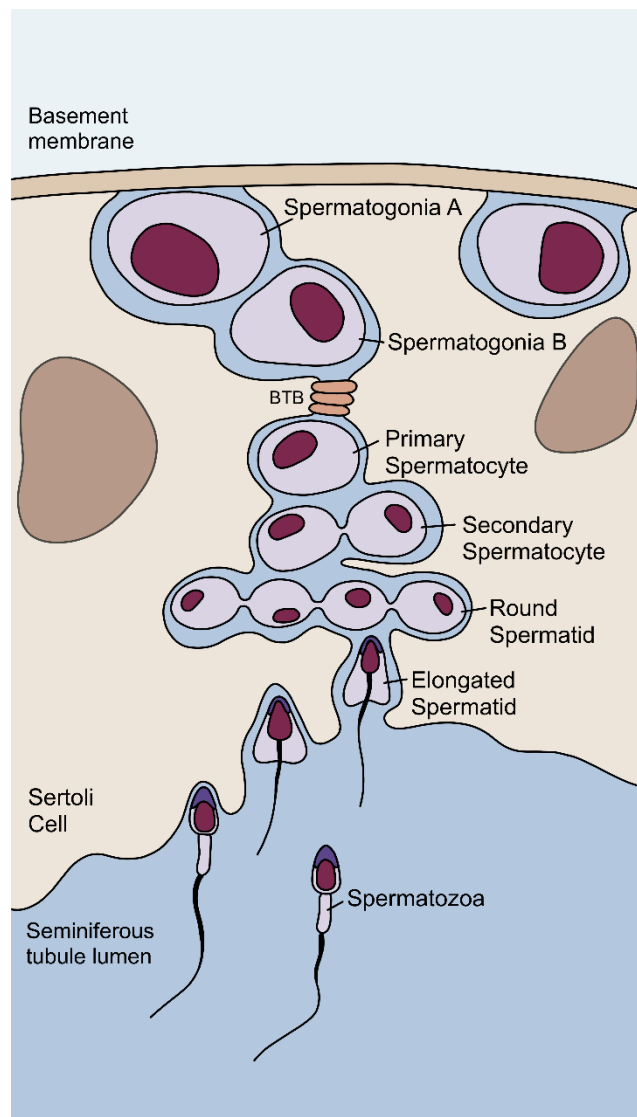


Fig. A2.1 – Schematic illustration of mammalian spermatogenesis. Spermatogonia A are located near the basement membrane and undergo mitosis to produce Spermatogonia B, which will differentiate into spermatozoa. Spermatogonia B differentiate into primary spermatocytes, cross the blood-testis barrier (BTB), and enter the first meiotic division for production of secondary spermatocytes. Round spermatids are formed as a result of the second meiotic division and elongated spermatids are produced as a result of spermiogenic processes, with these gametes being released into the lumen as immature spermatozoa in a process known as spermiation. Immature spermatozoa are transported to the epididymis, where maturation is completed.

Spermatogenesis is a very complex and finely regulated process that is still not well understood from a molecular perspective. The testis is regarded as a highly active metabolic and endocrine organ, sensitive to several pathophysiological conditions and there are a myriad of environmental or nutritional factors that are associated with compromised testicular function and impaired spermatogenesis and ultimately animal reproduction (Oliveira et al., 2017). In this review, there is elaboration on three major timely topics that are of great interest to the scientific community. The reason for this interest is that these factors affect animal reproductive functions as a result of having modulatory effects on hormonal control of spermatogenesis. The specific factors focused on in this review article are the endocrine-disrupting chemicals (EDCs), metabolic regulation, and epigenetic and transgenerational factors involved in spermatogenesis.

A2.2. Endocrine modulators of spermatogenesis

A2.2.1. Hormonal control of spermatogenesis

Spermatogenesis is a complex and precisely regulated process that requires the actions of molecules from several classes, including hormones, peptides, lipids, or metabolites. Spermatogenesis is a process that is very sensitive to fluctuations of the seminiferous milieu, including metabolic, ionic, or hormonal alterations (Bernardino et al., 2019). Although hormonal control of spermatogenesis has been intensively studied, there continues to be need for enhanced understanding of this process. The hormonal regulation is the result of the coordinated actions of the hypothalamus-pituitary-gonadal (HPG) axis, which is conserved to some extent among vertebrate species. In brief, in the hypothalamus there is production of kisspeptins which regulate the release of hypothalamic gonadotropin-releasing hormone (GnRH). The GnRH peptide is released in pulses into the hypophyseal portal vessels and stimulates the anterior pituitary to release the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both gonadotropins enter the circulatory system and

are transported to the testis, where LH stimulates the production of testosterone by Leydig cells which has a combined function with FSH in regulation of Sertoli cell functions and spermatogenesis (Braga et al., 2020; Carrageta et al., 2019). Testosterone is also converted to 17 β -estradiol as a result of aromatase functions and both of these hormones have a negative feedback effect on the hypothalamus and pituitary, regulating the release of GnRH. Consequently, both gonadotropins contribute for the maintenance of hormonal concentrations in the homeostatic ranges in the organism (Zirkin et al., 2011). Sertoli cells also produce inhibin B in response to FSH and testosterone, which also modulate the negative feedback mechanism for regulation of FSH secretion from the anterior pituitary (Meachem et al., 2001).

A2.2.2. Endocrine-disrupting chemicals (EDCs)

Endocrine-disrupting chemicals (EDCs) are toxicants widely dispersed in the environment that potentially disrupt the endocrine system and consequently affect reproductive tissue functions. According to the European Union Scientific Committee of Toxicity, Ecotoxicity and Environment (EUSCTEE), an EDC is “an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)population” (Damstra et al., 2002). Currently, there are approximately 1000 compounds identified as EDCs (Street et al., 2018). The EDCs include several compound groups, including derivatives produced in the plastic industry (bisphenol A, phthalates, nonylphenol ethoxylates), preservatives (parabens, triclosan), biocides (chlorotriazine herbicides, glyphosate, dichlorodiphenyltrichloroethane, vinclozolin, tributyltin compounds, among others), combustion-related products (benzo[a]pyrene, polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, and dioxins), and natural compounds such as phytoestrogens (e.g., genistein) (Sharma et al., 2020). Because these substances are widely dispersed in the environment (naturally or due to human activity), wildlife, livestock, and domestic animals are affected by these compounds (Tubbs & McDonough, 2018). The EDCs are in large concentrations in species that are higher in the trophic ranking hierarchy being greatest in the apex predators due to biomagnification of content of the EDCs through the food chain. Polluted waters or fish residing in EDC-polluted waters (Bursian et al., 2013; Meijer et al., 1999; Rhind et al., 2010), animals located near fire or plant incineration enterprises (Ingelido et al., 2009), or commercially available diets for which there has been a large amount of processing (Cerundolo et al., 2004; Court & Freeman, 2002; Kang & Kondo, 2002; Venier & Hites, 2011) may be sources of the larger quantities of EDCs.

The endocrine system is a complex network that relies on the integration and coordination of the functions of multiple enzymes, proteins, and receptors. The known and potential targets of EDCs, therefore, are vast (Fig. A2.2).

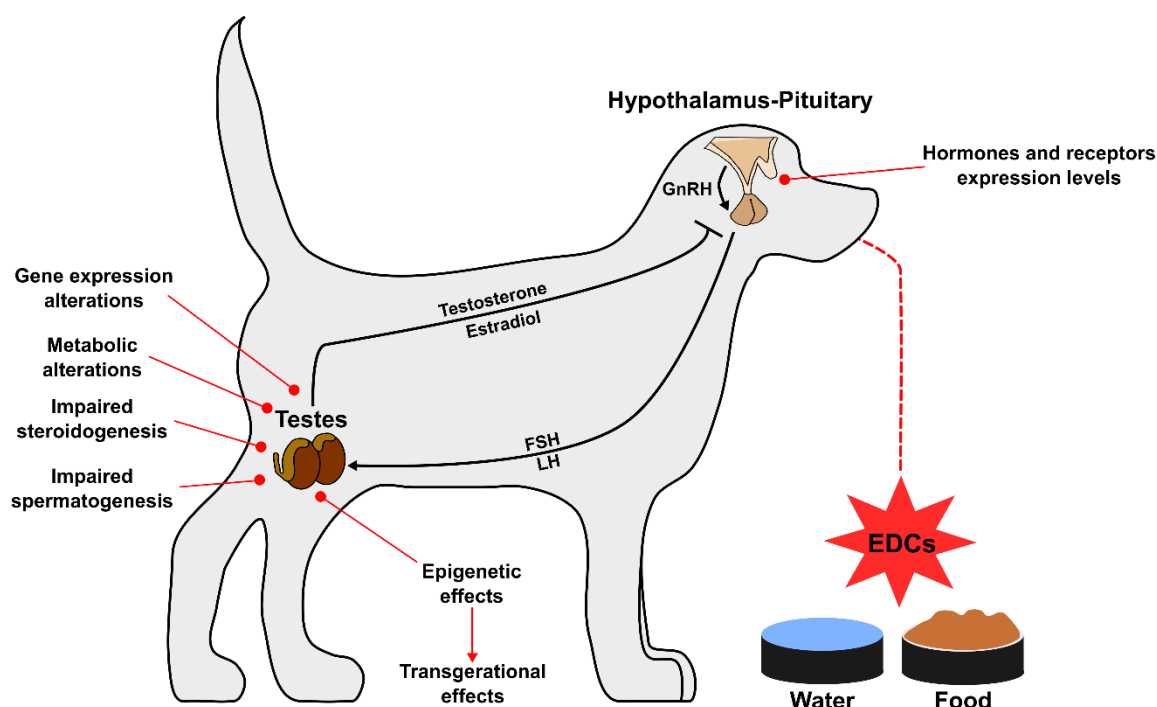


Fig. A2.2 – Effects of endocrine-disrupting chemicals (EDCs) on the hormonal control and reproductive function. EDCs are widely dispersed in the environment, including food and water sources. EDCs disrupt the hypothalamus-pituitary-gonads axis functions through modulation of hormones and the receptor functions at the hypothalamus/pituitary leading to a decrease in steroidogenesis in the testes, leading to impaired spermatogenesis and reduced reproductive function. In addition, EDCs have been associated with transgenerational reproductive damage.

Compelling evidence indicates EDCs interact with hormonal receptors either having agonistic or antagonistic effects or through the alteration of natural hormone synthesis and degradation, leading to the hypothalamic-pituitary-gonadal axis dysregulation (Zoeller et al., 2012). The EDCs can induce harmful effects on animals even at concentrations considered safe for humans. Some EDCs can have marked effects in very small quantities during gestation and early postnatal periods, highlighting that not only the quantity but also the physiological state is an important factor regarding effects of EDCs (Street et al., 2018). The EDCs may also induce species-specific functions, although the functions of the HPG axis is highly conserved among vertebrates (Tubbs & McDonough, 2018). The reported effects of EDCs are as vast as the potential tissue functions that can occur at various tissues in

different animals, including asthma, cognitive problems, cancer, obesity and metabolic syndrome, bone and immune disorders, and infertility (Palanza et al., 2016; vom Saal et al., 2008). In the majority of the studies, there was a focus on the EDC effects on the female reproductive tract and effects of EDCs on pregnancy. Although due to findings in many studies, there can be conclusions regarding the effects of EDCs on the male reproductive tract and testis function, more studies with male subjects are needed to more definitively determine EDC effects in males. In the subsequent sections of this review manuscript, there is highlighting of the main advances in the study of the most commonly evaluated and disseminated EDCs – phytoestrogens, bisphenol A, and glyphosate.

A2.2.2.1. Phytoestrogens

Phytoestrogens are plant-derived nonsteroidal compounds that have functions as endocrine disruptors as a result of the capacity to mimic the conformational structure of estradiol (Kuiper et al., 1997; Kuiper et al., 1998). There are phytoestrogens in vegetables and are very abundant in soybean and soy-derived products, which are included in large quantities in commercially available diets fed to animals (Cederroth et al., 2010a). Genistein and daidzein are the most common phytoestrogens. Both are abundant in soybeans as β -D-glycosides (genistin and daidzin, respectively), which are biologically inactive. Bacterial β -glucosidases present in the intestinal wall, however, are responsible for the conversion to the bioactive form and these forms can be absorbed through the wall of the intestinal tract (Setchell, 1998).

Results from studies of the effects of phytoestrogens on male reproduction and spermatogenesis are inconsistent (Table A2.1). There are detrimental effects of phytoestrogens on reproductive functions on male pups. For example, a daily dose of 1250 ppm genistein included in the diet of pregnant Sprague-Dawley rats, starting at day 7 of the gestational period and sustained throughout the entire gestational period, led to a smaller ventral prostate weight and aberrant or delayed spermatogenesis in the offspring when these animals were reproductively mature (Delclos et al., 2001). Interestingly, there were fewer litters produced per female that were vaginal plug-positive that were treated with genistein than of females not treated with genistein. In resulting offspring of pregnant Wistar rats treated with incrementally larger doses of genistein (0, 2, 20, 100 mg/kg/day) from day 12–19 of the gestational period, there was a genistein dose-dependent reduction in sperm production and epididymal sperm concentration and quality.

Table A2.1. Effects of phytoestrogens on male reproductive functions and transgenerational consequences.

Compound	Dose	Duration of treatment	Administration	Animal model	Main findings/outcomes	Reference
Genistein and vin-clozolin mixture	1 mg/kg/day of genistein and vin-clozolin mixture	Gestational day 1 to postnatal day 21 (group 1) Postnatal day 21 to postnatal day 100 (group 2)	Oral gavage	Wistar rats (n = 24, 12 per group)	Gestational/lactational exposition led to delayed puberty onset Gestational/lactational exposition altered mRNA expression of genes related to steroidogenesis but no alterations in testosterone production Prepubertal exposure decreased sperm production in the father and their offspring	(Eustache et al., 2020)
Genistein	20 mg/kg/day	2-3 weeks	Subcutaneous injection	Streptozocin-induced type 2 Diabetes mellitus Parkes mice (n = 40, 10 per group)	Improved diabetes-related changes in spermatogenesis Increased circulating 17 β -estradiol and testosterone levels Improved insulin sensitivity	(Verma et al., 2019)
Genistein	2, 20, or 100 mg/kg/day	12th to 19th day of gestation	Intraperitoneal injection	Pregnant Wistar rats (n = 32, 8 per group)	Dose-dependent reduction in sperm production and epididymal sperm density and quality Morphological alterations in the seminiferous tubules Decreased steroidogenesis Mean number of implantations and live foetuses per dam was reduced	(Meena et al., 2017)
Genistein and/or daidzein	17 or 170 mg/kg/day genistein 12 or 120 mg/kg/day daidzein Combination of low or high dose of both compounds	6 weeks	Oral gavage	Prepubertal male Wistar rats (n = 175, 25 per group)	Low doses (alone or mixture) delayed puberty onset High doses (alone or mixture) arrested puberty onset Lower epididymal sperm counts	(Caceres et al., 2015)
High soy-based diet	22–26 mg/kg/day of genistein and daidzein (estimated)	6 months	Oral ingestion	CD-1 male mice (n = 39, 18 in the high soy-based diet group)	Reduced proportion of haploid germ cells Decreased epididymal sperm counts Reduced litter size	(Cederroth et al., 2010b)

Table A2.1. Effects of phytoestrogens on male reproductive functions and transgenerational consequences (cont.).

Compound	Dose	Duration of treatment	Administration	Animal model	Main findings/outcomes	Reference
Genistein and vinclozolin alone or mixture	1 or 10 mg/kg/day of genistein 1 or 30 mg/kg/day of vinclozolin Combination of low or high dose of both compounds	Gestational day 1 to postnatal day 21	Oral gavage	Pregnant Wistar Han rats (n = 80, 10 per group)	Low dose of mixture and high dose of vinclozolin decreased litter size and increased post implantation loss Decreased pups' sperm count and motility Altered pups' testicular mRNAs expression involved in protein biosynthesis and FSH-related genes	(Eustache et al., 2009)
Soy formula milk	25.5 mg aglycone/L (estimated)	6 weeks	Oral ingestion	Newborn mammoset monkey (<i>Callithrix jacchus</i>) (n = 14, 7 per group)	Increased testis weight Increased number of Sertoli and Leydig cells per testis	(Tan et al., 2006)
Genistein	0.1, 0.5, 2.5, or 10 mg/kg/day	Gestational day 12 to postnatal day 20	Oral gavage	Pregnant C57BL/6 mice (n = 45, 10–13 per group)	No alterations on testis weight or sperm count in the pups were observed In vitro fertilizing ability of epididymal sperm was increased in the higher dose	(Fielden et al., 2003)
Genistein	0.4 or 4 mg/kg/day	Gestational day 6 to postnatal day 20 (interruption on postnatal day 1–2)	Oral gavage	Pregnant Sprague-Dawley rats (n = 60, 9–12 per group)	No alterations on the testis weight or sperm count in the pups were observed	(Kang et al., 2002)
Genistein	0.5, 25, 100, 250, 625 or 1250 ppm	Gestational day 7 to postnatal day 50	Oral ingestion	Pregnant Sprague-Dawley rats (n = 10)	Reduced number of litters, pups' weight, and pups' ventral prostate weight at highest concentration Pups presented aberrant or delayed spermatogenesis at highest concentration	(Deldos et al., 2001)

In addition, results from histological examinations highlighted that there was a deterioration in the seminiferous tubule structure. There was also less steroidogenesis and fewer fetal implantations and live fetuses per genistein-treated female as compared with dams of pups in the control group (Meena et al., 2017). When there was perinatal treatment of male mice with smaller doses of genistein (0.1–10 mg/kg/day), however, there were no adverse effects on testis weight or sperm count (Fielden et al., 2003). Consistent with these results, when pregnant Sprague-Dawley rats were treated with genistein (0.4–4 mg/kg by oral gavage) there were no alterations in the testis weight or sperm counts in male rat offspring (Kang et al., 2002). Considered together, these results indicate genistein effects on male fertility may occur when there are larger quantities of genistein present to have effects on the male reproductive system.

