Mecanismos de modulação da inflamação e angiogénese

por polifenóis naturais

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Dissertação de candidatura ao grau de Doutora em Metabolismo, Clínica e Experimentação, apresentada

à Faculdade de Medicina da Universidade do Porto

Porto · 2011

Artigo 48º, Parágrafo 3º: "A Faculdade não responde pelas doutrinas expendidas na dissertação" (Regulamento da Faculdade de Medicina do Porto – Decreto-Lei nº 19337, 29 de Janeiro de 1931).

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Doutor Valdemar Miguel Botelho dos Santos Cardoso

Doutor Walter Friedrich Alfred Osswald

Ao Pedro

Para a Francisca, a Teresa e a Isabel

Aos meus Pais, aos meus Irmãos

AGRADECIMENTOS

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Este projecto de doutoramento não foi um trabalho individual. Foi um trabalho de equipa. De uma equipa diversificada e heterogénea, onde me inseri e onde tive o gosto de trabalhar ao longo destes anos pelo ambiente saudável, descontraído e de inter-ajuda, associado a uma dinâmica de exigência e responsabilidade, que me permitiram realizar com satisfação este trabalho. Durante os anos em que tenho trabalhado no Departamento de Bioquímica, muitas pessoas contribuíram com o seu tempo, trabalho e dedicação para permitir que este trabalho fosse realizado. A todos expresso hoje a minha profunda gratidão.

Em 1998, o Professor Hipólito-Reis e a Professora Isabel Azevedo proporcionaram-me a oportunidade de fazer parte da equipa docente do Departamento de Bioquímica, tendo iniciado a partir daí também a minha actividade como investigadora. Ao Professor Hipólito-Reis e à Professora Isabel Azevedo agradeço o entusiasmo com que me transmitiram o gosto pelo ensino da Bioquímica e pela investigação.

À Professora Raquel Soares, minha orientadora e actual Directora do Departamento de Bioquímica, agradeço reconhecida o gosto que desenvolveu em mim pela investigação na área da angiogénese, a confiança que sempre depositou em mim e as oportunidades que me proporcionou para me enriquecer e desenvolver como investigadora. À Professora Raquel Soares devo muito do que sou como investigadora. Agradeço ainda a imensa paciência que teve, nos momentos mais difíceis em que o trabalho parecia não andar e a presença amiga ao longo destes anos. Tem sido um gosto enorme trabalhar na equipa de investigação que lidera com inteligência, exigência e entusiasmo pelos desafios que vão surgindo.

À Professora Isabel Azevedo, minha co-orientadora, gostaria de expressar a profunda gratidão e admiração que tenho por si. É para mim um exemplo de vida como pessoa e como académica. Agradeço reconhecidamente tudo que aprendi consigo, o cuidadoso acompanhamento deste trabalho e o entusiasmo que sempre me transmitiu. Realço com especial agradecimento a presença próxima e atenta, a capacidade agregadora e conciliadora, o entusiasmo e enorme gosto pela investigação e pelo ensino, e a inteligência e verdade com que durante muitos anos dirigiu o Departamento de Bioquímica. À Professora Isabel Azevedo devo muito do que sou pessoal e profissionalmente.

Ao Professor Hipólito Reis, que me conduziu no Departamento de Bioquímica, gostaria de lhe expressar a consideração imensa que lhe reservo e de lhe agradecer o muito que aprendi consigo e a amizade que me dedicou ao longo destes anos.

À Professora Maria João Martins com quem iniciei o meu trabalho de investigação no Departamento de Bioquímica, agradeço tudo o que me ensinou e a presença amiga, com que sempre me acompanhou.
Ao Professor Manuel Alçada agradeço a ajuda imprescindível ao longo destes anos sobretudo na parte informática e a amizade que tem por mim.
Ao Professor Tiago Guimarães agradeço a colaboração nalguns trabalhos desta dissertação e a presença amiga desde há muitos anos.
À Dra Luisa Guardão agradeço a ajuda experiente nos trabalhos com animais e os bons momentos de trabalho em conjunto.
A todos os meus colegas docentes e investigadores do Departamento de Bioquímica, Alejandro Santos, Ana Cristina Branco, Ana Faria, Ana Sofia Rocha, Ana Pirraco, Ângela Castela, Carla Costa, Cidália Almeida, Clara Lemos, Conceição Calhau, Diogo Pestana, Diana Teixeira, Duarte Torres, Elisa Keating, Fátima Martel, João Araújo, João Tiago Guimarães, Júlio Rocha, Laura Ribeiro, Manuel Nuno Alçada, Manuela Meireles, Maria Cavaleiro, Maria João Martins, Mario Mendanha, Pedro Gomes, Pedro Gonçalves, Raquel Costa, Rosário Monteiro, Rui Fontes, Susana Guerreiro, Tiago Gomes, Tiago Gregório, dedico uma palavra de gratidão pelo ambiente acolhedor e de aprendizagem que proporcionam.
Um agradecimento especial ao Diogo Pestana, à Diana Teixeira e à Ana Pirraco pela paciência com que sempre me esclareceram dúvidas e pela disponibilidade para me ajudarem.
Ao Delfim Duarte, à Raquel Costa e ao Tiago Taveira, agradeço reconhecida e com muita amizade o tempo que dedicaram, sempre com entusiasmo, espírito crítico e boa disposição, ao trabalho que fazemos juntos. Aprendi imenso com cada um. Obrigada pela imensa paciência que têm comigo.
Dedico um agradecimento muito especial à Elisa Keating, à Conceição Calhau, à Rosário Monteiro e à Ana Faria pela amizade que tenho por cada uma e pelo estímulo e incondicional ajuda. Obrigada também pela oportunidade de discussão agradável e crítica do trabalho científico.
Aos funcionários do Serviço de Bioquímica agradeço a simpatia e colaboração, especialmente à Srª Dª Fátima Maio pela presença discreta e disponibilidade com que sempre nos apoia. À Luisa Vasques agradeço a ajuda que me deu, a amizade que me dedica e o exemplo de que tudo se pode fazer bem e com tranquilidade. Ao Sr. Couto agradeço a disponibilidade e simplicidade com que me ensinou a trabalhar com os animais de laboratório.
A todos os meus colegas investigadores e funcionários do Instituto de Farmacologia e Terapêutica agradeço a disponibilidade e simpatia com que sempre me receberam.
Ao iBeSa e ao Sr. Engº Machado Cruz um obrigada pelo apoio na concretização deste projecto.

Ao meu grupo CVX, ao P. Belchior, à Clara, à Isabel, à Ana, à Joana, à Inês, à Marta, à Nicha, à Catarina, ao Paulo, ao Manel e ao Pedro, meus amigos em Cristo, agradeço a amizade profunda que nos une há muitos anos e a presença atenta. Obrigada por comigo viverem o desejo de alcançar o"mais" e de em tudo amar e servir.

À Clara agradeço com muito carinho a amizade que temos há tantos anos e a constante disponibilidade para me ajudar com as minhas filhas ao longo destes anos.

Um agradecimento muito especial à Inês pela amizade franca, discreta e muito atenta, por me "pôr na ordem" muitas vezes, e pelas muitas mais vezes em que "adoptou" as minhas filhas, para me dar tranquilidade.

Aos meus queridos Pais, o meu porto de abrigo, obrigada por me terem transmitido um sentido de vida e de família, e pelo que de mim fizeram. Obrigada também pela disponibilidade imensa que têm com as nossas filhas e por nos ajudarem a fazê-las mais felizes.

Aos meus queridos sogros agradeço a presença atenta na nossa vida e o carinho e a atenção que sempre me dedicaram. Obrigada ainda pela ajuda permanente e pelos imensos mimos que dão às nossas filhas.