In reproductively mature animals, there is evidence that chronic dietary inclusion of phytoestrogens or consumption of soy diet containing large quantities of phytoestrogens are detrimental to reproductive functions of these animals. Chronic treatment of Wistar Hans rats with a relatively small dose (1 mg/kg/day) of genistein and vinclozolin (antiandrogenic fungicide) or a large dose of vinclozolin (30 mg/kg/day) resulted in a lesser sperm concentration, motility and altered testicular abundance of mRNA transcripts that encode for specific proteins especially proteins modulated by FSH (Eustache et al., 2009). Mice fed from conception until reproductively mature a large quantity of soy had a lesser proportion of haploid germ cells, 25% less epididymal sperm counts, and a 21% smaller litter size. In addition, there were lesser abundances of mRNA transcripts encoding for androgen-responsive proteins in Sertoli cells and mRNA transcripts for genes encoding for proteins related to sperm bioenergetics and motility (Cederroth et al., 2010b). The duration of treatment is important because of differing effects when there are differing durations of phytoestrogen treatments. In another study, there were treatments of Wistar rats with relatively small, as compared with some other studies, doses of genistein and/or vinclozolin (1 mg/kg/day) during the gestational/lactational period and there were comparisons of effects when this treatment was applied during the prepubertal to reproductively mature period. Treatments during the gestational/lactational period only resulted in an altered abundance of mRNA transcripts encoding for proteins modulating steroidogenesis, however, there were no treatment effects on testosterone concentrations. Prepubertal treatments, nonetheless, had effects on epididymal weight and sperm count in the treated animals and male offspring of the treated animals (Eustache et al., 2020). Consistent with these results, there was a lesser epididymal sperm count after oral administration of genistein (17 or 170 mg/kg/day) and/or daidzein (12 or 120 mg/kg/day) during the prepubertal to reproductively mature period (Caceres et al., 2015). In addition, puberty onset was delayed when there was administration of the smaller doses and there was not an onset of reproductive functions (*i.e.*, no

pubertal onset) when there was treatment with the larger doses. In several studies, however, there were no adverse or even positive effects on reproduction when there was treatment with phytoestrogens. In marmoset monkeys (*Callithrix jacchus*) fed soy formula milk for 6 weeks, there were no adverse reproductive effects, although there were testis size and cell composition differences as a result of treatment with soy formula (Tan et al., 2006).

Treatment of streptozotocin-induced diabetic Parkes mice with genistein (20 mg/kg/day) for 14 or 28 days reduced the type 2 diabetes-mediated regression effects on spermatogenesis, circulating estrogen and testosterone concentrations, and insulin sensitivity (Verma et al., 2019). Although in several studies there has been a focus on the effects of phytoestrogens on the reproductive tract structures and functions, there are inconsistencies in the results probably because of a lack of standardization of experimental designs. There was a very informative review of this problem previously (Cederroth et al., 2010a), however, this problem still persists when conducting studies evaluating phytoestrogenic effects on reproduction.

A2.2.2.2. Bisphenol A

Bisphenol A (BPA) is widely recognized to be an EDC that originates from the plastic industry and plastic food packaging, being present in the environment due to pollution or sometimes in commercially available diets (Cariati et al., 2019; Street et al., 2018). The BPA compound is considered one of the most consensual EDCs because of the capacity of BPA to bind to estrogen receptors, resulting in a small amount of estrogen activity and disruption of the functions in the HPG axis (Vom Saal, 2016). In addition, BPA also has the capacity to bind to androgen receptors and the result is less androgenic effects (Lee et al., 2003; Wang et al., 2017).

There has been extensive study of BPA and there is compelling evidence of disruptive effects on spermatogenesis and male reproductive functions (Table A2.2). For example, Sprague-Dawley rats intraperitoneally injected with BPA (2, 10, and 50 mg/kg every other day) for 20 days had a lesser epididymal semen quality and count that was BPA dose-dependent as a result of oxidative stress (Ansoumane et al., 2014). Oral administration of BPA (2 µg/kg/day) for 14 days to reproductively mature rats led to lesser sperm counts and more germ cells undergoing apoptosis. In addition, the concentrations of serum testosterone and FSH, as well as GnRH mRNA transcript abundance were less in treated rats (Jin et al., 2013).

Table A2.2. Effects of bisphenol A (BPA) on male reproductive functions and transgenerational consequences.

Compound	Dose	Duration of treatment	Administration	Animal model	Main findings/outcomes	Reference
Bisphenol A	50 µg, 5 mg or 50 mg/kg/day	Embryonic day 7–14	Oral gavage	Pregnant CD-1 mice (n = 40, 10 per group)	Dose-dependent decrease in sperm count, motility, and intracellular ATP Decreased average litter size Altered sperm proteome	(Rahman et al., 2017)
Bisphenol A	5 or 25 mg/kg/day	40 days	Oral gavage	Wistar rats (n = 30, 10 per group)	Reduced sperm production Increased levels of morphologically abnormal sperm Significant damage to the acrosome and sperm plasma membrane Reduced sperm mitochondrial activity Reduced serum testosterone, LH and FSH, and increased estradiol Increased GnRH receptor, LH-beta, FSH-beta, estrogen receptor beta and androgen receptor transcripts in the pituitary Reduced estrogen receptor alpha transcripts in the hypothalamus	(Wisniewski et al., 2015)
Bisphenol A	2, 10 or 50 mg/kg every other day	20 days	Intraperitoneal injection	Sprague-Dawley rats (n = 24, 6 per group)	Reduced epididymal semen quality and sperm count in a dose-dependent manner Increased oxidative stress in epididymal sperm	(Ansoumane et al., 2014)
Bisphenol A	0.5, 1.75, 2.5, 5 or 10 µg/L	2 h	Diluted in culture medium	Sterlet (<i>Acipenser ruthenus</i>) sperm (n = 6)	Decreased sperm motility Decreased intracellular ATP content Increased DNA fragmentation and oxidative stress	(Hulak et al., 2013)

Table A2.2. Effects of bisphenol A (BPA) on male reproductive functions and transgenerational consequences (cont.).

Compound	Dose	Duration of treatment	Administration	Animal model	Main findings/outcomes	Reference
Bisphenol A	2 µg/kg/day	14 days	Oral ingestion	Sprague-Dawley rats (n = 50, 10 per group)	Reduced sperm count and germ cell apoptosis Decreased levels of serum testosterone, FSH and GnRH mRNA	(Jin et al., 2013)
Bisphenol A	10, 100, or 1000 nmol/L	Gestational day 0 to postnatal day 21	Oral ingestion	Pregnant ICR mice (n = 60 female mice, 8 male pups per group)	Increased cell apoptosis Alterations in mitochondria and endoplasmic reticulum in germ, Sertoli, Leydig and peritubular myoid cells	(Liu et al., 2013)
Bisphenol A	0.005, 0.5, 50, or 500 µg/kg/day	45 days	Oral gavage	Wistar rats (n = 36, 6 per group)	Increased plasma glucose and insulin Decreased testicular glucose Dose-dependent increase in H ₂ O ₂ Decreased hexokinase and phosphofructokinase activity and IRS-2 and GLUT-8 expression	(D'Cruz et al., 2012)
Bisphenol A	2 µg, 20 µg, 200 µg, 2 mg or 200 mg/kg every other day	2 weeks	Oral ingestion	White Leghorn male chicks (n not mentioned)	Testis growth arrest Severe inhibition of the development of seminiferous tubuli and spermatogenesis	(Furuya et al., 2006)
Bisphenol A	3 mg/kg/day	5 weeks	Subcutaneous injection	Wistar rats (n = 21, 11 in BPA group)	Reduced plasma testosterone Reduced epididymal sperm counts No significant effects on sperm motility Increased plasma IGF-1 and ventral prostate gland weight	(Herath et al., 2004)

Treatment of reproductively mature rats (90 days post-birth) for 45 days with relatively small doses of BPA (0.005, 0.5, 50, and 500 µg/kg/day), compared to doses in some other studies resulted in a dose-dependent increase in the concentrations of H₂O₂ and a marked decrease in the BPA-treated rats abundance of insulin receptor substrate 2 (IRS-2) and glucose transporter 8 (GLUT8), and in activity of hexokinase and phosphofructokinase in the testis (D'Cruz et al., 2012). In addition, plasma glucose and insulin concentrations were greater whereas testicular glucose concentrations were less in BPA-treated compared with rats not treated with BPA (D'Cruz et al., 2012).

Similar to phytoestrogens, the period for duration of treatment is also important. Results from other studies highlight that treatments with BPA during the gestational period have harmful and permanent effects on the offspring. When pregnant ICR mice were treated with BPA (through water containing 10, 100 and 1000 nmol/L) from day 0 of the gestational period until pup weaning at 21 days subsequent to birth the male offspring had greater cell apoptosis and alterations in the mitochondrial and endoplasmic reticulum of gametes, as well as Sertoli, Leydig and peritubular myoid cells (Liu et al., 2013). The effects of BPA during the gestational period (50 µg, 5 mg, or 50 mg/kg/day) on pregnant CD-1 mice during embryogenesis (days 7–14) were a lesser average litter size whereas sperm count, motility, and intracellular ATP concentrations decreased in a dose-dependent manner in the reproductively mature offspring. Additionally, it was reported that there was an alteration of the spermatozoa proteome, particularly in proteins related to ATP generation, oxidative stress response and ultimately fertility potential (Rahman et al., 2017).

A2.2.2.3. Glyphosate

Glyphosate and glyphosate-based herbicides are extensively used herbicides due to inhibitory effects on the biosynthesis of essential aromatic amino acids in plants (Benbrook, 2016; de Araujo-Ramos et al., 2021). Glyphosate and the primary breakdown product aminomethylphosphonic acid (AMPA) are widely abundant in the environment, from where there can be intake as a result of consumption of water or food containing these compounds (Connolly et al., 2020; Van Bruggen et al., 2018). The thoughts regarding EDC capacity of glyphosate are inconsistent due to there being data that vary for food and water source concentrations. The EDC capacity of glyphosate is related to the activation of estrogen receptor alpha as a result of a ligand-independent mechanism of action, but only when there are large concentrations of glyphosate consumed (Mesnage et al., 2017). According to the

Endocrine Disruptor Screening Program (EDSP) of the United States Environmental Protection Agency and the European Food Safety Authority, there is no sufficient acceptable evidence to classify glyphosate as an EDC (Safety, 2015; USEPA, 2015).

In some studies, there has been a focus on the potential effects of glyphosate and glyphosate-based herbicides on the endocrine and reproductive system (Table A2.3). For example, Roundup® administration (5, 50, or 250 mg/kg/day) orally to prepubertal male Wistar rats for 30 days resulted in there being lesser serum testosterone concentrations and morphological alterations in the testis (Romano et al., 2010). Surprisingly, in another study by the same group the treatment with Roundup® (50 mg/kg) during the gestational period of rats resulted in greater serum LH, testosterone, and 17 β -estradiol concentrations of the male offspring (Romano et al., 2012). In addition, from this study, it was reported that there was a larger total sperm count in the testes and epididymis, which was associated with the increase in the height of the germinal epithelium of seminiferous tubules. These latter results were considered rather controversial and some questioned both the results and conclusions (DeSesso & Williams, 2012). Results from some studies, however, indicate glyphosate impairs male reproductive functions. Effects of oral consumption of Roundup® (0.5% in water) when administered for 8 days to reproductively mature male Sprague-Dawley rats have been evaluated and there was a greater abundance of aromatase in the rats of the treated than control group (Cassault-Meyer et al., 2014). In addition, there was a greater percentage of sperm abnormalities even though there were similar values for sperm concentration and motility in the rats of the Roundup®-treated as compared with those of the control group. The effects of Roundup® administered by oral gavage (5 or 100 mg/kg/day) for 15 days to drakes (*Anas platyrhynchos*) were evaluated and there was a dose-dependent decrease in plasma concentrations of testosterone and 17 β -estradiol, changes in the abundance of androgen receptors in the testis, and morphological alterations in the seminiferous tubules, proximal efferent and epididymal ducts (Oliveira et al., 2007). Treatment with glyphosate diluted in water (5 or 10 mg/L) led to a lesser sperm motility but there were no alterations in sperm concentration in reproductively mature zebrafish (*Danio rerio*) (Lopes et al., 2014). In addition, the treatment with the largest concentration (10 mg/L) resulted in lesser mitochondrial functionality and membrane and DNA integrity.

Table A2.3. Effects of glyphosate and glyphosate-based formulations on male reproductive functions and transgenerational consequences.

Compound	Dose	Duration of treatment	Administration	Animal model	Main findings/outcomes	Reference
Roundup Ultra Plus® Glyphosate Polyoxyethyleneamine	0.01, 0.005, or 0.0025% (v/v) Equivalent doses	1 h	Diluted in culture medium	Boar spermatozoa (n = 5–6)	Decreased sperm motility in a dose-dependent manner Increased plasma membrane disorganization Altered motility-related signaling cascades	(Torres-Badía et al., 2021)
Glyphosate Roundup Bioflow®	0, 5, 25, 50, 100, or 360 µg/mL Glyphosate-equivalent doses	1–3 h	Diluted in culture medium	Pietrain boar spermatozoa (n = 10)	Decreased sperm motility, viability, mitochondrial activity, and acrosome integrity at 3 h upon treatment with the highest concentration of glyphosate Roundup Bioflow® had similar effects with lower concentrations (5 µg/mL glyphosate-equivalent) and shorter incubation times (1 h)	(Nerozzi et al., 2020)
Roundup Flex®	160 mg/kg/ day	50 weeks	Oral ingestion	Male Japanese quails (<i>Coturnix japonica</i>) (n = 47, 23–24 per group)	Decreased plasma testosterone levels No alterations on the testis were observed	(Ruuskanen et al., 2020)
Roundup®	1 mg/L	1–3 h	Diluted in buffer solution	Human spermatozoa (n = 66)	Decreased sperm progressive motility Decreased mitochondrial function	(Anifandis et al., 2018)
Roundup®	1 mg/L	1 h	Diluted in buffer solution	Human spermatozoa (n = 30)	Decreased sperm progressive motility No DNA fragmentation was observed	(Anifandis et al., 2017)
Glyphosate Glyphonova® 450 Plus	2.5 or 25 mg/ kg/day 25 mg/kg/day	2 weeks	Oral gavage	Prepubertal male Sprague-Dawley rats (n = 40, 10 per group)	No effects on testis or testosterone production Upregulation of steroidogenic genes upon treatment with the Glyphonova® 450 Plus	(Johansson et al., 2018)
Roundup Original® Roundup Transorb® Roundup WG®	0.5, 1, or 5 mg/L of glyphosate equivalent	24–96 h	Diluted in water	Adult killifish (<i>Jenynsia multidentata</i>) (n = 50, 5 per group)	Roundup Transorb® increased ROS production Decreased sperm motility at all studied concentrations	(Sanchez et al., 2017)

Table A2.3. Effects of glyphosate and glyphosate-based formulations on male reproductive functions and transgenerational consequences (cont.).