Aos meus irmãos, Tiago, Tocá e Leonor, Diogo e Vanda, Francisco e Raquel, Tomás e Filipa, Margarida e André, agradeço a presença próxima, confortável, cúmplice e amiga. Foi com cada um que aprendi o valor da vida em família e da entrega pelo outro.

Ao Pedro, meu querido marido, obrigada pela paciência e pelas flores e por contigo viver em louvor, entendimento e encantamento. Obrigada por fazeres de mim uma pessoa melhor e mais feliz.

Às nossas filhas, Francisca, Teresinha e Isabel, os "nossos projectos" mais bonitos, mais desafiantes e mais gratificantes, um obrigada muito especial pela paciência e pelos muitos mimos que recebi. Vocês são quem mais me faz feliz.

Esta investigação foi realizada no Departamento de Bioquímica da Faculdade de Medicina da Universidade do Porto.

A candidata realizou o trabalho experimental com o apoio de uma bolsa de Doutoramento (SFRH/BD/41888/2007) atribuída pela Fundação para a Ciência e a Tecnologia e com financiamento dos projectos PTDC/SAU-OSM/102083/2008 e PEst-OE/SAU/UI0038/2011.

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Doutor António Taveira Gomes, Professor Associado da Faculdade de Medicina da Universidade do Porto

A candidata declara que teve uma contribuição determinante na realização do trabalho experimental (programação, execução de protocolos e elaboração técnica), bem como na interpretação e discussão dos resultados apresentados em todos os artigos desta dissertação. Além disso, contribuiu activamente na redacção dos artigos apresentados.

Abreviaturas

Ang - angiopoietinas

Cat - catequina

EC - células endoteliais

ECM - matriz extracelular

EGCG - epigalocatequina-3-galato

EGF - factor de crescimento epidérmico

eNOS - síntase do óxido nítrico endotelial

EPC - células precursoras endoteliais

FGF - factor de crescimento fibroblástico

FLTR - células de músculo liso vascular de aorta fetal humana

HASMC - células de músculo liso vascular de aorta humana

HIFα - factor alfa induzido pela hipóxia

HIFβ - factor beta induzido pela hipóxia

HUVEC - células endoteliais de cordão umbilical humano

ICAM1 - molécula de adesão intracelular 1

IL - interleucina

iNOS - síntase do óxido nítrico indutível

IXN - isoxanto-humol

NADPH - fosfato de dinucleótido de nicotinamida e adenina reduzido

NRP - neuropilina

NFκB - factor nuclear kapa-B

NO - óxido nítrico

NOX - oxídases do NADPH

8PN - 8-prenilnaringenina

PDGFB - factor B de crescimento derivado de plaquetas

PDGFRβ - receptor β do factor de crescimento derivado de plaquetas

PHD - prolil-hidroxílases

PLGF - factor de crescimento placentário

RNS - espécies reactivas de azoto

ROS - espécies reactivas de oxigénio

TGFβ - factor beta transformante do crescimento

Tie2 - receptor 2 das angiopoietinas

TNFα - factor alfa de necrose tumoral

VEGF - factor de crescimento do endotélio vascular

VEGFR - receptor do factor de crescimento do endotélio vascular

VHL - proteína von Hippel-Lindau

VSMC - células de músculo liso vascular

XN - xanto-humol

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INTRODUÇÃO

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1. Os vasos sanguíneos

Os vasos sanguíneos formam uma complexa rede tubular que irriga o organismo e que transporta tudo o que as células necessitam para o seu regular funcionamento: oxigénio, nutrientes, hormonas, células implicadas na defesa imunológica, diversos compostos químicos, minerais e os produtos resultantes do metabolismo que necessitamos eliminar. Nenhuma célula fica a uma distância superior a 200 µm de um vaso sanguíneo, o que corresponde ao limite da capacidade de difusão do oxigénio através dos tecidos.

O funcionamento do sistema circulatório foi proposto inicialmente por William Harvey, em 1628, que sugeriu que o coração bombeava o sangue para todo o organismo através de artérias e que as veias conduziam o sangue de volta ao coração. Foi mais tarde, em 1661, que Marcello Malpighi identificou os capilares como estruturas envolvidas na ligação entre as artérias e as veias. O sistema cardiovascular é o primeiro sistema a desenvolver-se e a atingir o estado funcional no embrião, reflectindo a importância da rede vascular para o desenvolvimento fetal e para o crescimento dos vários órgãos [Coultas e colaboradores, 2005].

Os vasos sanguíneos são constituídos essencialmente por dois tipos de células: células endoteliais (EC), que formam a estrutura tubular do vaso e contactam directamente com o sangue, e células que revestem o endotélio, as células murais, responsáveis pela estabilização do vaso sanguíneo. As células murais podem ter fenótipos variáveis que vão desde os pericitos às células de músculo liso vascular (VSMC) [Sims, 2000]. Os pericitos aparecem isolados nos vasos de menor dimensão, os capilares, e revestem os vasos de dimensões intermédias, as arteríolas e as vénulas, com uma única camada, evitando o derrame do vaso. As VSMC envolvem as EC dos vasos intermédios ou de maior calibre, as artérias e as veias, com uma ou várias camadas celulares e regulam a compressão e vasodilatação do vaso sanguíneo [Gerhardt e Betsholtz, 2003].

2. Formação de vasos sanguíneos

A formação de vasos sanguíneos pode ocorrer por diferentes mecanismos. Os primeiros vasos sanguíneos formados durante a embriogénese têm origem nos angioblastos que se diferenciam em EC, organizando-se depois e dando origem a um plexo vascular primário, num processo designado por

vasculogénese (formação *de novo* de EC) [Coultas e colaboradores, 2005]. Diversos estudos têm sugerido que a vasculogénese também pode ocorrer no indivíduo adulto, nomeadamente em processos associados ao crescimento tumoral ou à cicatrização, sendo recrutadas células precursoras endoteliais (EPC) que podem ter origem nas células de tecidos embrionários ou na medula óssea e que se diferenciam no novo endotélio [Sata e Nagai, 2004]. A rede vascular inicialmente formada no embrião sofre posteriormente um processo de expansão e remodelação originando um sistema vascular maduro mais complexo e hierarquizado, constituído por uma rede organizada de vasos de calibre maior que se ramificam em vasos mais pequenos. Este processo envolve a formação de novos vasos sanguíneos a partir de outros pré-existentes e é designado por angiogénese [Folkman, 1971]. Através da arteriogénese, ocorre depois um espessamento da camada de VSMC, que reveste os vasos, conferindo ao vaso maior estabilidade e controlo da perfusão sanguínea [Semenza, 2007]. Os tecidos podem ainda ser vascularizados por outros mecanismos tais como intussuscepção, e no caso de cancro por co-opção, mimetização vascular e mosaicismo, mas a relevância desses processos ainda não é conhecida [Carmeliet e Jain, 2011]. Recentemente foi descrito um novo processo de vascularização de tecidos em cicatrização, o *looping* angiogénico [Kilarski e colaboradores, 2009]. Neste processo, os fibroblastos e os miofibroblastos presentes na zona em cicatrização promovem a rápida vascularização das lesões forçando a contracção do tecido de granulação e atraindo para o local de cicatrização vasos maduros que se encontram na proximidade da lesão e que se vão alongando para o tecido de granulação, formando *loops*.