Compound	Dose	Duration of treatment	Administration	Animal model	Main findings/outcomes	Reference
Glyphosate	5, 50, or 500 mg/kg/day	5 weeks	Oral gavage	Adult male Sprague-Dawley rats (n = 32, 8 per group)	Decreased sperm production at highest concentration No alterations at serum testosterone and estradiol levels No alterations on oxidative stress biomarkers	(Dai et al., 2016)
Glyphosate	5 or 10 mg/L	24–48 h	Diluted in water	Adult male zebrafish (<i>Danio rerio</i>) (n = 81, 27 per group)	Decreased sperm motility Decreased mitochondrial functionality, membrane and DNA integrity at the highest concentration	(Lopes et al., 2014)
Roundup Grand Travaux Plus®	0.5% in water	8 days	Oral ingestion	Adult Sprague-Dawley rats (n = 30, 15 per group)	Increased aromatase expression Alterations in the blood-testis barrier Higher percentage of sperm with abnormal morphology	(Cassault-Meyer et al., 2014)
Roundup Transorb®	50 mg/kg/day	Gestational day 18 to post-natal day 5	Oral gavage	Pregnant Wistar rats (n = 12, then 8 pups per dam)	Increased serum testosterone and estradiol in the male offspring Increased serum LH in the male offspring Increased total sperm production	(Romano et al., 2012)
Roundup Transorb®	5, 50 or 250 mg/kg/day	30 days	Oral gavage	Prepubertal Wistar rats (n = 68, 16–18 per group)	Dose-dependent delay on puberty onset Dose-dependent reduced testosterone production Alterations in seminiferous tubules morphology	(Romano et al., 2010)
Roundup®	5 or 100 mg/kg/day	15 days	Oral gavage	Adult drakes (<i>Anas platyrhynchos</i>) (n = 18, 6 per group)	Dose-dependent decrease in plasma testosterone and estradiol levels Altered androgen receptors expression Morphological alterations in the seminiferous tubules, proximal efferent and epididymal ducts	(Oliveira et al., 2007)

There has been evaluation of different Roundup® formulations and treatments with 0.5 mg/L was sufficient to induce a lesser sperm concentration and motility in the fish *Jenynsia multidentata*. Interestingly, toxicity varied between formulations, which highlights that other components than glyphosate are also responsible for these harmful effects (Sanchez et al., 2017). There are findings that indicate other compounds present in Roundup® exacerbate the potential harmful effects. There was a lesser sperm motility in a concentration-dependent manner, increased plasma membrane disorganization, and decreased phosphorylation of glycogen synthase kinase 3 beta (GSK3 β) and two protein kinase A (PKA) substrates when boar spermatozoa were treated with Roundup® (0.01%, 0.005%, or 0.0025%) or equivalent concentrations of the surfactant polyoxyethyleneamine, but not with equivalent concentration of glyphosate (Torres-Badia et al., 2021).

Compelling evidence also highlights that glyphosate-based herbicides are harmful to human spermatozoa. Direct treatment of human spermatozoa with Roundup® (1 mg/L) resulted in lesser sperm progressive motility when there was 1 h of incubation. In addition, there was mitochondrial dysfunction as a result of this treatment (Anifandis et al., 2017). In another study conducted by the same group there was also an effect of this treatment on sperm progressive motility, however, there was no effect on DNA fragmentation (Anifandis et al., 2018). In some studies, however, there was some distinct results. When there was oral ingestion of glyphosate (50 mg/kg/day) for 2 weeks in Sprague-Dawley rats, there were no effects on the testis or androgen concentrations. In addition, there was no indication that glyphosate was an EDC (Johansson et al., 2018). Consistent with these results, glyphosate (5–500 mg/kg/day for 5 weeks) had no effects on the serum concentration of testosterone and 17 β -estradiol or oxidative stress in adult Sprague-Dawley rats, although total sperm production was less when there was administration of the largest glyphosate concentration (Dai et al., 2016). Male Japanese quail (*Coturnix japonica*) were treated with Roundup Flex® (160 mg/kg/day) from 2 to 52 weeks subsequent to hatching and testosterone concentrations were less but there were no adverse effects on reproductive functions (Ruuskanen et al., 2020). Overall, there is a lack of consensus and standardization of experiments, where not only the dose but also the route of administration and formulation are different among studies. In addition, in a large number of studies there were improper experimental designs, making it difficult for valid conclusions to occur.

A2.3. Metabolic regulators of spermatogenesis

A2.3.1. Functions of Sertoli cells on the metabolic regulation of spermatogenesis

In all vertebrate species, the success of spermatogenesis depends on the close association between the Sertoli cells and germ cells. The sperm production capacity of a specific species is mainly determined both by the number of Sertoli cells in the testis and number of germ cells supported by each Sertoli cell. Hence, the establishment of the mature Sertoli cell populations is an important aspect of male fertility. In most mammals, Sertoli cell proliferation starts during fetal development and extends until puberty. Postnatal Sertoli cell division differs among species. In rats, sheep, and cattle, most mitotic activity occurs during fetal life; in rams, bulls, pigs, and humans, there is a secondary increase in Sertoli cell proliferation before puberty (Franca et al., 2000; Hochereau-de Reviers et al., 1987). During the peripubertal period, Sertoli cells mature, and there is establishment of the Sertoli cell barrier (the most important component of the BTB), and new functions develop that are supportive of the development of germ cells (Hochereau-de Reviers et al., 1987; Lara et al., 2020; Lara et al., 2018).

Spermatogenic germ cells are dependent on Sertoli cells for necessary nutrients and metabolic intermediates for development. This process is essential for the spermatogenesis and the lack of adequate energy metabolites leads to germ cell degeneration and apoptosis (Erkkila et al., 2002; Jutte et al., 1981). Synergistic metabolic functions among testicular cells is essential for spermatogenesis. In addition to the physical and nutritional support, Sertoli cells also secrete a variety of other molecules necessary to germ cell development, including hormones, peptides, vitamins, and metal ions (Alves et al., 2013; Mruk & Cheng, 2004). Adjacent Sertoli cells form tight junctions, creating the BTB, which physically separates the seminiferous epithelium into the basal and adluminal compartment (Mruk & Cheng, 2015). The BTB allows there to be a protected seminiferous tubule milieu in the adluminal compartment that is conducive for development of germ cells, and the composition of this milieu is precisely regulated by Sertoli cells. Germ cells located inside the BTB rely on the production of metabolites (Jutte et al., 1982) and selective transport of substances through the Sertoli cells (Setchell, 2009).

Sertoli cells have a Warburg-like metabolism, in which only 25% of the pyruvate produced from glucose is oxidized as a result of Krebs cycle processes (Grootegeod et al., 1986). This peculiar aspect of Sertoli cell metabolism is essential for the maintenance of spermatogenesis because it allows for the production and secretion of large quantities of lactate, the central substrate for energy production in spermatocytes and spermatids

(Grootegeod et al., 1986; Oliveira et al., 2015) (Fig. A2.3). Glucose is an essential substrate for spermatogenesis in vivo (Zysk et al., 1975). It, however, is present in the seminiferous tubular fluid in very small quantities, probably due to the rapid metabolizing to a variety of subproducts. Although always in small quantities, the ratio between glucose concentration in the tubular fluid and the plasma differs between mammalian species, ranging from less than 2% to about 75% (Robinson & Fritz, 1981). Glucose is the primary source for lactate production by the Sertoli cells (Jutte et al., 1982). Glucose uptake is the first of a series of processes involved in lactate production and is mediated by the high-affinity glucose transporter (GLUTs) isoforms, GLUT1 and GLUT3, which are present in the Sertoli cell plasma membrane (Burant & Davidson, 1994; Galardo et al., 2008; Ulisse et al., 1992). Glucose is subsequently metabolized to pyruvate in the cytoplasm as a result of aerobic glycolytic processes. The resulting pyruvate can be further metabolized in three pathways: conversion to lactate (1) or alanine (2) in the cytoplasm, or be transported into the mitochondria and converted to acetyl-CoA (3) (Rato et al., 2012).

In Sertoli cells, the vast majority of pyruvate produced as a result of glycolytic pathway processes is metabolized resulting in the production of large quantities of lactate, which are exported to the seminiferous tubular fluid through the monocarboxylate transporter 4 (MCT4) (Galardo et al., 2007). Lactate is subsequently transported into the developing germ cells as a result of the MCT2 transporter where there is utilization for metabolic processes (Boussouar et al., 2003). Other than lactate, Sertoli cells also produce large quantities of acetate, which might be utilized by germ cells to sustain lipid metabolic processes, including lipid synthesis (Alves et al., 2012). Under unfavorable conditions, Sertoli cells adapt metabolically to ensure adequate lactate production for the support of spermatogenesis. Sertoli cell viability is maintained and lactate production occurs when glucose concentrations are low or there is no glucose present. When there are these circumstances, there is an increased abundance of GLUT1 and MCT4 in Sertoli cells, to maximize glucose intake and lactate secretion (Galardo et al., 2007; Riera et al., 2009). Even though there is a large amount of glycolytic activity, glucose is not the main substrate utilized for ATP synthesis in Sertoli cells. Most of the energy transformed by Sertoli cells into ATP comes from lipids derived from apoptotic germ cells and residual bodies, through the oxidation of fatty acids. Briefly, upon entering the mitochondria, fatty acids are metabolized to acetyl-CoA (via β -oxidation), and acetyl-CoA is subsequently processed in the Krebs cycle and oxidized to CO_2 . The electrons produced during these metabolic processes (β -oxidation and Krebs cycle) are transferred to the electron transport chain, resulting in the synthesis of ATP (Xiong et al., 2009). Additionally, there can be utilization of several amino acids by Sertoli cells, including glutamine, glycine, and branched-chain amino acids, and also glycogen to ensure

adequate nutritional support for germ cell development and for sustaining of metabolic processes (Kaiser et al., 2005; Rato et al., 2015; Slaughter & Means, 1983).

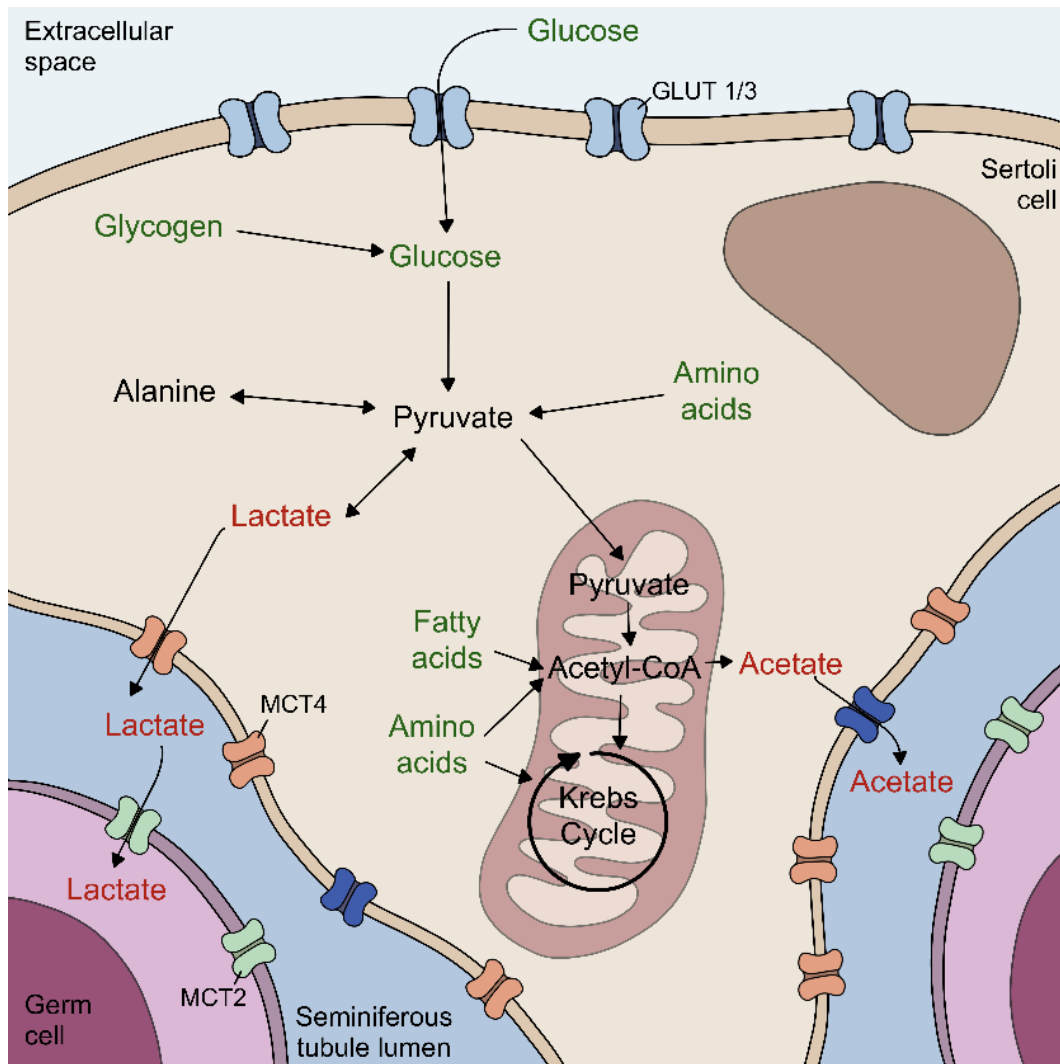


Fig. A2.3 – Metabolic pathways of Sertoli cells. Glucose enters the Sertoli cell through the high-affinity glucose transporters (GLUTs) isoforms GLUT1 and GLUT3. Inside the cytoplasm, glucose is converted to pyruvate, as a result of aerobic glycolytic processes. Pyruvate can then be metabolized to (1) lactate, (2) alanine or (3) be transported into the mitochondria to be oxidized to acetyl-CoA. In Sertoli cells, most of the glucose is converted to lactate, which is released into the seminiferous tubule lumen, by the monocarboxylate transporter (MCT) 4. Lactate is taken up by the developing germ cells, via the MCT2, to meet energetic requirements. Sertoli cells also secrete acetate to the seminiferous tubule fluid, which might be used by germ cells to support their lipid metabolism. Additionally, Sertoli cells metabolize several amino acids, glycogen, and fatty acids to sustain metabolism and meet energetic requirements.

A2.3.2. Metabolic needs of germ cells and spermatozoa

Spermatids, which are located at the adluminal compartment, have the greatest Krebs cycle activity of all germ cells and are dependent upon the lactate produced by the Sertoli cells for ATP production (Bajpai et al., 1998). The utilization of lactate for germ cell development is not only for energy production. Lactate is also known to regulate germ cell survival (Erkkila et al., 2002) and has protective effects in germ cells in vivo (Courtens & Ploen, 1999). Furthermore, lactate modulates the activity of various biosynthetic pathways in germ cells, such as the biosynthesis of RNA and proteins (Jutte et al., 1981; Nakamura et al., 1981).

Spermatozoa have metabolic flexibility and can utilize a wide variety of substrates to maintain metabolic functions. Also, metabolism is compartmentalized because oxidative phosphorylation is restricted to the midpiece and glycolysis mainly occurs in the fibrous sheath of the principal piece, although there are smaller quantities of glycolytic enzymes in the sperm head (du Plessis et al., 2015; Ferramosca & Zara, 2014). Hence, spermatozoa can use a variety of metabolic pathways for energy production, with the predominant energy source being dependent on animal species. Spermatozoa are known to have the greatest glycolytic activity and the least Krebs cycle activity of all germ cells (Bajpai et al., 1998). Nevertheless, oxidative phosphorylation is thought by some to be the most important source of energy transfer in some species due to large yield of ATP molecules per glucose molecule metabolized (du Plessis et al., 2015). Most of the ATP utilization occurs at the dynein ATPases located in the flagellum, which allows for sperm motility. Even though some species have a system to transfer high-energy phosphate from the mitochondria to the principal piece, this shuttle system is poorly developed or absent in most mammals. Glycolysis occurring in the principal piece, therefore, is thought to sustain sperm motility (Travis et al., 2001). Glucose and fructose are the main substrates for energy transfer in spermatozoa (Bajpai et al., 1998). Nonetheless, spermatozoa also have the capacity for metabolizing fatty acids, glycerol, and even glycogen to meet metabolic requirements (Ballester et al., 2000; Jones & Bubb, 2000; Mohri & Masaki, 1967; White, 1957).

The preferred pathway for sperm energy transfer apparently varies among animal species. These differences might be related to different phenotypes of sperm from different species. For example, boar spermatozoa have little motility and have a short period of survival subsequent to ejaculation and there is mostly glycolytic processes for energy transfer, whereas dog spermatozoa have rapid motility and a longer survival period subsequent to ejaculation with there being primarily anabolic metabolism, possibly the pentose phosphate pathway for energy transfer (Ferramosca & Zara, 2014; Marin et al., 2003). Furthermore, the metabolic flexibility of spermatozoa is essential for oocyte fertilization because sperm adapt to survive in the different milieu present in the female reproductive tract. The energy

production pathway might be determined by the conditions in the oviduct of the female, because in the cow oviduct there are small concentrations of glucose (ranging from 50 to 100 μM) (Carlson et al., 1970), whereas the concentration of glucose in the mouse oviduct is much larger (ranging from 3 to 4 mM) (Gardner & Leese, 1990). It appears that the functionality of metabolic pathways of spermatozoa varies from species to species and may be dependent upon the nutrients and conditions in the female reproductive tract (du Plessis et al., 2015; Storey, 2008).

A2.4. Epigenetic regulators of spermatogenesis

A2.4.1.Rediscovery of Lamarck’s principles

At the beginning of the 19th Century, the French naturalist Jean-Baptiste Lamarck presented the first systematic Evolution Theory of the History of Biology (Lamarck, 1809). Lamarck’s views on the Evolution of the Species, however, were later supplanted by the Law of Natural Selection proposed by Charles Darwin (Darwin, 1859). The inheritance mechanisms studies performed by Gregor Mendel (Bateson, 1913; Mendel, 1866) would help popularize Darwin’s finding and these principles were the foundation principles for artificial selection of animal characteristics. Lamarck’s principles, however, were never abandoned, notably as a result of the studies of Waddington, who even presented the first definition of “epigenetics” of the molecular biology era (Waddington, 1939). There is considerable evidence that the DNA sequence, the genome, could not explain all the traits of an organism because of the cell diversity of pluricellular organisms. If all somatic cells in an organism have the same genome which is clonally replicated during meiosis, the phenotype must be changed by other molecular mechanisms besides the changes in DNA sequence (Felsenfeld, 2014). This premise led to research in the processes of cellular differentiation and gene expression, eventually leading to the discovery of important epigenetic mechanisms, such as DNA methylation and chromatin condensation (Deans & Maggert, 2015) (Fig. A2.4).

These principles would set the first modern definition of “epigenetics” (Holliday, 1994; Riggs et al., 1996), and, consequently, Lamarck’s Principles continue to be relevant in the 21st Century. A definition of “epigenetics” and “epigenetic factors” has not reached a consensus among scientists, and is often overstated (Greally, 2018). One of the critical aspects of this definition is the inheritance of epigenetic changes in the structural configuration of DNA, a central aspect for neo-Lamarckists. The inheritance as a result of epigenetic factors

that can be ascertained by evaluating environmentally-induced markers could support Lamarck's Inheritance of Acquired Traits. This hypothesis led to a new field of study – Transgenerational Epigenetic Inheritance. The recent expansion of this subject, its historical context within epigenetics and implications have been extensively reviewed (Cavalli & Heard, 2019; Crisostomo et al., 2021b; Villota-Salazar et al., 2016). In this section of this review article, there is a narrow scope of evaluation of epigenetics that being the epigenomic changes that occur in the germline and during embryonic development, and how these changes may be applied in mammalian breeding.

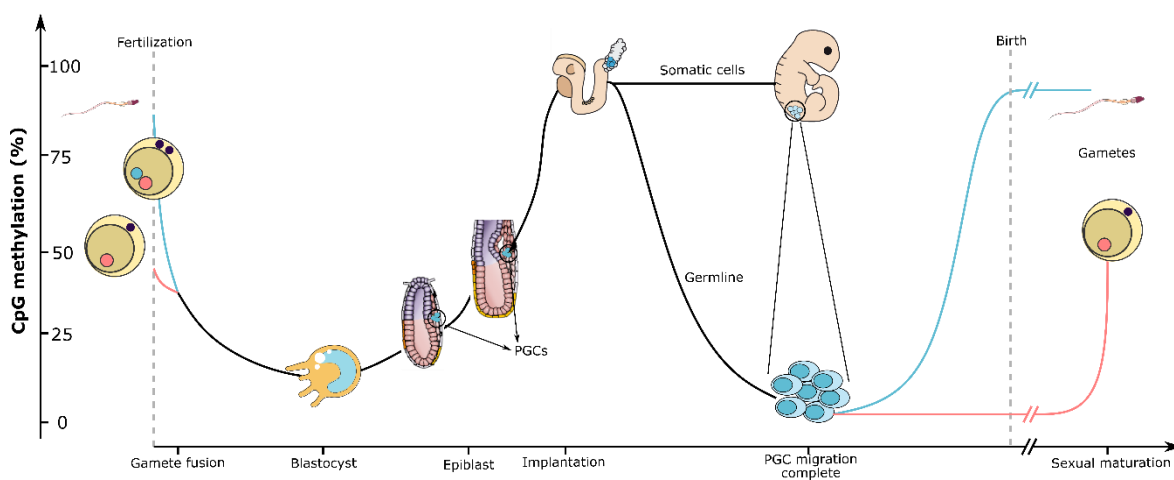


Fig. A2.4 – Timeline of the major demethylation processes in mammals. Before fertilization, gamete DNA is at in the most methylated state ($\approx 85\%$ spermatozoon, $\approx 40\%$ oocyte). After fertilization, DNA is rapidly demethylated, a process that continues even after gamete fusion and during the first ovum divisions. When the embryo developed to the blastocyst stage, DNA methylation is at a minimum ($\approx 15\%$). Due to “genomic imprinting”, some loci are not demethylated and so DNA demethylation is not complete. Embryo DNA is remethylated during the epiblast phase, as tissues start to differentiate. During this phase, the cells that will potentially become gametes start to differentiate – the primordial germ cells (PGCs). Soon after embryo implantation, embryonic cells are in a maximally DNA methylated state ($\approx 90\%$). Somatic cells will not be further demethylated, whereas PGCs start migrating towards the reproductive ridge and undergo additional DNA demethylation with there being a lesser minimum ($\approx 7\%$). After PGC migration is complete, in a female embryo, PGCs differentiate into oogonia and are clonally expanded to produce the future ovarian reserve. Oogonia remain with the same DNA extent of methylation as PGCs until there is entry into the ovarian cycle, after sexual maturation. In male embryos, PGCs differentiate into spermatogonia and there is extensive DNA methylation before birth ($\approx 85\%$).

A2.4.2. Twins who are not: epigenetics and animal husbandry

Epigenetic factors can define the phenotype of genetically similar mammals, such as the inbred rodent strains used in scientific research. One of the earliest known examples of epigenetic inheritance is the Agouti gene in mice. The Agouti gene is responsible for the fur pigment pattern commonly known as “agouti”, which is the wild-type fur color for several mammals. This coloration results from yellow and black pigment bands overlaid in a single hair, whence changing color according to the angle of the observer and the animal’s pattern of movement. The Agouti gene contains several hair-cycle-specific promoters and non-coding exons, which induce the switch between the production of the black or yellow pigment by the melanocytes. Four co-dominant alleles of the Agouti gene, however, contain an upstream intra-cisternal A particle retrotransposon that when activated induces the expression of the yellow pigment independently of the hair growth cycle (Morgan et al., 1999). Mice with this retrotransposon are considered “pseudoagouti” because their fur color can range from being completely yellow to completely “agouti” depending on the methylation state of the retrotransposon (Argeson et al., 1996). Intrauterine environment and body weight was suggested to regulate the activity of the retrotransposon; yet, it also induced ectopic expression of the Agouti gene, causing confounding pleiotropic effects such as increased susceptibility to obesity, diabetes and tumors (Duhl et al., 1994). Morgan et al. have rebuked the intrauterine environment hypothesis and reported that the “pseudoagouti” phenotype was caused by an epiallele – an allele with a specific methylation pattern – inherited from the “pseudoagouti” ancestor. This methylation pattern was preserved through the germline “epigenetic reset” and could be inherited from either the female or the male progenitor; which could express the “pseudoagouti” phenotype if the other inherited allele were the wild-type Agouti or the recessive “all black” allele (Morgan et al., 1999).

In 1996, the first mammal generated by somatic-cell nuclear transfer (SCNT) was born (Campbell et al., 1996). “Dolly”, the sheep, which was a landmark in genetics and reproductive sciences, but it also illustrates the importance of epigenetics in embryo viability and development of the organism. Even though Dolly resulted from a normally developed embryo and there was a typical postnatal development pattern for “Dolly”, she died at a relatively young age of six, due to a lung infection which, according to the team who cloned her, had no connection to the cloning technique (Sinclair et al., 2016). More than 20 other species have been successfully cloned after the sheep cloning was reported, but developmental abnormalities and shorter lifespan often occur (Long et al., 2014). Besides, the efficiency of the SCNT is small with only 5–10% of SCNT-derived embryos resulting in viable offspring (Long et al., 2014). Because there are no gametes involved in SCNT, any organism produced by conducting this technique has the same genome as the nucleus donor, at the time

of cell collection. This means that this offspring has 1) the same telomere length (Shiels et al., 1999a; Shiels et al., 1999b) and 2) the same epigenome (Long et al., 2014). In subsequent studies, there has been confirmation of the (Akagi et al., 2011; Doherty et al., 2002; Seisenberger et al., 2013), aberrant gene imprinting (Santos et al., 2003) and dysregulation of histone modifications (Akagi et al., 2011; Lee et al., 2011) that are associated with SCNT-derived embryos.

A2.4.3. Increasing importance of epigenetic factors in livestock management and breeding

Epigenetics has the potential to revolutionize practices in livestock management. Surprisingly, few studies on the inheritance of acquired traits have been conducted and in even fewer there have been descriptions of epigenetic mechanisms (Feeney et al., 2014; Gonzalez-Recio et al., 2015). In intensive farming enterprises, cows are often lactating while being pregnant. Gonzalez-Recio et al. reported that this long-standing and unethical practice results in less efficient dairy cows. They have observed that female calves born from lactating cattle had a shorter lifespan, produced less milk with a lesser nutritional value than calves produced by non-lactating dams (Gonzalez-Recio et al., 2012). Although there was not characterization of any epigenetic mechanisms, previous results suggest that methylation of CpG islands in the upstream promoter of α S1-casein gene in the mammary epithelial cells decreases milk production (Singh et al., 2010; Singh et al., 2008). Yet, this effect is related to a greater extent by a direct action on the embryo rather than an effect on an ancestor of the embryo.

Epigenetics has been studied in pigs due to their physiological similarities with humans, especially with regard to environmental effects on gene expression (Feeney et al., 2014). There have been reports of the effects of sulforaphane on muscle growth (Fan et al., 2012). In this study, this naturally occurring vegetable compound inhibits myostatin gene expression in cells of pigs by the inhibition of histone deacetylase. Myostatin inhibits muscle and bone growth in mammals (McPherron et al., 1997), thus inhibition of myostatin gene expression may promote muscle growth, increasing meat production. Pigs have also been used to study germline cell epigenetic programming. In pigs, the major epigenetic programming has been reported to occur in primordial germ cells during embryonic development (Hyldig et al., 2011).

The few studies addressing epigenetic regulation in spermatogenesis, in mammals, have been mostly focused on the inheritance of acquired traits or transgenerational epigenetic effects in rodents. Compelling evidence highlights that EDCs alter the epigenome

leading to transgenerational effects and disease (reviewed by Skinner (2014)Skinner (2014)Skinner (2014)Skinner (2014)Skinner (2014)Skinner (2014)Skinner (2014)Skinner (2014)). Interestingly, in some studies EDCs had specific transgenerational effects on the endocrine and reproductive function. Anway et al. reported that the transient treatment of female rats with vinclozolin or methoxychlor (antiandrogenic and estrogenic compounds, respectively) during the gonadal sex determination period of the gestational period led to a decreased spermatozoa number and viability on the male offspring (Anway et al., 2005). Alarming, this damage was correlated with alterations in the DNA methylation patterns and persisted through all the generations in which evaluations occurred (total of four generations), which were sub-fertile or infertile. In pregnant female mice administered BPA orally (50 µg to 50 mg/kg/day) from day 7–14 of the gestational period, the larger doses of BPA led to disrupted testicular germ cell organization and impaired spermatogenesis with decreased sperm count, motility, and fertility in both the F1 and F2 generation but not the F3 generation of offspring. These alterations were associated with alterations in the sperm DNA methylation patterns (Rahman et al., 2021). There have been few studies conducted regarding epigenetic effects during spermatogenesis and further research is needed to clarify transgenerational alterations in spermatogenesis. Some other studies also focused on other EDCs and pollutants which have been associated with an epigenetic fingerprint of germline cells (Guerrero-Bosagna et al., 2012; Guerrero-Bosagna et al., 2010; Manikkam et al., 2013; Skinner et al., 2018; Skinner et al., 2013). Although these studies were not focused on the epigenetic control of spermatogenesis, the results from these studies highlight the persistence of the epigenetic fingerprints due to environmental factor effects on ancestors for as many as three generations, the transmission via the male lineage, and defects in sperm functions and/or morphology.

The association between nutritional state and reproductive function has also been widely suggested (Ashworth et al., 2009; Crisóstomo et al., 2018; Moreira et al., 2017; Oliveira & Alves, 2015). This association has been studied in the context of animal production to hasten sexual maturity and improve the breeding of commercially valuable species. An interesting and recent study in this area has been performed in fallow deer (Ros-Santaella et al., 2019). In this study the researchers supplemented pasture with barley to advance sexual maturation of deer by one breeding season, comparing to littermates maintained on pastures without there being dietary concentrate supplementation. Such differences in timing of sexual maturation represent a major improvement for the economic value of the species (Ros-Santaella et al., 2019). Also, premature sexual maturation had no negative effect on sperm quality or the viability of the animals, although there was no follow-up on their health after the breeding season and on their offspring. Results from a series of studies have indicated there is an association between the feeding of a diet with a large

quantity of fat and lesser sperm quality in rodents (Crisostomo et al., 2019; Crisostomo et al., 2020). These findings in these studies have allowed for the description of a testicular metabolic fingerprint and abnormal lipid composition after the feeding of a diet with a large fat content. Interestingly, the switch to a diet that did not contain the large quantity of fat, during the early post-pubertal period, did not result in restoration of testicular metabolism, lipid composition and values for sperm variables, indicating there is a critical developmental period when male germline cells are most susceptible to environmental factors. In another study, results were considered to indicate there was a testicular “inherited metabolic memory” when there was feeding of ancestors of offspring a diet with large quantities of fat. The feeding of this diet was associated with lesser values for sperm variables for several generations, especially in the male grand-offspring of the mice fed the diet with a large quantity of fat (Crisostomo et al., 2021a). Nevertheless, while these studies indicate a potential epigenetic inheritance via the male gamete, no epigenetic mechanisms for these effects have been described.

A2.5. Conclusion and future perspectives

Farming and animal breeding have been integral for development of modern societies in past millennia and our modern communities would not have been possible without the first farming communities having been established during the Neolithic period (Nikitin et al., 2019). Farming and breeding practices have changed markedly throughout historical periods, but the goals have not changed - optimize the yield from natural resources to feed the largest number of people. The greatest challenges of farming have not changed either - maintaining profitability for the farmers, providing reliable food products to the consumers and being sustainable from the perspective of natural resources. Our increasing scientific knowledge has allowed humankind to advance all those aspects of farming but, notably after World War II, the balance between profit, yield, and sustainability has been disrupted. Spermatogenesis is a central aspect of farming: this biological process is essential for renewing animal resources (as part of Reproduction Sciences). Progress for farmed species would not be complete without the artificial selection of animals, selecting for those characteristics that better fit the needs of food and fiber of the animal producers and, ultimately, of the consumers of these products. The knowledge of biological processes is foundational for the empiric farming practices, but there remains a lack of understanding of how some characteristics are inherited. Also, the knowledge base is just starting to be developed about how the environment factors that are often controlled by farmers modulate gene expression and, therefore, animal performance.

The latest advances in the molecular biology of spermatogenesis have resulted in an enhanced understanding of how there were previously unknown negative effects of present farming and breeding practices on the reproductive efficiency of livestock. This recently developed knowledge base has also enhanced the awareness about the environmental (epigenetic) information transmitted to the offspring via the paternal lineage, that can have detrimental effects on the quality of the meat used for human consumption for several generations. Although this knowledge could be transferred to farming practices immediately, more research is needed to improve the understanding of the environment-spermatogenesis interaction to implement preferred practices and re-establish the profit, yield, and sustainability in farming.

A2.6. References

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A3. Signatures of metabolic diseases on spermatogenesis and testicular metabolism

Abstract

Diets leading to calorie overload are linked to metabolic disorders and reproductive function impairment. Metabolic and hormonal abnormalities stand out as defining features of metabolic disorders, exerting a significant impact on the functionality of the testis. This review will delve into the effects of high-fat diets and metabolic disorders on testicular metabolism and spermatogenesis, crucial elements for male reproductive function. It will particularly emphasize the intricate mechanisms through which metabolic disorders instigate testicular metabolic dysfunction, chronic inflammation, and oxidative stress. The disruption of gastro-intestinal, pancreatic, adipose tissue, and testicular hormonal regulation can also contribute for the state of compromised fertility. Recent studies underscored the influence of metabolic disorders on the epigenome of male gametes and its potential role at transmitting phenotype traits across generations. In the pursuit of truth, the well-known saying “We are what we eat” might now be expanded to include “We are what we eat and our ancestors ate”. Notwithstanding, the existing evidence strongly underscores the unmet need of further understanding of the mechanisms responsible for transgenerational paternal inheritance of male reproductive function impairment related to metabolic disorders. This knowledge could be useful for devising targeted interventions to prevent, counteract, and most of all break the perpetuation chain of male reproductive dysfunction associated with metabolic disorders across generations.