3. Angiogénese

O termo angiogénese foi usado pela primeira vez em 1787 por John Hunter, um cirurgião britânico, para descrever o crescimento de novos vasos sanguíneos em chifres de rena. Só muito mais tarde, em 1935, Arthur Tremain Hertig descreveu a observação do processo angiogénico na placenta de primatas. Actualmente, a angiogénese é reconhecida como um processo complexo que decorre em várias etapas e envolve a interacção entre diferentes tipos de células, a matriz extracelular (ECM), diversas citocinas e factores de crescimento, sendo estritamente regulado pelo balanço entre factores pró-angiogénicos e inibidores da angiogénese. A angiogénese tem início quando um estímulo pró-angiogénico é libertado num local e se difunde através da ECM na sua vizinhança, activando as EC próximas, que libertam

proteases, iniciando-se a degradação da membrana basal e da ECM. Durante este processo, as células murais destacam-se das EC, permitindo a sua proliferação e migração em direcção ao gradiente de VEGF formado, num processo designado por crescimento angiogénico ou *sprouting* angiogénico (figura 1).

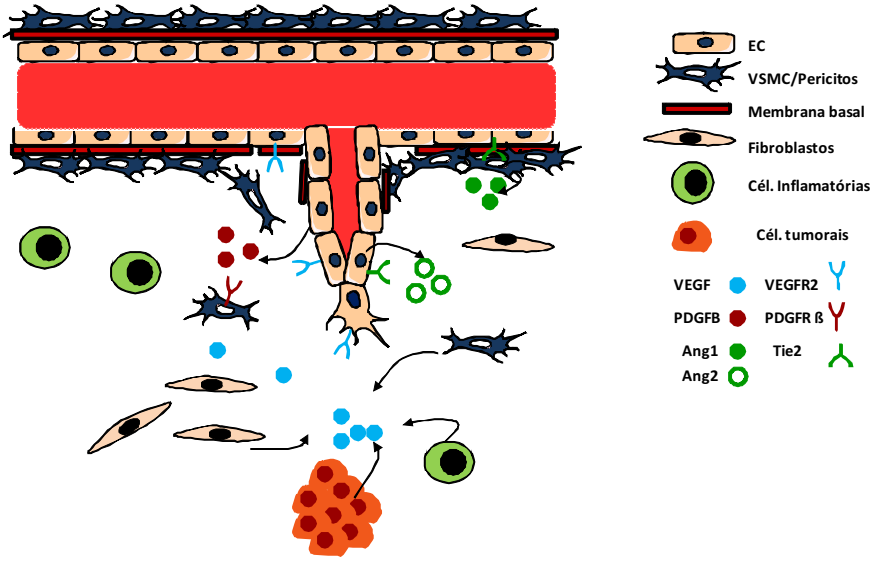

Figura 1 – *Sprouting* angiogénico

Foi recentemente descrito que durante o *sprouting* angiogénico ocorre uma especialização das EC que adquirem funções diferentes e cuja articulação é necessária de modo a formar um vaso funcional [De Smet e colaboradores, 2009]. As EC que se encontram na extremidade dos vasos sanguíneos em formação, as células *tip*, possuem evaginações da membrana citoplasmática, os *filopodia* (figura 2). Os *filopodia* são sensíveis e respondem a gradientes de diversos factores na sua proximidade que controlam a migração destas EC. Imediatamente a seguir às células *tip*, surgem as células *stalk*. Estas células proliferam, alongam o vaso e formam um lúmen, estabelecendo a circulação. O outro tipo de células envolvido neste processo são as células *phalank*, as EC mais quiescentes, de vasos consolidados, responsáveis pela manutenção da estabilidade do vaso sanguíneo.

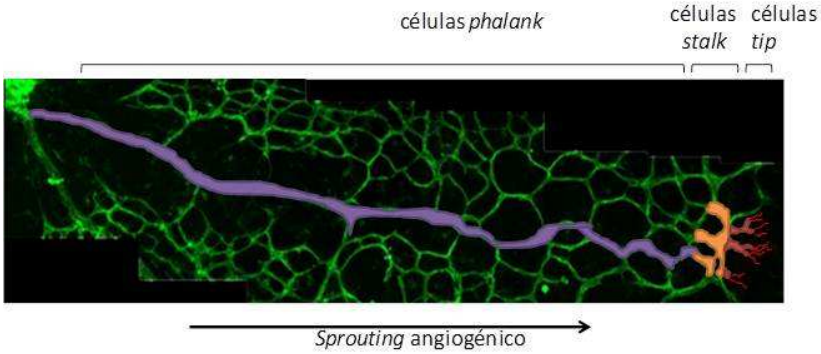

Figura 2 – Diferenciação das células endoteliais durante o *sprouting* angiogénico

Após a anastomose, isto é a fusão de novos vasos em crescimento, forma-se um novo capilar, que vai sendo recoberto pela membrana basal e rodeado por células murais, originando um vaso mais estável e conduzindo à extensão da rede vascular [Carmeliet, 2000]. Durante as últimas fases de maturação vascular, os novos vasos formados vão sendo cortados ou "podados" (*pruning* angiogénico), um mecanismo fisiológico que regula a perfusão em função das necessidades metabólicas do tecido, evitando a formação de uma rede vascular demasiado densa pelos novos vasos e tornando-a mais eficaz.

3.1. Estímulos angiogénicos

Hipóxia

O funcionamento normal dos tecidos e órgãos do organismo depende de uma irrigação sanguínea eficiente o que implica a existência de uma vasculatura intacta e madura. Quando o fornecimento de sangue não é suficiente, as áreas pouco irrigadas entram em isquemia e, consequentemente, em hipóxia. Esta constitui um dos principais estímulos à ocorrência de angiogénese, que restituirá a irrigação sanguínea a esse tecido [Fraisl e colaboradores, 2009].

O mecanismo molecular subjacente ao efeito pró-angiogénico da hipóxia está bem estudado. As células possuem sensores de oxigénio, tais como as prolil-hidroxílases (PHD), que, na presença de oxigénio, hidroxilam o factor alfa induzido pela hipóxia (HIFα). Esta hidroxilação induz a interacção do HIFα com a proteína von Hippel-Lindau (VHL), o que conduz à degradação proteolítica do HIFα. Em situação de hipóxia, não ocorre a hidroxilação do HIFα que deixa assim de ser degradado. Como consequência, as sub-unidades HIFα associam-se à subunidade HIFβ, insensível ao oxigénio e expressa constitutivamente,

originando um factor de transcrição de diversos genes associados à angiogénese, como, por exemplo, do factor de crescimento do endotélio vascular (VEGF) [Forsythe e colaboradores, 1996]. Existem três factores induzidos pela hipóxia, HIF1α, HIF2α, HIF3α. O HIF1α é ubiquamente expresso, e parece responder a situações graves de hipóxia, estimulando a expressão de genes envolvidos na glicólise. No entanto, a estabilização do HIF1α pode ocorrer na presença de concentrações normais de oxigénio, através de um mecanismo que envolve o factor nuclear kapa-B (NFκB), um factor de transcrição central na regulação do processo inflamatório, estabelecendo uma ligação entre o processo inflamatório e o HIF1α [Rius e colaboradores, 2008]. A modulação da função vascular e da angiogénese em resposta à hipóxia é especialmente regulada pelo HIF2α, cuja expressão está aumentada nas EC, onde regula a expressão de genes específicos destas células como o receptor 2 das angiopoietinas (Tie2), e o receptor 2 do VEGF (VEGFR2) [Hu e colaboradores, 2003; Mowat e colaboradores, 2010]. A função do HIF3α ainda não está completamente esclarecida. Estudos recentes sugerem que a remodelação vascular induzida pela hipóxia pode ser regulada pela actividade de enzimas modificadoras da ECM, afectando a disponibilidade de factores de crescimento associados à ECM [van Hinsbergh e Koolwijk, 2008].

Inflamação

A inflamação é um processo biológico complexo, mediado pelo sistema imunológico, que envolve diferentes tipos de células e factores que actuam coordenadamente de forma a proteger o organismo de agressões patogénicas, traumáticas ou tóxicas. O processo inflamatório é regulado por factores pró- e anti-inflamatórios, sendo auto-limitado, o que permite prevenir eventuais danificações no tecido. No entanto, se o estímulo persistir ou o processo se desregular, estabelece-se a inflamação crónica.

A inflamação crónica e a angiogénese são dois processos que ocorrem muitas vezes associados. De facto, as células inflamatórias libertam factores que promovem e regulam o crescimento vascular. Os leucócitos estão directamente implicados na angiogénese, participando na condução das células *tip*, na anastomose e no *pruning* vascular [Fantin e colaboradores, 2010; Ishida e colaboradores, 2003]. Foi também recentemente descrito que a exposição de macrófagos à hipóxia aumenta a sua maturação e a expressão de factores angiogénicos de uma forma significativa [Staples e colaboradores, 2011]. Por outro lado, os novos vasos formados sustentam o processo inflamatório, uma vez que levam até ao local da inflamação oxigénio e nutrientes adequados às necessidades metabólicas das células no local da

resposta inflamatória. Os novos vasos permitem ainda a migração de células do sistema imunológico para a zona de inflamação [Costa e colaboradores, 2007; Mor e colaboradores, 2004].

As células inflamatórias produzem uma diversidade de factores inflamatórios e angiogénicos, entre os quais VEGF, interleucina (IL) 1β, IL6, IL8, factor alfa de necrose tumoral (TNFα), angiopoietinas (Ang), factor básico de crescimento fibroblástico (bFGF), factor beta transformante do crescimento (TGFβ) e o factor B de crescimento derivado de plaquetas (PDGFB).

O TNFα é uma citocina produzida sobretudo na fase aguda da reacção inflamatória. O efeito pró-inflamatório do TNFα envolve a activação do NFκB, um factor de transcrição de acção rápida, presente nas células numa forma inactiva, que permite uma resposta imediata a um estímulo agressor. O NFκB regula os processos inflamatório e angiogénico, mas também o stresse, a proliferação e a apoptose em resposta a diferentes estímulos, controlando a expressão de mais de 200 genes. Um destes genes é o VEGF [Baldwin, 1996]. O NFκB é um factor de transcrição regulado em parte pelo estado redox da célula, sendo sobre-expresso num estado pró-oxidativo [Brar e colaboradores, 2003].

Stresse oxidativo

O conceito de stresse oxidativo foi recentemente redefinido por Dean Jones e Helmut Sies como um desequilíbrio entre oxidantes e anti-oxidantes a favor dos oxidantes, originando a desregulação da sinalização redox e do seu controlo e/ou a danificação molecular [Jones, 2006; Sies e Jones, 2008].

Diversos estudos indicam que as espécies reactivas de oxigénio (ROS) e de azoto (RNS) activam vias de sinalização intracelular relacionadas com a angiogénese [Soares e Costa, 2009; Thannickal e Fanburg, 2000]. Foi já referido que o estado redox da célula tem impacto na regulação do NFκB, que por sua vez regula a expressão de diversos factores inflamatórios e angiogénicos, entre os quais o VEGF e o óxido nítrico (NO) [Baldwin, 1996; Brar e colaboradores, 2003]. A família das oxídases do fosfato de dinucleótido de nicotinamida e adenina reduzido (NADPH) (NOX) produz superóxido a partir de oxigénio molecular. Estas enzimas existem na maioria das células, nomeadamente nas EC e VSMC, o que demonstra a importância das ROS para a homeostasia celular. As NOX regulam indirectamente a expressão de diversos genes associados à apoptose, proliferação, inflamação e angiogénese por regulação redox do NFκB. Mecanismos sensíveis ao estado redox podem também promover a estabilização do HIF1α, mesmo em condições de normoxia, levando ao aumento da expressão de genes

como o VEGF, entre outros, sugerindo que o processo é independente da hipóxia, mas regulado pelas ROS [Abe e Berk, 2002; Semenza, 2009].

O NO é um radical livre e também um importante factor inflamatório, produzido nas células inflamatórias pela síntase do NO indutível (iNOS) e no endotélio pela síntase do NO endotelial (eNOS). O NO regula a angiogénese directa e indirectamente, e estimula a vasodilatação e a permeabilidade vascular, levando à libertação de células inflamatórias no local da inflamação. O NO induz a síntese e activação do HIF1α, que promove a expressão do VEGF e este a do NO [Kimura e Esumi, 2003].

As ciclo-oxigenases e a oxídase da xantina são também enzimas que produzem ROS e que estão envolvidas na angiogénese [Costa e colaboradores, 2002; Rojas e colaboradores, 2008].

3.2. Factores reguladores da angiogénese

A angiogénese é um processo complexo, regulado por factores pró- e anti-angiogénicos.

Os inibidores endógenos da angiogénese protegem da activação da angiogénese em condições patológicas e limitam a angiogénese fisiológica. O interferão-α foi descrito em 1980 como o primeiro inibidor da angiogénese [Brouty-Boye e Zetter, 1980]. Muitos outros inibidores foram depois sendo descritos como o factor 4 plaquetário, o tetra-hidrocortisol, a angiostatina, a endostatina e a trombospondina-1 [Folkman, 2007a]. Foi na década de 1990 que surgiram os primeiros fármacos anti-angiogénicos, mas apenas em 2004 foi aprovado, pela *Food and Drug Administration*, o primeiro fármaco desenvolvido para terapia anti-angiogénica, o bevacizumab [Hurwitz e colaboradores, 2004]. Os inibidores endógenos da angiogénese, e os novos fármacos anti-angiogénicos que têm sido descobertos, regulam os estimuladores endógenos da angiogénese [Folkman, 2007a].

VEGF

O VEGF é um potente estimulador do aumento da permeabilidade dos vasos sanguíneos e da angiogénese. Foi descoberto em 1989 por Ferrara e colaboradores [Leung e colaboradores, 1989]. A família do VEGF consiste num conjunto de factores como o VEGFA (referido normalmente apenas como VEGF), considerado o factor angiogénico mais importante e potente, e também o VEGFB, o VEGFC, o VEGFD e o factor de crescimento placentário (PlGF). O VEGF tem várias isoformas, formadas por *splicing* alternativo ou processamento proteolítico, sendo o $VEGF_{121}$, o $VEGF_{165}$ e o $VEGF_{189}$ as isoformas mais

produzidas pela maior parte das células humanas. As diferentes isoformas têm afinidade diferente para o sulfato de heparano, um proteoglicano da ECM. A sinalização pelas várias moléculas de VEGF ocorre via três receptores transmembranares do VEGF (VEGFR), com actividade de cínase de tirosina, o VEGFR1, o VEGFR2 e o VEGFR3 [Tammela e colaboradores, 2005]. O VEGF estimula a angiogénese fisiológica e patológica, sobretudo através da sinalização via VEGFR2. As neuropilinas (NRP) 1 e 2 são co-receptores do VEGFR2, promovendo a ligação do VEGF ao VEGFR2 [Soker e colaboradores, 1998]. Recentemente, foi sugerido que a NRP1 é capaz de regular a migração e o *sprouting* das EC, independentemente do VEGFR2, e que o $VEGF_{121}$ pode interagir directamente com a NRP1, sem a formação do complexo NRP1-VEGFR2 [Pan e colaboradores, 2007]. O VEGF também se liga ao VEGFR1. O VEGFR1 existe nas formas membranar e solúvel e foi considerado durante muito tempo apenas um regulador negativo da angiogénese, através da sequestração do VEGF, reduzindo a sinalização via VEGFR2 [Gille e colaboradores, 2000]. No entanto, evidências recentes sugerem que o VEGFR1 pode regular a angiogénese e a inflamação através de sinalização própria. Este receptor está sobre-expresso em situações de isquemia e inflamação e em diversos tumores. É ainda expresso no endotélio de vasos em crescimento e remodelação, embora também seja detectado em EC quiescentes [Fischer e colaboradores, 2008]. O VEGF produzido pelo endotélio em níveis reduzidos mas sustentados, por longos períodos de tempo, promove a viabilidade das EC em condições normais. Perante um estímulo angiogénico diversos tipos de células como as inflamatórias, tumorais e murais libertam VEGF que aumenta a permeabilidade vascular, a proliferação e o *sprouting* das EC, originando muitas vezes uma vasculatura desorganizada e sem células de suporte. O VEGF inibe o recrutamento de pericitos para os vasos em formação [Greenberg e colaboradores, 2008] e recruta células provenientes da medula-óssea em circulação que são retidas na proximidade dos vasos em formação. Estas células aumentam a proliferação das EC no local, através da produção de factores angiogénicos distintos dos produzidos pelas células locais [Grunewald e colaboradores, 2006].