A3.1. Introduction

The past few decades have seen remarkable technological, economic, and social progress that has significantly altered the lifestyle of modern societies. The availability of electricity has led to the emergence of new career opportunities and increased flexibility in work schedules. Simultaneously, the popularity of motor vehicles and a wide range of services has led to a more sedentary lifestyle. In response to the fast-paced and stressful urban environment, traditional carbohydrate and fiber-rich diets have been replaced by calorie dense foods often rich in refined sugars and fats. Regrettably, this trend has contributed for the upswing of metabolic disorders like overweight/obesity, type 2 diabetes, and cardiovascular diseases, which exhibit a positive correlation with the index of social development (Saklayen, 2018). According to the World Health Organization, the prevalence of worldwide obesity nearly tripled between the years 1975 and 2016 (WHO). Moreover, as high-income countries have disseminated this modern lifestyle to middle- and lower-income countries,

the prevalence of obesity and other metabolic disorders has tended to grow exponentially (WHO). However, it is important to acknowledge that the swift rise in the incidence of metabolic disorders is not solely attributable to the lifestyle of modern society. While a high-calorie diet and a sedentary lifestyle are significant factors, metabolic disorders have a multifactorial etiology that includes genetic alterations and predisposition. In recent decades, several genetic mutations have been linked to the development of metabolic disorders. For example, mutations in genes associated with circadian rhythm regulation have been shown to cause a wide range of metabolic abnormalities (Rudic et al., 2004). It is also worth mentioning monogenic obesity, a disorder caused by a single defective gene on the autosomes, often related to the regulation of the leptin/melanocortin pathway, a condition that leads to a phenotype of overweight/obesity (Huvenne & Dubern, 2014). Additionally, various genes associated with the risk of obesity development, commonly known as obesity-related genes (ORG), have been identified. These encompass the fat mass and obesity gene (*FTO*), transmembrane protein 18 (*TMEM18*), glucosamine-6-phosphate isomerase 2 (*GNPDA2*), and melanocortin 4 receptor (*MC4R*) (Hotta et al., 2009; Leon-Mimila et al., 2013).

Alarming, the number of children and young adults with metabolic disorders is rapidly increasing, and this escalating trend is poised to yield significant adverse health consequences in the near future (Oliveira et al., 2017). Metabolic imbalance during childhood development can result in severe and enduring consequences that persist into adulthood. One often overlooked and silent comorbidity of metabolic disorders is the impairment of the endocrine and reproductive systems. Clinical and experimental evidence demonstrates a clear negative association between obesity and healthy male reproductive function. It is estimated that approximately 20% of male subfertility and infertility cases are directly linked to overweight and obesity (Du Plessis et al., 2010; Liu & Ding, 2017). The most commonly reported effects are related to poor sperm parameters. In a study conducted by Chavarro et al. in 483 subfertile couples, it was reported that overweight men defined by a body mass index (BMI) higher than 25 kg/m² had lower sperm count when compared to normal-weight men (Chavarro et al., 2010). In support of these findings, a study on 1558 male military recruits reported a negative association between BMI and sperm count (Jensen et al., 2004). Stronger evidence was obtained in the meta-analysis performed by Sermondade et al., which reported that, in a sample of 13077 men attending fertility counseling, men with overweight and obesity had an increased prevalence of oligospermia or azoospermia (Sermondade et al., 2013). In addition, obesity was also associated with asthenozoospermia (low sperm motility) (Hammoud et al., 2008; Hofny et al., 2010; Wang et al., 2017). Interestingly, in non-Western countries where the prevalence of obesity is lower, the incidence of male infertility is also lower, providing additional epidemiological evidence on the association between obesity and healthy male reproductive function (Swan et al., 2000).

In addition to the negative consequences for male reproductive health, recent studies have highlighted that an abnormal metabolic state can imprint metabolic signatures in male gametes. The epigenetic imprinting induced by metabolic disorders has only recently begun to be unveiled, although there is already clear evidence that this will be a major topic of research and a challenge in the years to come (Pereira et al., 2020). It is estimated that in 2025 more than 300 million people will suffer from metabolic diseases and associated complications (Reuter & Mrowka, 2015). A disturbing yet crucial question has arisen: could the modern western lifestyle of fathers be jeopardizing the health of future generations? As the molecular mechanisms of transgenerational inheritance of metabolic disorders are being unveiled, it is becoming increasingly clear that protecting the health of future generations will require more than a simple change in individual lifestyle. It is essential to understand the molecular pathways through which metabolic diseases are inherited by offspring and to prevent the predisposition of individuals to develop such disorders. In this comprehensive review, we will delve into the mechanisms elucidating how the systemic metabolic and metabolic-related hormonal status in men intricately regulate testicular function and spermatogenesis. It will also discuss the effects of high-fat diet consumption and metabolic diseases on male fertility, which is often overlooked comorbidity. Additionally, we will discuss novel data concerning metabolic-related epigenetic signatures imprinted in spermatozoa and the evidence of paternal transgenerational inheritance of metabolic disorders.

A3.2. Effects of high-fat diets and metabolic disorders on testicular hormonal and metabolic regulation

The testes are very specialized organs where the production of spermatozoa is dependent on the metabolic state of the male individual. The somatic Sertoli cells (SCs) integrate many of these metabolic cues, which are later reflected in spermatogenesis due to its functions: nutritional support and formation of the blood-testis barrier (BTB) (Mruk & Cheng, 2004, 2015). The BTB plays a crucial role in promoting a controlled and immunologically safe environment for the developing germ cells, while also selectively allowing the passage of solutes and nutrients. SCs are responsible for forming the BTB and providing nutritional support to the developing germ cells. These cells also limit the expansion of the spermatogonia population, and each SC is able to support 30 to 50 germ cell (Alves et al., 2013; Weber et al., 1983). Besides the immunological and mechanical support, SCs metabolism represents a central role in spermatogenesis. The human germ cell line exhibits a unique metabolism, where lactate is used as the preferential energy source even though these cells possess glucose transporters (GLUTs) and the molecular machinery for glycolysis

(Kokk et al., 2004; Oliveira et al., 2012). To ensure that germ cells are supplied with the adequate lactate levels necessary for its correct development, SCs developed several metabolic strategies to prioritize the production of lactate above any other metabolic pathways. SCs present a “Warburg-like” metabolism, as detailed by (Oliveira et al., 2015). The high production of lactate by SCs starts with the uptake of glucose from the interstitial space by high-affinity GLUTs, mainly GLUT1 and GLUT3 (Oliveira et al., 2015). Glucose is then preferentially metabolized into pyruvate and then into lactate. Interestingly, the unique metabolism of SCs can be compared to cancer cells, although the high glycolytic rate is not associated with rapid energy production or cellular proliferation. As a matter of fact, the population of human SCs remains roughly the same throughout an individual’s life (Oliveira et al., 2015). The majority of lactate produced by SCs is necessary to fuel spermatogenesis, and thus, exported to the intratubular fluid of seminiferous tubules by the specific proton/monocarboxylate transporter 4 (MCT4). There, developing germ cells obtain lactate mainly through MCT2 and MCT1, which is once again converted to pyruvate, fueling its metabolic needs (Boussouar & Benahmed, 2004). SCs also export high amounts of acetate into the intratubular fluid. Although the exact role of acetate in spermatogenesis remains to be elucidated, it is proposed that this metabolite is used by germ cells during *de novo* lipid synthesis, which is in accordance with the high demand for lipids by these cells (Oliveira et al., 2015).

SCs play a critical role in the regulation of spermatogenesis, acting as both “nurse” cells and “gatekeepers” of the seminiferous tubules. SCs integrate metabolic and hormonal cues from the human body, which are then translated into the germ cell line. As a result, a complex network is responsible for regulating SCs’ metabolism, enabling it to adapt its metabolism based on the individual’s energy state. SCs exhibit high metabolic plasticity, allowing the continuous production and export of lactate by metabolizing a wide range of energetic substrates, even under abnormal metabolic conditions (Oliveira et al., 2015). In a shortage of glucose, SCs can use ketone bodies, fatty acids, and glycogen to keep up the metabolic chain and maintain lactate production (Alves et al., 2013). However, SCs functions are susceptible to high concentrations of circulating fatty acids, a common features associated with high-sugar and high-fat diets, as well as several metabolic disorders.

A3.2.1. How high-fat diets and metabolic disorders disrupt testicular metabolism

Human studies have provided further evidence that high-fat diets are associated with impaired male reproductive health. The association between dietary fatty acids intake and

asthenozoospermia (low motility) was studied by Eslamian et al. in 235 age-matched men. The authors observed that a high intake of total saturated fatty acids, total trans-fatty acids, palmitic acid, and stearic acid was positively associated with the incidence of asthenozoospermia (Eslamian et al., 2015). Interestingly, a high intake of omega-3 polyunsaturated, such as docosahexaenoic fatty acid, was regarded as protective against asthenozoospermia. These results are concordant with an earlier study conducted by Attaman et al., which reported that higher saturated fat intake was negatively correlated with total sperm count and concentration but omega-3 polyunsaturated fatty acids were correlated with improved sperm morphology (Attaman et al., 2012). However, while some evidence from human studies is available, the majority of data found in the literature was obtained using animal studies. Nevertheless, compelling evidence from these studies clearly demonstrates the negative effects of high-fat diets on male reproductive health. Fan et al. fed male C57BL/6 mice with a high-fat diet for 10 weeks and observed decreased total and progressive sperm motility, whereas morphological defects were increased (Fan et al., 2015). In addition, morphological analysis of the testes revealed an atrophic seminiferous epithelium and BTB disruption. Collectively, the fertility of these mice was significantly impaired, as evidenced by low pregnancy rates when mated to normal females. In support of these results, Ye et al. also observed the disruption of the BTB and impaired fertility in mice fed a high-fat diet for 8 weeks, which was ameliorated when mice were treated with metformin (Ye et al., 2019). In addition, a recent proteomics study conducted by Jarvis et al. on the testis of C57BL/6 mice fed with a high-fat diet reported altered expression of proteins related to BTB integrity, oxidative stress, and lipid homeostasis (Jarvis et al., 2020). The accumulation of lipids and the subsequent lipotoxicity in the testis are hypothesized to be associated with alterations in the BTB structure. Hu et al. fed male C57BL/6 mice with a high-fat diet for 16 weeks and observed decreased sperm concentration although no alterations in motility were noted (Hu et al., 2018). In addition, these authors reported lipid accumulation and increased reactive oxygen species (ROS) production in the testis, which was associated with decreased SCs number due to higher apoptotic rates. *In vitro* studies with the SC line TM4 and primary cultures of mouse SCs revealed that high concentrations of palmitic acid (0.1-0.8 mM) induced apoptosis in a time and dose-dependent manner (Hu et al., 2018). Contrastingly, omega-3 polyunsaturated fatty acids were associated with positive effects and protected SCs against palmitic acid-induced damage. Similar results were obtained by Luo et al., as these authors fed male Wistar rats with a high-fat diet and observed a decreased sperm concentration and decreased number of pups per litter (Luo et al., 2020). Then, Luo et al. exposed primary cultures of rat SCs to lower concentrations of palmitic acid (0.5 μ M), which also promoted intracellular lipid accumulation and increased β -oxidation, despite increased glycolytic rates and lactate production were observed (Luo et al., 2020).

The increase in β -oxidation was associated with increased ROS production and mitochondrial dysfunction, which led the authors to hypothesize that an insufficient energy supply for SCs would impair their function and, consequently, spermatogenesis. In a study by Crisóstomo et al., a different approach was taken to study the effects of high-fat diets. The authors investigated the effects of a transient high-fat diet then switched to a standard diet during early adulthood in C57BL6/J mice (Crisostomo et al., 2019). The study showed that although metabolic syndrome was reversed, and mice lost weight, exposure to a high-fat diet during pubertal development resulted in permanent deterioration of sperm parameters such as viability, motility, and morphology. Moreover, the metabolomics analysis of the testis showed that there were permanent alterations in testicular metabolome, which were not restored through diet switch or weight loss. In a follow-up study, these authors reported a permanent alteration in testicular lipid composition, even after diet correction (Crisostomo et al., 2020). More specifically, the authors reported that saturated fatty acids were the most abundant lipids in the testicular lipidome of the control and the transition diet group. For the group fed with a high-fat diet, polyunsaturated fatty acids were the most abundant lipids in the testicular lipidome. The high-fat diet and transitional diet groups also presented an increased abundance of monosaturated fatty acids, including an accumulation of oleic acid (Crisostomo et al., 2020). These authors further reported a strong correlation between the overaccumulation of proinflammatory omega-6 polyunsaturated fatty acids and poor sperm parameters. Taken together, a diet high in lipid content promotes lipid accumulation in the testis, which can negatively impact the metabolism, mitochondrial function, and mechanical function of supporting SCs by disrupting the BTB. In addition, lipid accumulation is highly associated with inflammation and increased oxidative stress, both of which are known to be primary contributors to male infertility. Hence, meticulous control of lipid intake through diet is imperative for sustaining a healthy male reproductive function.

A3.2.2. Testicular fat accumulation, inflammation, and oxidative stress trigger male reproductive dysfunction

Metabolic disorders cause lipid accumulation in the testis, which can have multiple detrimental effects. An abnormal distribution of scrotal fat can cover the veins and the spermatic cord, impairing testicular thermoregulation and resulting in a rise in scrotal temperature (Shafik & Olfat, 1981). This condition can negatively impact the development of germ cells, which are particularly vulnerable to heat stress (Zhang et al., 2012). Furthermore, the compression of cord veins by excess fat promotes testicular ischemia, further impairing repro-

ductive function (Shafik & Olfat, 1981). Additionally, obesity and the consequent accumulation of fat in the testis have also been positively linked to altered testicular morphology, including reductions in seminiferous epithelium height, diameter, and germ cell proliferation, which ultimately affect spermatogenesis (Campos-Silva et al., 2015). Finally, increased fat content promotes the accumulation of liposoluble toxic compounds, such as some pesticides that are absorbed through the diet and stored within fat droplets. Some of these compounds are known to be endocrine disruptors. The direct impact of pesticides on the male reproductive system has been previously discussed (for review please consult (Moreira et al., 2021)).

The increased accumulation of fat in adipose tissue is often accompanied by a state of chronic inflammation (Fig. A3.1). As an endocrine organ, adipose tissue secretes various inflammatory mediators in response to fat accumulation in adipocytes, predisposing a pro-inflammatory state (Coelho et al., 2013; Ellulu et al., 2017). Exacerbated levels of saturated fatty acids, such as palmitic acid, can also interact with cell pattern-recognition receptors, which are involved in the innate immune system and play a crucial role in the chronic inflammatory state observed in individuals with obesity and type 2 diabetes. This chronic inflammatory state can further decrease cells' sensitivity to insulin and leptin, leading to resistance (Konner & Bruning, 2011). Both a pro-inflammatory state and the accumulation of fat in the testis are inextricably linked to OS. OS and oxidative damage are significant contributors to male infertility, as germ cells have limited intracellular antioxidant defenses and are highly susceptible to ROS damage (Dutta et al., 2021; Sabeti et al., 2016). Clinical studies reported that 30% to 80% of infertile men have increased levels of ROS (Iwasaki & Gagnon, 1992; Shekarriz et al., 1995; Tremellen, 2008). OS is linked to disrupted spermatogenesis and alters sperm physiology. This can lead to lower sperm concentration, reduced motility, more abnormal shapes, and DNA damage (Agarwal et al., 2003; Aziz et al., 2004; Moustafa et al., 2004; Said et al., 2004). However, ROS play a dual role in spermatozoa function. During human spermatozoa capacitation, mitochondrial activity and ROS production increase significantly (Carrageta et al., 2020). At controlled levels, ROS are essential for spermatogenesis, sperm function, and fertilization, acting as a secondary messenger in several pathways, including spermatozoa capacitation, sperm-oocyte fusion, acrosome reaction, and sperm head decondensation (Carrageta et al., 2020; Du Plessis et al., 2015; Monteiro et al., 2021). As such, the regulation of ROS in the male reproductive system is currently a topic of intense research, with efforts focused on identifying compounds that can ameliorate ROS-induced damage and novel ROS-related molecular targets (Martin-Hidalgo et al., 2019). Recent studies have shed light on potential molecular targets that can address the intersection between metabolic disorders and male infertility. For instance, a promising target are mitochondrial uncoupling proteins (UCPs), which play a crucial role in regulating

ROS production and mitochondrial oxidative phosphorylation. Dysfunctional or altered UCP function is associated with the onset of metabolic disorders (Monteiro et al., 2021). Our group recently conducted a study that identified the expression of UCP1, UCP2, and UCP3 homologs in human spermatozoa. Remarkably, our findings showed that inhibiting UCPs using a selective inhibitor caused irreversible motility loss by impairing the glycolytic and mitochondrial metabolism of spermatozoa (Carrageta et al., 2023). This discovery underscores the potential of targeting UCPs in addressing male infertility associated with metabolic disorders. Overall, the consumption of high-fat diets and the incidence of metabolic diseases leads to an oxidative imbalance. However, these disorders are multifaceted and can affect every biological system, including endocrine signaling. This disruption of the endocrine system can have cascading effects on the reproductive system, impacting both metabolic and hormonal functions.