O VEGFB e o PlGF só têm elevada afinidade para o VEGFR1. O VEGFB é expresso em diversos tecidos, sendo particularmente abundante no coração, no músculo esquelético e no tecido adiposo castanho. A função do VEGFB parece estar ligada ao metabolismo energético. O VEGFB parece redundante para a angiogénese fisiológica e o seu papel na angiogénese patológica permanece por esclarecer [Bellomo e colaboradores, 2000; Fischer e colaboradores, 2008]. O PlGF estimula a migração e a sobrevivência das

EC, aumentando também a proliferação de fibroblastos e de VSMC, o recrutamento de EPC e de macrófagos. Este factor estimula a angiogénese, em parte por libertação do VEGF do VEGFR1, aumentando a sinalização VEGF-VEGFR2, podendo também formar dímeros com o VEGF. O PlGF também estimula a expressão de VEGF, FGF2, PDGFB e metaloproteínases da ECM. Apesar disso, a expressão do PlGF é redundante no desenvolvimento vascular e manutenção dos vasos sanguíneos em situações fisiológicas. O PlGF é, no entanto, abundantemente expresso em situações patológicas, tendo sido sugerido como um marcador específico da angiogénese patológica [Fischer e colaboradores, 2008].
O VEGFC e o VEGFD podem ligar-se ao VEGFR2 e ao VEFGR3. O aumento da permeabilidade vascular induzida pelo VEGFC e pelo VEGFD foi sempre associado à activação do VEGFR2. A activação do VEGFR3 tem sido relacionada com a estimulação da linfangiogénese [Achen e colaboradores, 1998; Joukov e colaboradores, 1996]. Tammela e colaboradores demonstraram recentemente que o VEGFR3 é altamente expresso nas EC dos *sproutings* angiogénicos, sustentando a proliferação, a migração e a sobrevida das EC de um modo independente do VEGFR2, implicando o VEGFR3 na regulação do processo angiogénico [Tammela e colaboradores, 2008].

PDGF

Um dos passos fundamentais na angiogénese, necessário à estabilização e funcionalidade dos vasos sanguíneos, é o recrutamento de pericitos para a vasculatura em formação. Os pericitos são inicialmente recrutados pelo PDGFB, segregado pelas EC, que fica retido na proximidade do endotélio, e que promove a proliferação e a migração das células murais, guiando-as e atraindo-as até às EC, dado que as células murais expressam o receptor β do PDGFB (PDGFRβ) [Lindblom e colaboradores, 2003]. À medida que rodeiam as EC, os pericitos produzem VEGF necessário à sobrevivência das EC, protegendo-as. No entanto, elevadas concentrações de VEGF inibem a sinalização via PDGFRβ e originam deficiências na muralização, tornando os vasos permeáveis e tortuosos, levando a hemorragias e à perda de funcionalidade.

Angiopoietinas

A interacção entre endotélio e células murais envolve outras vias de sinalização como é o caso da família das Ang Esta família consiste num conjunto de quatro factores de crescimento, Ang1, Ang2 e seus

ortólogos Ang3 (Ratinho) e Ang4 (Homem). Todas as Ang são ligandos do Tie2 e a Ang1 e Ang4 podem activar também o Tie1 [Saharinen e colaboradores, 2005]. A Ang1 é considerada o principal agonista do Tie2 [Suri e colaboradores, 1996], enquanto a Ang2 é, na maioria dos contextos, antagonista do Tie2, competindo com a Ang1 [Maisonpierre e colaboradores, 1997]. A acção da Ang3 e da Ang4 não é ainda bem conhecida, mas a Ang3 tem sido descrita como antagonista e a Ang4 como agonista do Tie2 [Valenzuela e colaboradores, 1999]. A Ang1 é produzida por pericitos, pelas VSMC e células tumorais, e tem grande afinidade para a ECM, enquanto a Ang2 é produzida pelas células endoteliais. O Tie2 é expresso predominantemente nas EC, embora alguns tipos de macrófagos e células tumorais também o possam expressar [Armulik e colaboradores, 2005]. Foi identificada uma forma solúvel do Tie2 no plasma, que pode estar envolvida na captação de ligandos sem que ocorra sinalização [Reusch e colaboradores, 2001]. Quando o vaso sanguíneo está formado, a Ang1 induz a localização do Tie2 na zona das junções intercelulares, mantendo o vaso quiescente. A Ang1 também estimula a muralização do vaso e a deposição da membrana basal, promovendo a sua estabilização. No entanto, após a ruptura de ligações intercelulares no endotélio, estimulada pelo VEGF, a Ang1 promove a localização do Tie2 para a zona de contacto EC-ECM, acelerando a angiogénese e cooperando com o VEGF [Saharinen e colaboradores, 2008]. Na presença de factores inflamatórios e angiogénicos, como o VEGF, as células *tip* do endotélio libertam Ang2, que antagoniza a sinalização Ang1/Tie2, promovendo a desmuralização dos vasos, a permeabilidade vascular, a proliferação e o *sprouting* angiogénico [Fiedler e colaboradores, 2006; Oh e colaboradores, 1999]. No entanto, na ausência de VEGF, a Ang2 estimula a regressão vascular. Dependendo do contexto, a Ang2 pode ainda funcionar como agonista do Tie2 em situações de estimulação prolongada [Saharinen e colaboradores, 2010].

Existem ainda muitos outros factores reguladores da angiogénese como por exemplo o Notch, uma família de proteínas transmembranares que, durante o estímulo angiogénico, previne o *sprouting* angiogénico excessivo, promovendo um desenvolvimento ordenado de vasos funcionais [Hellstrom e colaboradores, 2007]. O TGFβ parece estimular a libertação de citocinas angiogénicas pelas células inflamatórias, induzir a expressão de VEGF e promover a angiogénese tumoral [Gaengel e colaboradores, 2009], podendo também ter efeitos anti-angiogénicos, dependendo do contexto e dos receptores presentes [Carmeliet e Jain, 2011]. O FGF estimula directa e indirectamente a angiogénese,

promovendo a libertação de factores angiogénicos por outros tipos de células [Beenken e Mohammadi, 2009]. Concentrações baixas de FGF mantêm a integridade vascular, enquanto que a produção excessiva de FGF promove a angiogénese tumoral.

3.3. Angiogénese fisiológica e patológica

A angiogénese é um processo fisiológico vital durante o desenvolvimento embrionário e o crescimento do organismo. Na idade adulta, a angiogénese ocorre apenas em situações fisiológicas reguladas em que os vasos sanguíneos, apesar de estarem já formados, proliferam durante fenómenos como a cicatrização e regeneração dos tecidos, o ciclo menstrual da mulher, a gravidez, o crescimento capilar e em situações de isquemia ou de inflamação tecidular [Folkman, 2007b].

A observação de que o aparecimento de tumores está associado ao aumento da vascularização peritumoral foi descrita por Goldman e data de há 100 anos (1907). Mas foi há cerca de 40 anos que Judah Folkman propôs que os tumores dependem dos vasos sanguíneos na sua proximidade para sobreviverem, e que a destruição desses vasos conduziria à destruição do tumor [Folkman, 1971]. Estas descobertas iniciaram uma nova área de estudo: a terapia anti-angiogénica como supressora do crescimento tumoral. O conceito de patologias dependentes da angiogénese surgiu em 1972, com o reconhecimento de que patologias não neoplásicas associadas a um estado de inflamação crónica e muitas vezes de stresse oxidativo, como a psoríase, estão também associadas à angiogénese crónica, que amplifica o processo inflamatório [Folkman, 1972]. Seguiram-se desde então descobertas de muitas outras patologias associadas à angiogénese [Folkman, 2007b].

A angiogénese patológica assenta nos mesmos mecanismos moleculares básicos que intervêm na angiogénese fisiológica. No entanto, contrariamente ao que acontece na angiogénese fisiológica, na angiogénese patológica há um desequilíbrio entre os mecanismos reguladores pró- e anti-angiogénicos e o processo não regride, tornando-se persistente. São exemplo de patologias associadas a uma excessiva angiogénese o cancro, a aterosclerose, a psoríase, a doença de Crohn, a fibrose pulmonar, a doença de Alzheimer, a endometriose, a artrite reumatóide, a asma, a esclerose múltipla, a nefropatia diabética e a retinopatia diabética [Costa e colaboradores, 2007; Folkman, 2007a; Soares e Costa, 2009]. Paradoxalmente, na diabetes ocorre simultaneamente uma deficiência na angiogénese a nível periférico associada ao desenvolvimento de úlceras de difícil cicatrização. São exemplos ainda de patologias

associadas a uma deficiente angiogénese a isquemia do miocárdio, a doença arterial periférica, a pré-eclâmpsia e algumas patologias neurológicas [Carmeliet, 2005].

Nos últimos anos tem sido desenvolvida uma intensa investigação na procura de agentes pró-angiogénicos e anti-angiogénicos, na tentativa de intervir em diversas patologias, tendo sido já descritos vários reguladores da angiogénese que são usados como agentes terapêuticos [Folkman, 2007a].

4. Alimentação e saúde - interface com o meio ambiente

A alimentação constitui uma importante forma de influência do meio ambiente sobre o organismo humano, exercendo um impacto profundo na saúde do indivíduo. O modo como nos alimentamos determina também, de uma forma considerável, o funcionamento do mundo, tendo repercussões ecológicas, sociais e económicas. Alimentarmo-nos responsavelmente é entender e regular esta complexa relação [Pollan, 2011].

A recente transição em massa das populações das regiões rurais para zonas preferencialmente urbanas, já com cerca de 50% da população mundial, gerou alterações significativas dos hábitos alimentares [Porter e colaboradores, 2011]. Em termos gerais, poder-se-á assumir que o fenómeno da globalização económica trouxe também uma globalização alimentar e, em parte relacionada com esta, uma globalização no padrão de doenças [Calhau, 2011]. O tipo de alimentação actual tem sido associado à prevalência de doenças como a diabetes tipo 2, a obesidade, as doenças cardiovasculares, o cancro e as doenças neurodegenerativas, que se encontram associadas a inflamação crónica e à angiogénese. A abundância alimentar a que grande parte das sociedades urbanas está sujeita actualmente alia-se à carência de alguns constituintes alimentares vitais para o desenvolvimento e manutenção da saúde humana, como por exemplo os minerais e as vitaminas, e está ainda associada a uma menor ingestão de compostos bioactivos, como os polifenóis, presentes nos hortofrutícolas [Calhau, 2011]. A descoberta de que a saúde depende em grande parte da alimentação e a revelação da excelência da dieta mediterrânica, na qual o vinho, o azeite, as frutas e legumes têm o seu papel, abriram novas perspectivas de investigação exploradas por diversos autores [Hipólito-Reis, 2008].

5. Polifenóis

Os polifenóis são constituintes da dieta com especial interesse, dada a evidência epidemiológica que sugere que o desenvolvimento de determinadas patologias como as doenças cardiovasculares, a diabetes tipo 2, as doenças neurodegenerativas e o cancro, diminui com o consumo de dietas ricas em legumes, frutos e certas bebidas como sumos de fruta, chá, vinho e cerveja. Esta protecção tem sido atribuída à presença de um conjunto de compostos com estruturas químicas muito diversas, designados por polifenóis, tendo sido descritos já mais de 8000 [Bravo, 1998; Soleas e colaboradores, 1997; Stevens e Page, 2004].

Os polifenóis são sintetizados pelas plantas como resultado do seu metabolismo e como resposta a estímulos exteriores, desempenhando na planta um papel protector da radiação e de outros agentes agressores, intervindo na pigmentação, no crescimento e na reprodução das plantas, e são por estas razões componentes essenciais na sobrevivência das espécies [Soleas e colaboradores, 1997]. A composição e concentração em polifenóis dependem da planta em causa, do local em que é cultivada, do clima e da exposição solar, mas também do processamento e condições de armazenamento dos alimentos, entre outros factores [Bravo, 1998].

Estes compostos manifestam uma actividade biológica vasta, incluindo actividade anti-bacteriana, anti-vírica, anti-oxidante, anti-inflamatória, anti-carcinogénica e anti-obesidade [Araújo e colaboradores, 2011; Monteiro e colaboradores, 2009; Oak e colaboradores, 2005; Rahman e colaboradores, 2006; Stevens e Page, 2004], sendo por isso objecto de interesse sobretudo para a indústria alimentar, cosmética e farmacêutica. Os polifenóis estão directa ou indirectamente relacionados com a qualidade dos alimentos, contribuindo para a sua cor, sabor, odor, adstringência e estabilidade oxidativa [Naczk e Shahidi, 2004].

Os polifenóis englobam um variado número de compostos quimicamente muito diferentes entre si e nem sempre os polifenóis ingeridos em maior quantidade pela sua abundância nos alimentos e bebidas são necessariamente os mais absorvidos e os mais bioactivos [Manach e colaboradores, 2004]. Embora não sejam classificados como nutrientes essenciais e não se conheçam manifestações clínicas associadas à sua deficiência, os polifenóis contribuem, sem dúvida, para o bem-estar físico e para a promoção da saúde. Estima-se que a ingestão diária média de polifenóis seja cerca de 1 grama por dia [Scalbert e Williamson, 2000].

6. Flavonóides

Dentro de todos os grupos de compostos que constituem os polifenóis, o grupo dos flavonóides é o mais representativo. Estes compostos têm em comum o núcleo flavânico, representado na figura 3, que consiste numa estrutura C6-C3-C6, com 2 anéis aromáticos A e B, ligados por uma cadeia de 3 carbonos (o anel C). Os flavonóides podem ser divididos em várias subfamílias, como os flavonóis, os flavanóis, as flavonas, as flavanonas, as isoflavonas, as antocianidinas e as chalconas, de acordo com o nível de oxidação do anel C e com os seus substituintes [Scalbert e Williamson, 2000].

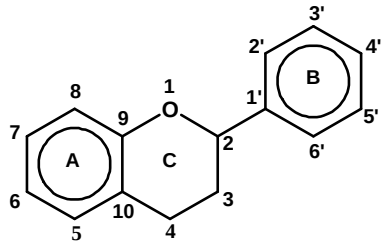

Figura 3 – Representação da estrutura química do núcleo flavânico

6.1. Xanto-humol, isoxanto-humol e 8-prenilnaringenina

O xanto-humol (XN), o isoxanto-humol (IXN) e a 8-prenilnaringenina (8PN) são flavonóides prenilados (ver figura 4).