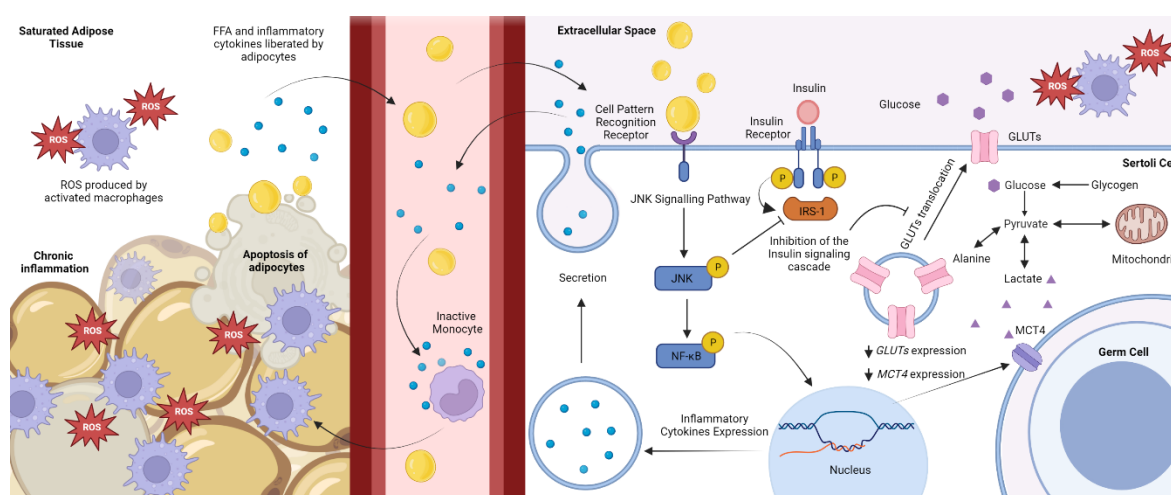


Fig. A3.1 – Schematic illustration of the chronic inflammation in the adipose tissue as a consequence of fat accumulation and its impact on testicular metabolism. The adipose tissue is an endocrine organ, capable of producing and releasing hormones and cytokines. Fat accumulation promotes resident macrophage activation and pro-inflammatory cytokines secretion, predisposing the adipose tissue to a chronic low-grade inflammatory state. Moreover, excessive fat accumulation also triggers the apoptosis of adipocytes, resulting in the increased release of free fatty acids and pro-inflammatory cytokines. Upon adipocyte apoptosis, a large influx of macrophages infiltrates the tissue, generating an inflammatory state and oxidative stress. In turn, increased circulating lipids are a known cause of insulin resistance, which further promotes the systemic inflammatory state and impairs testicular metabolism. *Abbreviations:* FFA – free fatty acids; GLUT – glucose transporter; IRS-1 – insulin receptor substrate 1; JNK – c-Jun N-terminal kinase; MCT4 – monocarboxylate transporter 4; NF-κB - nuclear factor kappa light chain enhancer of activated B cells; ROS – reactive oxygen species.

A3.2.3. Metabolic diseases, gut hormones, and testicular energy homeostasis: a vicious cycle of hormonal dysregulation

Testes are not only metabolically regulated but also hormonally through the hypothalamic-pituitary-gonadal (HPG) axis. In brief, the gonadotropin-releasing hormone (GnRH) is synthesized and released in pulsatile fashion by the hypothalamus, which stimulates the anterior pituitary to produce and release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both these hormones regulate the testicular function, although through different targets – LH stimulates steroidogenesis and the release of testosterone in Leydig cells; FSH modulates the function of SCs and spermatogenesis. In turn, testosterone and inhibin, produced by Leydig and SCs, respectively, regulate the activity of the hypothalamus-pituitary glands in a negative feedback mechanism (Clavijo & Hsiao, 2018).

In addition to the hormonal control via the HPG axis, the testes also respond to an individual's metabolic status through hormonal regulation. Thus, metabolic disorders severely impact male reproductive function by disrupting the HPG axis and causing hormonal dysregulation (Fig. A3.2). This disruption leads to the development of hypogonadism, particularly secondary hypogonadism due to low levels of circulating testosterone. In turn, the lower levels of testosterone also promote increased fatty acids uptake and adiposity. Taken together, male obesity and hypogonadism constitute a vicious cycle (Carrageta et al., 2019).

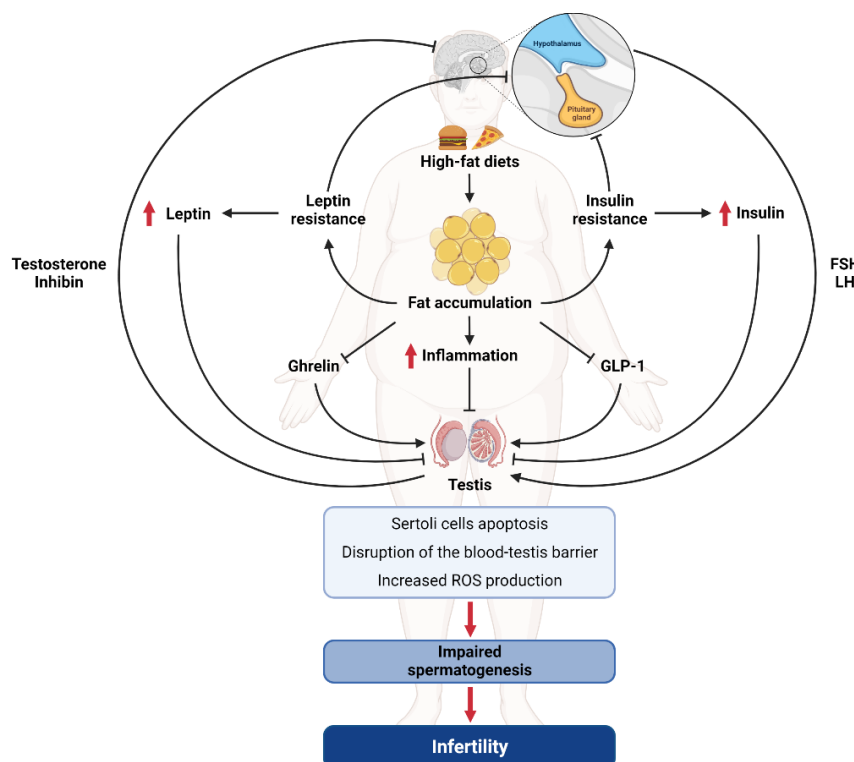


Fig. A3.2 – High-fat diet-induced metabolic and hormonal testicular dysregulation. The accumulation of fat induces hormonal dysregulation and chronic inflammation, which affects the testis

directly and indirectly through the disruption of the hypothalamus-pituitary-gonads (HPG) axis. Fat accumulation leads to leptin and insulin resistance, which impairs the release of gonadotropins by the hypothalamus-pituitary glands. Additionally, elevated levels of leptin and insulin are hypothesized to directly impair testicular function. In contrast, fat accumulation decreases the secretion of GLP-1 and ghrelin, two gut hormones that directly regulate testicular function. Overall, the testicular function is severely affected, resulting in impaired gamete production. *Abbreviations:* FSH – follicle-stimulating hormone; GLP-1 – glucagon-like peptide 1; LH – luteinizing hormone.

Gut associated and adipocyte hormones, which regulate food intake and energy balance, play a crucial role in this cycle. Among those hormones, ghrelin, glucagon-like protein 1 (GLP-1), insulin, and leptin are highlighted as particularly important for testis metabolism. As its receptors are widely expressed in brain and testicular cells, these hormones can coordinate both the metabolic state of the individual and the energetic demands of the reproductive system (Fig. A3.3). In a healthy individual, sex steroids, pituitary, gut, and adiposity signal hormones act together to maintain the energetic homeostasis of oneself (Oliveira et al., 2017). In the following sections, we will discuss how the imbalance of gut and adiposity signaling hormones can disrupt the energy balance in the testis, worsening the interaction between metabolic disorders and male infertility.

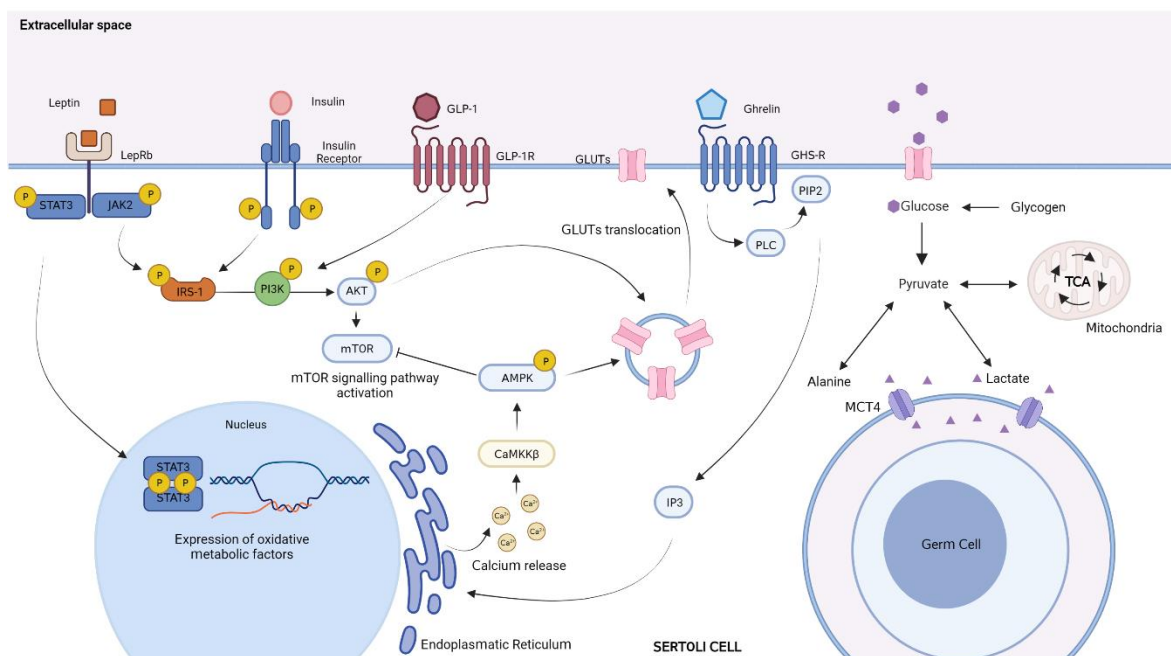


Fig. A3.3 – Putative signaling pathways involved in energetic regulation of human Sertoli cells (SCs) by gut and adiposity signal hormones. SCs are highly glycolytic, favoring the production of high quantities of lactate – the germ cell line preferential source of energy. For that purpose, SCs are able to orchestrate several metabolic and hormonal clues, indicating that the metabolic and hormonal

status of the individual are reflected in their reproductive health. Among the energy-related hormones, gut and adiposity signal hormones are known regulators of SCs metabolism, although the signaling pathways are not fully characterized. Among the gut hormones, glucagon-like peptide 1 (GLP-1) and ghrelin are known regulators of SCs function and metabolism. GLP-1 mediates its function through the GLP-1 receptor (GLP-1R), which is hypothesized to activate the phosphoinositide 3-kinases/ protein kinase B (PI3K/Akt) pathway. Ghrelin acts through the growth hormone secretagogue receptor (also known as ghrelin receptor, GHS-R). Ghrelin signaling is mediated through the phospholipase C (PLC)/inositol triphosphate (IP3) pathway, which stimulates the intracellular release of Ca^{2+} from the endoplasmic reticulum and the activation of the Calcium/calmodulin-dependent protein kinase kinase beta (CaMKK β). The later activates the AMP-activated protein kinase (AMPK). Among the adiposity signal hormones, leptin and insulin are the most widely studied. Leptin acts through the leptin receptor (LepRb), which canonically activates the janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway. However, leptin seems to modulate SCs function mainly through the insulin receptor substrate (IRS)/PI3K/Akt pathway, which is canonically associated with insulin and insulin receptor. All these pathways are hypothesized to promote the translocation of glucose transporters (GLUTs) into the plasma membrane, stimulating the glycolytic rate and controlling the supply of energy substrate for germ cells development. *Abbreviations:* MCT4 – monocarboxylate transporter 4; mTOR – mammalian target of rapamycin; PIP2 - Phosphatidylinositol 4,5-bisphosphate; TCA - tricarboxylic acid cycle.

A3.2.3.1. Gut hormones

Glucagon-like peptide 1 (GLP-1)

Glucagon -like protein 1 (GLP-1) is a gut peptide hormone encoded by the proglucagon gene and secreted into the bloodstream after tissue specific post-translational processing. GLP-1 has been extensively studied for its weight loss and glucose-lowering effects, as it plays a prominent role in regulating glucose homeostasis, gastrointestinal motility, and appetite. GLP-1 is released in response to the presence of nutrients, particularly sugars, and fats, in the small intestine, or through direct contact between nutrients and L-cells (Shah & Vella, 2014). The intrinsic significance of GLP-1 in orchestrating appetite regulation is widely acknowledged. GLP-1 mediates its function through the GLP-1 receptor (GLP-1R), which can activate different signaling pathways, including the phosphoinositide 3-kinases (PI3K)/ protein kinase B (PKB, also known as Akt), cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathways, depending on its target cell (Buteau et al., 2001; Rowlands et al., 2018; Smith et al., 2019). Within the pancreas, it triggers insulin secretion while curbing glucagon release through the activation of the cAMP/PKA pathway (Lester et al., 1997; Meloni et al., 2013). Simultaneously, within

the hypothalamus, GLP-1 fosters feelings of satiety, leading to a decrease in caloric consumption (MacDonald et al., 2002). Thanks to these attributes, GLP-1 analogs were formulated for effectively addressing diabetes and obesity (Hinnen, 2017).

Recent studies suggest that GLP-1 also plays a role in regulating the male reproductive system, although more research is needed to fully understand its mechanisms of action. In 2019, the GLP-1 receptor was first identified in human SCs. (Martins et al., 2019). Using primary cultures of human SCs, Martins et al. demonstrated that exposure to GLP-1 decreased glucose consumption and increased lactate production, indicating a shift in its metabolic activity (Martins et al., 2019). However, the alternative energetic source that fuels lactate production or the signaling pathways that mediate the action of GLP-1 remains to be identified in future studies. Additionally, some evidence suggests that GLP-1 may have an anti-apoptotic effect on developing germ cells (Martins et al., 2019). Recently, Rago et al. identified the GLP-1R in human spermatozoa (Rago et al., 2020). These authors treated human spermatozoa with a GLP-1 analog, exendin-4 (Ex-4), and found that progressive motility and cholesterol efflux were increased after 1 h of incubation, with 300 pM of Ex-4 being the most effective treatment. The beneficial effects of Ex-4 were reversed by incubation with Ex-4 plus H89, a PKA inhibitor, suggesting that Ex-4 acts via the GLP-1 receptor to regulate capacitation through a PKA-dependent signaling pathway (Rago et al., 2020). In addition, Ex-4 increased lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PDH), lipase, and acyl-CoA dehydrogenase activities, while sperm triglycerides content was decreased, suggesting that this hormone may have a lipolytic effect in human spermatozoa (Rago et al., 2020). These findings suggest that spermatozoa are a potential GLP-1 target, unveiling the perspective of an additional pharmacological use for this hormone on *in vitro* capacitation of spermatozoa.

Ghrelin

Ghrelin, known as the hunger hormone, is another gut peptide known for its wide physiological functions, including food intake, sleep, body weight, and inflammation (van der Lely et al., 2004). It is known that ghrelin promotes food intake both in rodents and in humans and reduces insulin secretion (Wren et al., 2001). Individuals with obesity tend to exhibit notably lower ghrelin levels compared to normal weight men. This lends supports to the idea that reduced ghrelin levels may counteract the uptake of energy (Tschöp et al., 2001). Ghrelin acts through the growth hormone secretagogue receptor (also known as ghrelin receptor, GHS-R). Two isoforms of the GSH-R have already been identified, GHS-R1a and GHS-R1b, although only GHS-R1a seems to be capable of transducing ghrelin signaling

(Yanagi et al., 2018). Ghrelin signaling is mainly mediated through the phospholipase C (PLC)/inositol triphosphate (IP₃) pathway, which stimulates the intracellular release of Ca²⁺ from the endoplasmic reticulum (Hedegaard & Holst, 2020). However, the GSH-R1a is also able to form heterodimer receptors with other G-protein-coupled receptors (GPCR), leading to the activation of diverse signaling pathways according to its counterpart and cell type. For instance, the GSH-R1a can form a heterodimer with the somatostatin receptor 5 in pancreatic β -cells and prevent insulin release through the suppression of Ca²⁺ influx and the inhibition of cAMP formation (Dezaki et al., 2007; Park et al., 2012).