O lúpulo (*Humulus lupulus* L.) é rico em flavonóides prenilados e o primeiro registo da sua utilização no fabrico da cerveja data do ano de 1079. No entanto, muito antes da utilização do lúpulo no fabrico da cerveja, já se utilizava o lúpulo como planta medicinal no tratamento de diversas manifestações patológicas tais como anorexia, indigestão, nervosismo, dor de cabeça, reumatismo e infecções [Moir, 2000]. O extracto de lúpulo é adicionado ao mosto utilizado durante a produção da cerveja, sendo responsável em parte pela sua conservação e pelas características organolépticas desta bebida. A cerveja tem cerca de 500-1000 mg/L de polifenóis [Scalbert e Williamson, 2000].

O XN é uma chalcona prenilada e o prenilflavonóide mais abundante no lúpulo. O XN tem sido considerado uma molécula promissora uma vez que lhe têm sido atribuídas propriedades anti-carcinogénicas, anti-inflamatórias, anti-angiogénicas, anti-oxidantes e reguladoras do metabolismo

glicídico e lipídico [Albini e colaboradores, 2006; Gerhauser e colaboradores, 2002; Monteiro e colaboradores, 2008; Nozawa, 2005], podendo contribuir para a prevenção de várias patologias de elevada incidência e mortalidade na civilização ocidental. Durante o processo de fabrico da cerveja, a maior parte do XN é convertido no seu isómero IXN, uma flavanona prenilada, com maior solubilidade na cerveja. A 8PN é também uma flavanona prenilada presente no lúpulo e na cerveja, sendo o fitoestrogénio mais potente isolado até hoje. Na realidade, a 8PN é considerada um agonista estrogénico [Milligan e colaboradores, 2002].

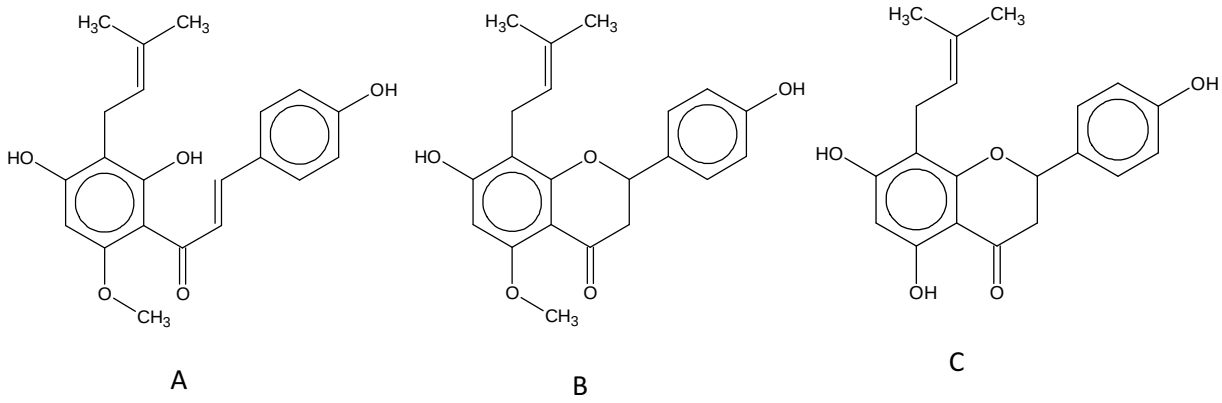

Figura 4 – Representação da estrutura química do xanto-humol (A), do isoxanto-humol (B) e da 8-prenilnaringenina (C)

6.2. Catequina

Na cerveja é ainda possível encontrar, entre muitos outros polifenóis, a catequina [Gerhauser, 2005]. A catequina é um flavanol, um dos grupos de flavonóides mais abundantes na alimentação humana. As unidades monoméricas dos flavanóis diferem entre si na hidroxilação dos anéis A e B e na estereoquímica da posição C3 (figura 5).

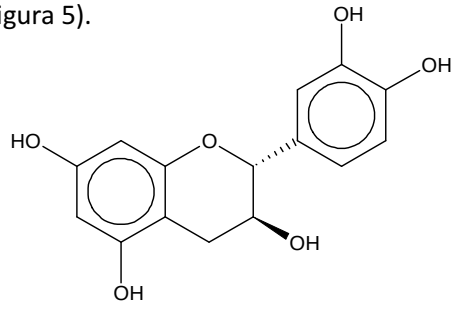

Figura 5 – Representação da estrutura química da (+)-catequina

A catequina existe em níveis elevados na casca e sementes de frutos como maçãs, uvas, pêssegos e cerejas assim como no vinho tinto, no chá verde e no chocolate [Bravo, 1998; Manach e colaboradores, 2004]. Embora a catequina e a epicatequina sejam os flavanóis mais abundantes nos frutos, outras catequinas como a epicatequinagalato, a epigalocatequina e a epigalocatequinagalato (EGCG), têm suscitado mais atenção da comunidade científica dada a sua elevada concentração no chá verde e as suas propriedades anti-carcinogénicas, anti-angiogénicas, anti-inflamatórias, anti-oxidantes e inibidoras da migração das VSMC [El Bedoui e colaboradores, 2005; Han, 1997; Tang e colaboradores, 2007]. Apesar de existirem poucos estudos especificamente sobre a catequina, também lhe têm sido atribuídas características anti-inflamatórias e protectoras da aterosclerose, inibidoras da angiogénese tumoral e anti-carcinogénicas [Auclair e colaboradores, 2009; Guruvayoorappan e Kuttan, 2008; Weyant e colaboradores, 2001], podendo vir a constituir uma molécula promissora dada a sua abundância em vários alimentos.

7. Polifenóis e angiogénese

O efeito anti-angiogénico de polifenóis naturais tem sido alvo de estudo nos últimos anos, dada a relação entre a angiogénese e um número crescente de patologias com grande prevalência actualmente, como por exemplo as doenças cardiovasculares, a diabetes tipo 2 e o cancro. Foi demonstrado que polifenóis existentes no vinho tinto e no chá verde diminuem a expressão de factores angiogénicos e de factores inflamatórios assim como a proliferação e migração de EC e VSMC, processos fundamentais para a formação de novos vasos sanguíneos [Barthomeuf, 2007; Iijima e colaboradores, 2002; Oak e colaboradores, 2005]. Concretamente, o resveratrol e as antocianinas existentes no vinho tinto diminuem a produção de citocinas e de VEGF [Dulak, 2005; Lamy e colaboradores, 2006]. A EGCG do chá verde inibe a formação de vasos tumorais em modelos animais, diminuindo a viabilidade e a proliferação das células tumorais e o desenvolvimento do tumor [Mojzis e colaboradores, 2008], e bloqueia a activação do HIF1α, diminuindo o VEGF, através das vias de sinalização do PI3K/Akt e Erk1/2 [Zhang e colaboradores, 2006]. A quercetina tem efeitos anti-carcinogénicos, inibindo várias etapas da angiogénese [Igura e colaboradores, 2001] e a genisteína diminui a expressão do VEGF e do factor de crescimento epidérmico (EGF), diminuindo o risco de formação de tumores [Lamartiniere e Wang, 1999].