Although studies concerning its effects on the male reproductive function are scarce, ghrelin is hypothesized to be capable of modulating the testis either directly, or indirectly through regulation of the HPG axis in the central nervous system. *In vitro* studies have reported that ghrelin can inhibit the testicular secretion of testosterone and suppress LH and FSH secretion by the pituitary (Dupont et al., 2010; Ishikawa et al., 2007). In the human male reproductive system, Gaytan et al. observed that ghrelin is expressed in both Leydig and Sertoli cells but not in the germ cell line (Gaytan et al., 2004). *In vitro* studies proposed that ghrelin can inhibit the proliferative activity of Leydig cells and immature germ cells, avoiding an excess build-up of germ cells which is crucial for spermatogonia survival (Barreiro & Tena-Sempere, 2004; Kheradmand et al., 2012). Martins et al. not only confirmed the existence of GSH-R in human SCs but also showed that ghrelin plays a role in modulating the metabolism of these cells (Martins et al., 2016). In this study, it was observed that the concentration of ghrelin found in healthy men (100 pM) decreased glucose and pyruvate consumption and acetate production as compared to the concentration found in men with obesity (20 pM). However, lactate production was not modified due to the increased LDH activity upon exposure to ghrelin levels found in healthy men. Based on these findings, these authors proposed that ghrelin might serve as an energy sensor for human SCs in a dose-dependent manner. However, further studies are needed to fully understand the implications of these results. As of now, there is a lack of research on the expression of GSH-R or the effects of ghrelin on human spermatozoa. However, there is evidence that ghrelin may promote capacitation in rat spermatozoa. Łukaszyk et al. reported that GHSR-1a is expressed in the acrosome region of rat spermatids and epididymal spermatozoa (Łukaszyk et al., 2012). In the same study, these authors showed that 1 μ M of ghrelin raised intracellular Ca²⁺ levels and enhanced progressive motility, indicating a possible involvement on sperm capacitation and acrosome reaction (Łukaszyk et al., 2012). However, it is still crucial for future research to show whether these animal model findings also apply to the human.

A3.2.3.2. Adiposity signal hormones

Insulin

Insulin is an anabolic hormone that is produced by the pancreatic β -cells. Insulin is primarily responsible for regulating glucose and lipid metabolism. It facilitates glucose entry into cells by translocating GLUT4 to the plasma membrane, while also promoting glycogenesis and lipogenesis (Kersten, 2001; M. M. Qaid et al., 2016). The action of insulin is mediated through the insulin receptor, which changes its conformation upon insulin binding to promote its tyrosine kinase activity and auto-phosphorylation. Then, the phosphorylation of the insulin receptor substrates (IRS) occurs, which activates the PI3K/Akt and MAPK signalling pathways to transduce its effects (Boucher et al., 2014; White & Kahn, 2021).

Insulin resistance is a hallmark of metabolic diseases and is strongly associated with the development of these disorders. In the case of obesity, it mainly occurs due to the accumulation of visceral fat and increased circulating free fatty acids, which impairs the ability of insulin to stimulate insulin-sensitive tissues (Barazzoni et al., 2018; Hardy et al., 2012). However, data on the effects of insulin and insulin resistance in the male reproductive system are scarce. Leisegang et al. observed that men with obesity had increased serum and seminal insulin levels, which were correlated with poor sperm parameters (Leisegang et al., 2014). These findings led the authors to hypothesize that elevated insulin levels might be detrimental to the male reproductive system. Insulin plays a crucial role in modulating the male reproductive system, both indirectly through the HPG axis and directly at the testis. In the central nervous system, insulin regulates the hypothalamus-pituitary complex in response to the metabolic and energetic state of the organism. It achieves this by directly influencing GnRH neurons or indirectly by regulating neuropeptide Y neurons which, in turn, have the capacity to modulate the activity of GnRH neuron (Pralong, 2010). When insulin levels are low or insulin resistance is present, there is a disturbance in the secretion of GnRH, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). This disruption affects the HPG axis, significantly impacting the reproductive system (Boukhliq et al., 1996; Burcelin et al., 2003; Natah et al., 2013). At the testis, insulin is known for its role in testicular development and regulation of sex-determination genes (Nef et al., 2003; Pitetti et al., 2013). Besides, insulin mediates the metabolic control of spermatogenesis both directly, due to its action in germ cells, and indirectly through the regulation of SCs metabolism (M.M. Qaid et al., 2016). In SCs, the insulin receptor was first identified by Oonk and Grootegoed, who suggested that insulin had specific functions in these cells maturation and activity (Oonk & Grootegoed, 1987). This hormone is reported to regulate SCs viability, differentiation, and metabolism, particularly by influencing the production of lactate and acetate (Alves

et al., 2012; Escott et al., 2013; Pitetti et al., 2013). Insulin-deprived SCs presented decreased transcript levels of genes associated with lactate metabolism (*LDH* and *MCT4*) and decreased glucose consumption and acetate production, suggesting that insulin resistance can impair the metabolic support of spermatogenesis (Alves et al., 2012; Oliveira et al., 2012). Insulin has also been widely studied for its capacity to modulate steroidogenesis. Notably, insulin resistance is associated with a decrease in testosterone secretion by Leydig cells, accomplished through the modulation of LH receptors or by affecting the population of robust and functional cells (Pitteloud et al., 2005). In addition, studies in rodent animal models showed that hyperglycemia is linked to hyperplasia of Leydig cells and increased risk of testicular tumor development, whereas insulin treatment decreases the number of hyperplastic cells and increases its viability and testosterone production (Leisegang & Henkel, 2018; Nakane et al., 2020). Nevertheless, these results are yet to be demonstrated in humans. Although the data on the human germ cell line are scarce and conflicting, there is evidence that insulin promotes spermatogonia differentiation and sperm capacitation, motility, and acrosome reaction, which are necessary for successful oocyte fertilization (Ando & Aquila, 2005; Lampiao & du Plessis, 2008). Lampiao and Plessis observed that the addition of insulin (0.35 ng/mL) to the media improved human spermatozoa total and progressive motility and acrosome reaction (Lampiao & du Plessis, 2008). Conversely, Wang et al. reported that higher concentrations of insulin (10 and 100 µg/mL) did not produce any effect on human spermatozoa motility, thus further studies are needed to clarify the effects of insulin on human spermatozoa (Wang et al., 2015). Interestingly, there is also evidence of insulin production by the human germ cell line, although the data is also conflicting and its function is poorly understood (Aitken et al., 2021; Aquila et al., 2005a). The results obtained by Aquila et al. suggest that insulin may modulate capacitation in an autocrine/paracrine manner, although further studies are required to confirm this hypothesis.

Leptin

Leptin is a peptide hormone produced mainly by white adipocytes, whose function is to decrease food intake through the feeling of satiety (Friedman & Halaas, 1998). Although leptin cannot induce satiety by itself, it can interact with other hypothalamic hormone pathways associated with appetite regulation (Friedman & Halaas, 1998; Rogge et al., 2008). The action of this hormone is modulated by the leptin (or obesity) receptor (LepR) (Tartaglia, 1997). From the several isoforms of the LepR already identified, the long isoform (LepRb) is considered the main signaling transducer (Lee et al., 1996). This receptor is widely expressed in the human organism and its effects are dependent on the target tissue and cell

type. The canonical signaling pathway attributed to LepR is the janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway (Evans et al., 2021).

The development of metabolic disorders is associated with leptin resistance, which results in increased levels of circulating leptin (Enriori et al., 2007; Frederich et al., 1995). High levels of leptin in individuals with obesity are thought to be promoted by adipose tissue expansion (Considine et al., 1996). Besides its regulatory role on energy intake, leptin also modulates the HPG axis in both the hypothalamus and the testes, which makes this hormone an important mediator between energy reserves, nutritional status, and the male reproductive system (Carrageta et al., 2019). In the hypothalamus, animal studies have demonstrated that leptin administration induces pulsatile GnRH release in a dose-dependent manner (Parent et al., 2000). Furthermore, leptin has been shown to increase LH and FSH secretion by stimulating the growth and differentiation of pituitary cells. This effect occurs regardless GnRH presence or absence (Tezuka et al., 2002). However, the effects of leptin on the reproductive system seem to be more pronounced than the regulation of the hypothalamus-pituitary complex (O'Rahilly, 1998; Strobel et al., 1998). In fact, leptin is one of the most widely studied hormones concerning metabolic disorders-induced male infertility, although the molecular mechanisms are poorly understood (Malik et al., 2019; Moreira et al., 2018). Animal studies demonstrated that leptin is essential for the reproductive system as inactivating mutation of the leptin gene, as observed in ob/ob mice, renders animals infertile (Barash et al., 1996). The causality has been demonstrated since leptin administration rescues the fertility of these animals, while a similar effect is not obtained in mice with LepR inactivating mutations (db/db mice) (Hoffmann et al., 2016; Mounzih et al., 1997). In humans, congenital leptin deficiency was also associated with hypogonadism, puberty arrest, and infertility both in male and female individuals (Strobel et al., 1998). In the same study, it was shown that administration of GnRH to men with congenital leptin deficiency rescued FSH and LH levels, whereas administration of human chorionic gonadotropin (hCG) rescued testosterone levels, further suggesting the role of leptin in the regulation of the hypothalamus (Strobel et al., 1998). In a follow-up study, it was demonstrated that leptin replacement therapy greatly improved his body composition, metabolism, and endocrinal and gonadal function (Licinio et al., 2004). Interestingly, the observed reproductive effects of leptin do not appear to be tied to the canonical signaling pathway. The knockout of STAT3 and STAT5 in LepRb-expressing cells led to obesity without affecting fertility (Singireddy et al., 2013). Instead, it seems to operate through the IRS/PI3K/Akt pathway (Garcia-Galiano et al., 2017).

Clinical studies in humans have demonstrated that obesity in men is associated with higher circulating levels of leptin, which in turn are correlated with poor sperm parameters (Hofny et al., 2010; Leisegang et al., 2014). Interestingly, leptin levels in the seminal fluid

are also related to poor sperm parameters. Guo et al. observed a higher leptin concentration in the seminal fluid of men with asthenozoospermia, although no differences were observed concerning serum leptin in this cohort (Guo et al., 2014). Seminal leptin levels were also negatively correlated with sperm progressive motility and serum total testosterone. In a meta-analysis, Mo et al. observed higher leptin levels in both serum and seminal fluid of infertile men. In addition, a negative correlation between both serum and seminal leptin levels and sperm counts and motility was reported (Mo et al., 2022). Animal studies support these findings, showing that leptin administration to normal-weight rodents leads to increased oxidative stress and poor sperm quality. These results suggest that high leptin levels are associated with poor male reproductive health and impaired spermatogenesis (Abbasihormozi et al., 2013; Almabhouh et al., 2015; Haron et al., 2010; Wang et al., 2018). In fact, compelling evidence highlights the regulatory role of leptin in the testis, although the molecular mechanisms are not fully disclosed (Soyupek et al., 2005). For instance, leptin can decrease testosterone production by inhibiting the transport of cholesterol to the mitochondria and interfering with the cAMP-PKA signaling pathway in Leydig cells (Lin et al., 2009). In human SCs, Martins et al. were the first to demonstrate the presence of the LepR (Martins et al., 2015). In this study, it was found that leptin at a concentration of 5 ng/mL, as usually detected in lean men, increased GLUT2 expression and LDH activity in SCs. However, this effect was not observed for leptin concentrations equivalent to circulating levels usually detected in men with obesity (25 and 50 ng/mL). While glucose consumption and lactate production did not show consistent alterations in all tested concentrations, exposure to leptin decreased acetate production in human SCs across all concentrations tested (Martins et al., 2015). There is also evidence from animal studies that exposure to high levels of leptin disrupts the BTB. Wang et al. treated seven-week-old male C57BL/6 mice with leptin (0.1, 0.5, or 3 mg/kg) and observed a dose-dependent disruption of the BTB through the downregulation of tight junction-related proteins in SCs. In addition, these authors also reported a dose-dependent decrease in sperm concentration and motility, whereas a higher percentage of morphologically abnormal sperm cells were observed after leptin treatment, suggesting impaired spermatogenesis (Wang et al., 2018). In support of these results, these authors observed that a 48 h treatment with leptin (100 nM, equivalent to 1.6 µg/mL) decreased the expression of tight junction-related proteins in TM4 cells (mouse SCs line). The expression of these proteins was partially rescued with the use of PI3K and MAPK signaling pathways chemical inhibitors, and to a lesser extent with the use of JAK2 chemical inhibitor, suggesting their involvement in the signaling transduction of leptin (Wang et al., 2018). Nevertheless, future studies are required to further elucidate which signaling pathways mediate the effects of leptin in SCs.

Leptin and LepR were identified in human spermatozoa by Jope et al (Jope et al., 2003). Interestingly, these authors identified the LepR both in the tail of human spermatozoa and a soluble isoform in the seminal fluid. Later, Aquila et al. observed that leptin is mainly expressed in the equatorial region of the head and midpiece in non-capacitated human spermatozoa (Aquila et al., 2005b). These authors also reported that leptin is released during capacitation, being responsible for both cholesterol efflux and protein tyrosine phosphorylation in an autocrine/paracrine fashion. Due to the pattern of expression of the leptin receptor, it was hypothesized that leptin can regulate human spermatozoa motility, however, conflicting results are reported in the literature. Lampiao and Plessis reported that leptin (20 nM, or 30.6 ng/mL) improved human spermatozoa total and progressive motility, and stimulated acrosome reaction (Lampiao & du Plessis, 2008). Contrastingly to these findings, Li et al. observed no effects on spermatozoa motility, capacitation, or acrosome reaction upon incubation with leptin (10-1000 ng/mL) (Li et al., 2009). Interestingly, it was found that high-motility spermatozoa expressed higher levels of leptin than the low-motility fraction. In addition, no correlation was observed between leptin or LepR seminal fluid levels and sperm parameters.

Collectively, these findings imply that the dysregulation of gut and adiposity signal hormones, often linked with metabolic disorders, can have adverse effects on testicular function and spermatogenesis. This could potentially be a significant contributor to male infertility. However, there is conflicting data in the literature regarding the molecular mechanisms that underlie these effects, which may be due to the wide range of concentrations used in these studies. Besides the metabolic and hormonal dysregulation, novel studies address the hypothesis that high-fat diets and metabolic diseases induce epigenetic alterations in male gametes, which not only regulates their function, but also may passively induce the inheritance of metabolic signatures through generations. These hypotheses are a key focus of current research in this field, and insights into the currently available data will be discussed in detail on the following topics.

A3.3. High-fat diets induce epigenetic changes in male gametes: transgenerational inheritance of metabolic traits

Epigenetics refers to mitotically stable molecular mechanisms that govern genomic activity independently of the DNA sequence. Epigenetic changes comprise both DNA and chromatin alterations which modulate gene expression (Klose & Bird, 2006; Li, 2002). These molecular alterations include the DNA methylation of CpG dinucleotides, one of the most well-studied epigenetic markers which are generally stable and long-lasting in somatic cells,

histone modifications, alterations in chromatin structure, RNA methylation, and the great majority of noncoding (nc)RNAs (Nilsson et al., 2018; Skinner, 2011). The epigenome is characterized by its plasticity, as it can undergo modifications in response to environmental stimuli, including metabolic and hormonal cues. These changes can result in phenotypic and gene expression alterations without altering the genetic code (King & Skinner, 2020). Therefore, the metabolic and hormonal changes associated with high-fat diets and metabolic diseases can be integrated and reflected in the epigenome of every cell in the body.

Although epigenetic research is still in its early stages, there is compelling evidence that the germline is highly regulated by its epigenetic profile (Fernandez-Perez et al., 2018; Gui & Yuan, 2021; Lehtiniemi & Kotaja, 2018). An increasing number of studies is highlighting the expression and regulatory function of small RNAs, such as small non-coding RNAs (sncRNAs), PIWI-interacting RNAs (piRNAs), tRNA-derived small RNAs (tsRNA), and microRNAs (miRNAs), in the human germline and spermatozoa (Baxter & Drake, 2019; Donkin & Barres, 2018; Schagdarsurengin & Steger, 2016). These small RNAs are known for their involvement in silencing processes and consequent regulation of gene expression (for a review see (Schagdarsurengin & Steger, 2016)). Besides, spermatozoa exhibit specific epigenetic mechanisms such as the replacement of the majority of DNA-binding histones by protamines (Steger et al., 1998). Protamination induces chromatin packaging, which greatly halts transcription in spermatozoa. As transcription is highly reduced, spermatozoa are, thus, hypothesized to rely on epigenetic regulation more than any other cell (Schagdarsurengin & Steger, 2016). Studies have suggested that the epigenetic profile of mature spermatozoa may play a crucial role in successful fertilization and embryo development, and alterations in the epigenetic signature have been linked to infertility (Carrell & Hammoud, 2010; Gunes & Esteves, 2021). For instance, spermatozoa carry both the precursor and the mature form of miR-34c. Liu et al. observed that the injection of a miR-34c inhibitor into zygotes inhibited DNA synthesis and significantly suppressed first cleavage division (Liu et al., 2012). Nevertheless, while studies suggest that sperm miRNAs contribute to the regulation of gene expression in the early embryo, the full extent of its contribution remains to be elucidated.

Metabolic disorders can induce several epigenetic modifications which can result in impaired spermatogenesis and/or affect spermatozoa function (Baldeon et al., 2015; Mahmoud, 2022). Consequently, investigating these epigenetic changes has become a critical area of interest in the field of reproductive biology (Guerra-Carvalho et al., 2022). Although data on metabolic-induced epigenetic alterations in Sertoli and Leydig cells are scarce, there is increasing evidence that metabolic disorders and high-fat diets induce epigenetic alterations in spermatozoa, which may have implications for embryo development. Specifically using the aforementioned example, miRNAs of the miR-34 family, which are

expressed in human spermatozoa and are important for embryonic development, are sensitive to the metabolic status of the organism (Baldeon et al., 2015; Liu et al., 2012). Obesity is also related to altered methylation patterns and expression of small ncRNAs in spermatozoa, although histone positioning was found to be similar. Interestingly, Donkin et al. reported that CpG hypomethylation changes between lean and obese individuals were almost exclusively found in protamine-associated DNA, then in histone-associated regions. The GO analysis revealed that the specific genome locations that were differently methylated were associated with the term “nervous system development”, including 274 genes that are appetite regulators, and genes associated with obesity (*MC4R*, *FTO*, carbohydrate sulfotransferase 8 (*CHST8*), and SH2 binding domain-containing protein 1 (*SH2B1*), among others) (Donkin et al., 2016). These authors also reported that bariatric surgery-induced weight loss led to the remodeling of sperm DNA methylation, prominently at genetic locations implicated in the central control of appetite (Donkin et al., 2016). ORGs, which are genes related to the onset of obesity and include the *FTO*, *MC4R*, *GNPDA2*, and *TMEM18*, have been reported to present altered methylation patterns in spermatozoa from lean and obese individuals, and before and after bariatric surgery-induced weight loss. Pereira et al. reported that *FTO*, *MC4R*, and *GNPDA2* transcripts are present in the mRNA pool of spermatozoa. Although the authors were not able to associate the abundance of these transcripts with the metabolic phenotype of individuals, *GNPDA2*, *MC4R*, and *FTO* mRNA abundance were associated with sperm quality, embryo development, and pregnancy rates (Pereira et al., 2021b). *FTO* encodes for a demethylase enzyme that is responsible for the (de)methylation of both DNA and RNA, interfering with the expression of several genes. Although the authors did not investigate the role of *FTO* in spermatozoa or embryo, it is reasonable to assume that changes in *FTO* expression due to obesity pose a substantial risk for altering the expression of other genes linked to *FTO* activity (Relier et al., 2022). In another study, *TMEM18* and *GNPDA2* were identified in human SCs and it was reported that its expression responds to hormonal cues associated with obesity (Pereira et al., 2021a). Protein acetylation patterns are also altered in response to the nutritional status. Palmer et al. observed that a high-fat diet reduced the expression of SIRT6 in mice spermatozoa, which led to increased levels of acetylated histone H3K9 (Palmer et al., 2011). Interestingly, McPherson et al. reported that some obesity-induced epigenetic alterations were partially reverted upon physical exercise intervention and the insulin sensitivity and adiposity of the mice were also improved (McPherson et al., 2015). Other obesity-induced detrimental effects on the testis, however, seem to be irreversible even upon weight loss (Crisostomo et al., 2019; Crisostomo et al., 2020).

Some authors argue that a direct effect of the diet on the spermatozoa epigenome would be unlikely because spermatozoa are fully differentiated cells that do not undergo further

division and have a protamine-packaged high-order nuclear chromatin and highly reduced transcriptional activity (Schagdarsurengin & Steger, 2016). In humans, the growth and differentiation of spermatogonia into spermatozoa require around 72-74 days, which is followed by a 2-week epididymal maturation phase (Heller & Clermont, 1963). As such, it was hypothesized that the primary targets would be the developing germ cells within the testis, through enzymes that modulate the epigenome of the DNA and histones, and not mature spermatozoa in the epididymis (Schagdarsurengin & Steger, 2016). This hypothesis would be later refuted with the discovery of epididymosomes and the study of its genetic content. Epididymosomes are extracellular microvesicles present in the epididymal fluid which are transferred to spermatozoa during their maturation phase (Sullivan, 2015). Epididymosomes carry miRNAs which are hypothesized to be involved in diverse regulatory mechanisms not only for spermatozoa but also for epithelial cells located downstream from its release site (Belleannee, 2015; Ding et al., 2021; Reilly et al., 2016). The epididymis, thus, is an important site in establishing the sperm epigenome (Nixon et al., 2015; Trigg et al., 2019). For an in-depth review of the contribution of the epididymis to sperm epigenetic, please see the review conducted by Chen and colleagues (Chen et al., 2021). Epididymosomes content seem to also be influenced by environmental stimuli, although diet-induced alterations in its content remain to be explored (Alshanbayeva et al., 2021; Reilly et al., 2016).

A3.3.1. Paternal transgenerational inheritance of metabolic disorders

The inheritance of epigenetic signatures present in the fertilizing spermatozoon has wide-ranging implications for offspring. These epigenetic markers can impact gene expression and contribute to the development of certain traits and diseases in the offspring (Gapp et al., 2014). The findings that the paternal epigenetic signatures could be transmitted to subsequent generations toppled the current theories of Evolutionism and were firstly rendered highly controversial due to the demethylation process of the zygotic paternal genome (Mayer et al., 2000; Noble et al., 2014). An hypomethylation state is necessary for the zygote to obtain a pluripotent stem cell state during its development (Paranjpe & Veenstra, 2015). During mammalian embryonic development, demethylation occurs, although it has been found that it is not complete. Research indicates that methylation levels are reduced by almost 70% during the first phase of demethylation, and subsequently reduced to a minimum of 8% after the second demethylation cycle (Gkountela et al., 2015; Tang et al., 2016). In addition, it was hypothesized that spermatozoa would not carry any epigenetic factors

due to its small cytoplasmatic volume and protamination. Following meiosis, histones undergo replacement with specialized protamines, a crucial step for achieving a highly compacted chromatin structure (Braun, 2001). It was thought that all epigenetic modifications would be removed during protamination, rendering the epigenetic inheritance to the mother (Wykes & Krawetz, 2003). Currently, it is known that about 5-15% of sperm chromatin is nucleosome-bound (van der Heijden et al., 2008; Wykes & Krawetz, 2003), which is enriched in imprinted gene clusters, miRNA, and other signalling factors (Hammoud et al., 2009). Therefore, there is a consensus that spermatozoa epigenetic imprints are transmitted to the oocyte (Miller, 2007). The extent to which spermatozoa epigenetic imprints influence the metabolic condition of the progeny can be inferred from early development to birth and even later into adulthood (Perez & Lehner, 2019). If spermatogenesis is affected during its critical development window, which is the embryonic gonadal development and germline differentiation, these epigenetic modifications may become permanent in the germ cell line epigenome and be inherited by future generations (Ding et al., 2015). Furthermore, the occurrence of metabolic disorders at an early age, especially during peak reproductive years, can lead to the transmission of this information to the next generation (Pereira et al., 2020), perpetuating a chain of early-onset metabolic disorders (Fig. A3.4). In other words, the popular saying “we are what we eat” may be further extended to “we are what we eat and what our ancestors ate”. While the exact molecular mechanisms are not yet fully understood, there is substantial evidence indicating that the offspring of male individuals with obesity or type 2 diabetes have a heightened risk of developing these metabolic disorders. Studies conducted in animal models have also demonstrated that the consumption of high-fat diets by animals in the F0 generation can lead to obesity, which in turn has long-term impacts on the health of their descendants. These findings suggest that there may be transgenerational effects of diet-induced obesity on metabolic health. Crisóstomo et al. conducted studies which revealed that obesity in male mice led to significant alterations in testicular metabolism. Interestingly, these metabolic changes were detectable in the testicular metabolome of their offspring and grandchildren, despite being fed standard chow (Crisóstomo et al., 2021). Furthermore, this transgenerational effect of obesity had a negative impact on sperm quality across all generations (Crisostomo et al., 2022b). These authors also reported obesity-induced alterations in the sperm miRNA content, which were also traceable in the miRNA content of descendants. Although this information is not enough to establish an epigenetic mechanism responsible for the inheritability of metabolic disorders, it supports the hypothesis that the sperm epigenome has the potential to strongly influence the offspring's health (Crisostomo et al., 2022a). In support of these results, Fullston et al. performed a similar transgenerational study and also concluded that sperm miRNAs are key mediators of the mechanism of metabolic inheritance (Fullston et al., 2013). Cropley et al.

also studied the transgenerational effects of paternal obesity and prediabetes in a mouse model. These authors observed that the offspring of fathers with obesity (F1 generation) had an impaired glucose and lipid metabolism, which persisted until the third generation, although largely attenuated in the latter. In addition, spermatozoa of F1 males exhibited an altered epigenetic signature including in diet-responsive small ncRNAs (Cropley et al., 2016). In another study, Barbosa et al. observed that the newborn offspring of fathers fed with a high-fat diet had reduced body weight and pancreatic beta cell mass. Curiously, adult female, but not male, F1 and F2 offspring exhibited glucose intolerance and were resistant to high-fat diet-induced weight gain. In addition, the epigenome of spermatozoa from both fathers with obesity and F1 male offspring exhibited similar DNA methylation patterns and diet-induced small ncRNAs expression signatures (de Castro Barbosa et al., 2016). More recently, Pepin et al. have proposed that sperm histone H3 lysine 4 tri-methylation (H3K4me3) levels could be used as metabolic sensor of paternal obesity (Pepin et al., 2022). The authors reported that obesity promoted an increase in H3K4me3 in spermatozoa from mice of two different strains (C57BL/6NCrl wild-type and KDM1A transgenic line). The gene ontology analysis of the DNA regions affected by the increase in H3K4me3 in spermatozoa from F0 animals was concordant with the disturbed metabolic phenotypes found in F0-F2. Building upon these findings, the authors extended their proposition that sperm H3K4me3 might constitute a component of the non-genetic mechanism linking paternal diet to offspring phenotypes (Pepin et al., 2022). Applying a different methodology, Grandjean et al. have shown the capacity of the RNAs extracted from the fathers to induce metabolic phenotypes in the progeny (Grandjean et al., 2015). These authors retrieved the RNAs either from the testis or spermatozoa from male mice fed a western-like diet and microinjected them into naïve one-cell embryos, which induced the metabolic phenotype observed in the fathers. Among the identified 22 miRNAs that were altered due to the diet, the increased expression of miR19b was highlighted as a potential inducer of the observed metabolic phenotype as its direct injection-induced similar metabolic alterations in the progeny (Grandjean et al., 2015).

Metabolic disorders are highly complex conditions that arise from a combination of genetic, environmental, and epigenetic factors, all of which contribute to an individual's unique metabolic phenotype. Understanding the intricate molecular pathways involved in the development and inheritance of metabolic disorders is essential to address the alarming increase in the prevalence of these conditions and associated complications. By gaining a deeper understanding of these pathways, we can develop more effective preventative measures and treatments to combat this growing epidemic.

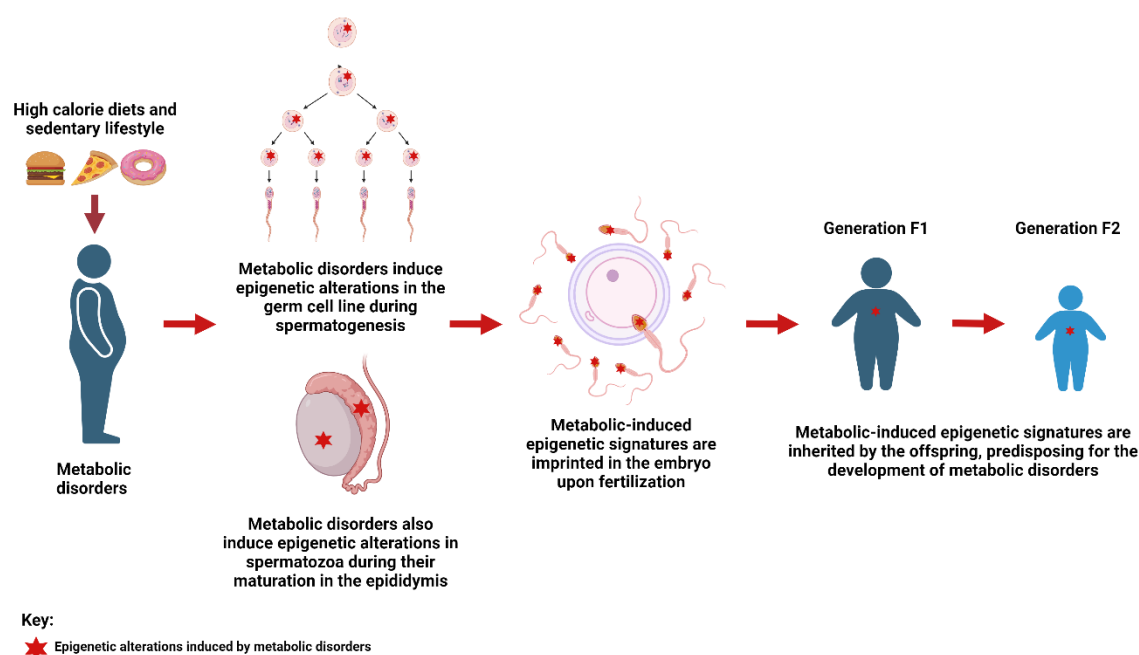


Fig. A3.4 – Overview of the proposed model of paternal inheritance of metabolic diseases.

High-fat diets and metabolic disorders cause epigenetic alterations in the male gametes, imprinting the metabolic signature of the father into the offspring. These epigenetic markers are then carried by the spermatozoon and imprinted in the embryo upon fertilization, potentially conditioning the health of the offspring towards the development of metabolic disorders.

A3.4. Conclusions and future perspectives

The testes are highly intricate organs that are strongly influenced by an individual's hormonal and metabolic status. Notably, testicular cells are highly responsive to gut hormones, meaning that the diet can have a significant direct impact on male reproductive health. Although the broad effects of metabolic disorders on the testes and male reproductive function are well-known, some questions regarding the underlying molecular mechanisms and signaling pathways still remain unanswered. In addition, we must highlight the cases of individuals with overweight/obesity that exhibit normal sperm quality parameters and fertility potential, unveiling the complexity of the association between metabolic disorders and male infertility (Al-Ali et al., 2014; Rybar et al., 2011; Wegner et al., 2010). As the field of Omics continues to evolve, we can expect to gain deeper insights into these processes. Multi-omics approaches, including proteomic, lipidomic, and metabolomic analyses, will offer valuable data for evaluating metabolic-induced alterations in reproductive function. These Omics can highlight not only biomarkers of metabolic disease-induced dysregulation but also

identify novel therapeutic targets for improving assisted reproductive technology (ART) outcomes. By taking advantage of these emerging platforms, we can better understand the impact of metabolic disorders on male fertility and develop more effective strategies for managing and treating these conditions.

The transgenerational inheritance of metabolic diseases is a fairly novel and crucial area of investigation. Not only do we need to address the growing prevalence of obesity and related health issues in Western societies, but we also need to understand how these conditions can be passed to future generations since the development of metabolic disorders can be perpetuated in a never-ending chain. The well-known saying "we are what we eat" takes on new meaning when we consider that our diets can affect the health of our descendants for at least two generations and thus can be extended to "we are what we eat and so is our progeny". However, studying the transgenerational inheritance of metabolic diseases through epigenetic alterations is a complex and challenging task. The epigenome is a highly sensitive and finely regulated network of signalling and regulatory mediators that respond to environmental stimuli. Small ncRNAs, methylation patterns, and acetylation patterns are just some of the factors that constantly change in response to external factors. While it is essential to identify altered epigenetic signatures, it is equally important to understand its regulatory functions. Another challenge is that the epigenome of male gametes is modulated not only during spermatogenesis but also by extracellular microvesicles found in the seminiferous tubules, epididymis, and seminal fluid. Currently, and to the best of our knowledge, no mechanistically study has demonstrated how the association between the metabolic and hormonal dysregulation conferred by metabolic disorders induce epigenetic alterations in male gametes. Despite these difficulties, studying the epigenome provides exciting opportunities. Through the characterization and manipulation of the epigenome, we can pave the way for innovative assisted reproduction technology (ART) protocols. This approach enables us to discern and select the most suitable spermatozoa for fertilization. Moreover, delving into the epigenetic content of microvesicles provides novel avenues to tackle the escalating challenge of male infertility. In summary, while studying the transgenerational inheritance of metabolic disorders through epigenetic alterations poses significant challenges, it is a critical area of research that provides exciting opportunities for improving human health.

A3.5. References

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Monteiro BS, Freire-Brito L, **Carrageta DF**, Oliveira PF, Alves MG (2021). Mitochondrial uncoupling proteins (UCPs) as key modulators of ROS homeostasis: a crosstalk between diabetes and male infertility? *Antioxidants*, 10(11): 1746. <https://doi.org/10.3390/antiox10111746>

Carrageta DF, Freire-Brito L, Guerra-Carvalho B, Ribeiro JC, Monteiro BS, Barros A, Oliveira PF, Monteiro MP, Alves MG. (2023). Inhibition of mitochondrial uncoupling proteins arrests human spermatozoa motility without compromising viability. *Antioxidants*, 2023; 12(2):409. <https://doi.org/10.3390/antiox12020409>

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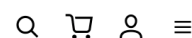
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