Relativamente aos polifenóis XN, IXN, 8PN e catequina, alguns estudos foram já realizados com o intuito de avaliar a modulação da angiogénese e da inflamação pelos referidos polifenóis. Albini e colaboradores demonstraram pela primeira vez que a administração subcutânea de XN diminui eficazmente a angiogénese e o crescimento de tumor mamário inoculado em ratinhos C57/Bl6N, por inibição do NFκB e da via do Akt nas EC [Albini e colaboradores, 2006]. A administração oral de XN resultou também na diminuição da inflamação e na redução da densidade vascular de células MCF7 de adenocarcinoma mamário inoculadas em ratinhos, confirmando que o XN é simultaneamente um agente anti-inflamatório, anti-angiogénico e anti-tumoral [Monteiro e colaboradores, 2008]. Bertl e colaboradores verificaram que o IXN diminui *in vitro* a proliferação, a migração e a diferenciação de células HMEC-1 microvasculares endoteliais [Bertl e colaboradores, 2004]. As características anti-angiogénicas da 8PN foram estudadas por Pepper e colaboradores que verificou que a 8PN diminui a angiogénese induzida pelo VEGF e pelo bFGF *in vitro* assim como *in vivo* usando o ensaio da membrana cório-alantóica [Pepper e colaboradores, 2004]. Estudos sobre a catequina revelaram que este polifenol actua sobre as VSMC, inibindo a sua migração [El Bedoui e colaboradores, 2005] e que possui propriedades inibidoras da angiogénese tumoral [Guruvayoorappan e Kuttan, 2008].

A possibilidade dos polifenóis diminuírem o risco de estabelecimento ou a evolução de patologias intervindo na angiogénese e/ou na inflamação é de facto interessante e tem sido explorada para os mais variados tipos de polifenóis. No entanto, apesar do estudo já realizado sobre estes polifenóis, a sua acção específica sobre o endotélio e sobre as VSMC não é ainda bem conhecida, sobretudo no que diz respeito à modulação da angiogénese por interferência na interacção entre estes dois tipos de células indispensáveis ao processo angiogénico. O efeito destes polifenóis na modulação da angiogénese tem sido também pouco explorado em ensaios *in vivo* que confirmem os resultados obtidos com culturas de células para alguns destes compostos, ou relativamente à angiogénese não tumoral, ou ainda no que diz respeito aos mecanismos celulares envolvidos nesta modulação.

OBJECTIVOS

Assim, abordámos, ao longo deste trabalho, as seguintes questões:

Capítulo 1

Atendendo a que só na última década se começaram a encarar as células murais como importantes na regulação e maturação do processo angiogénico, referindo-se a grande maioria dos estudos em angiogénese apenas a estudos em EC,

a) Será que os polifenóis exercem efeitos diferentes nas EC e VSMC?
b) Actuarão de modo semelhante nos vasos angiogénicos e nos vasos maduros?

Capítulo 2

a) Será idêntica a modulação da angiogénese e da inflamação pelo XN, IXN e 8PN?
b) Serão os efeitos destes três polifenóis na angiogénese e na inflamação *in vitro* semelhantes aos efeitos *in vivo*?

Capítulo 3

Atendendo a que a fortificação de alimentos com polifenóis tem sido alvo de interesse pela indústria alimentar, será que a ingestão destes alimentos pode intervir na modulação da angiogénese e da inflamação?

Capítulo 4

Alguns polifenóis classicamente considerados menos potentes no que respeita às suas propriedades anti-angiogénicas e anti-carcinogénicas, como a catequina, estão presentes em maior quantidade na dieta e em alimentos mais variados, do que outros considerados mais potentes.

a) Terá a catequina, nos modelos utilizados, eficácia na regulação da angiogénese e da inflamação?
b) Será o seu efeito semelhante em modelos de angiogénese fisiológica e patológica?

Capítulo 5

a) Como afectarão estes polifenóis a organização vascular durante o processo angiogénico?
b) Será que alteram a interacção EC-VSMC necessária à maturação do vaso em crescimento?

c) Que vias moleculares envolvidas na regulação da angiogénese e da inflamação serão moduladas por estes compostos?

Capítulo 6

A baixa biodisponibilidade atribuída de um modo geral aos polifenóis e a dificuldade de associar um efeito protector directamente a um composto têm levantado dúvidas sobre a eficácia dos polifenóis.

a) Será que os polifenóis poderão de facto vir a ser utilizados nutricional ou farmacologicamente na prevenção ou tratamento de determinadas patologias?

CAPÍTULO 1

Evidence for the effects of xanthohumol in disrupting angiogenic vessels, but not stable ones

Int J Biomed Sci 3: 279-286, 2007

Negrão MR, Íncio J, Lopes R Azevedo I, Soares R

INTERNATIONAL JOURNAL of BIOMEDICAL SCIENCE

Evidence for the Effects of Xanthohumol in Disrupting Angiogenic Vessels, but not Stable Ones

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ABSTRACT

Angiogenesis is a complex multistep process that comprises proliferation, migration, anastomosis of endothelial cells, followed by stabilization of the newly formed vessel through the attachment of support cells. This process is imbalanced in a huge number of disorders, including cardiovascular disease, diabetes and cancer. Evidence indicates that xanthohumol (XN), a prenylated chalcone present in beer, exerts anti-angiogenic properties. However, its precise effect within the angiogenic steps is not accurately established. The purpose of the present study was to examine which features of the angiogenic process can be disturbed by XN. Human umbilical vein endothelial cells (HUVEC) and human fetal aortic smooth muscle cells (SMC) were incubated with xanthohumol at 5 and 10 μM, and cell viability, apoptosis, invasion and capillary-like structures formation were addressed. Treatment with 10 μM XN significantly decreased viability and invasion capacity and increased apoptosis in both cell types as assessed by MTT, double-chamber assay and TUNEL assay respectively. The two concentrations of XN further led to a significant reduction in the number of capillary-like structures formation, when HUVEC were cultured on growth factor reduced-Matrigel-coated plates. Interestingly, XN exhibited the opposite effect whenever HUVEC were co-cultured with SMC, leading to an increase in the number of cord structures. In addition, incubation of both types of cells with XN resulted in reduced activity of NFκB, a transcription factor implicated in these cell fates. Given the absence of adverse effects in mature vasculature by XN, these findings emphasize the potential use of XN against pathological situations where angiogenesis is stimulated.

Keywords: angiogenesis; endothelium; matured vessels; neovessels; polyphenols; smooth muscle cells

INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a complex multistep process that involves extracellular matrix degradation, endothelial cell (EC) proliferation, migration and anastomosis, ending up by the recruitment and adhesion of pericytes or smooth muscle cells (SMC) that promote neovessel stability. This process is imbalanced in a vast series of pathological situations, such as cardiovascular disease, psoriasis, diabetic retinopathy, rheumatoid arthritis and cancer (1). Evidence has been gathered regarding the effects of diet flavonoids in preventing angiogenesis (2-9). One of these molecules, xanthohumol (XN), is a prenylated chalcone present in beer (2, 4, 8-10). Epidemiological and experimental evidence indicates that XN is able to prevent proliferation and migration (2-4), rendering this agent a useful chemopreventive cancer agent. XN was found to exert anti-pro-

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Note: This manuscript is supported by FCT (POCI/BM/55556), iBeSa and by "Investigação científica na pré-graduação" program funded by University of Porto.

liferative effects in human breast cancer MCF7 cells (10, 11) and in prostate epithelial cells (12, 13). Using human colon cancer cells, Pan et al (14) also showed that XN down-regulated bcl-2 expression, preventing, thus, caspase cascade activation. XN also exhibited anti-oxidant activity and anti-inflammatory properties (2-4, 10), preventing tumour progression. Oxidative stress and inflammation are two processes that cope with angiogenesis (1, 2). Therefore, we anticipate that in agreement with the effects of other polyphenols, XN is also capable of exerting anti-angiogenic effects. However, only a few reports focused on the direct effects of this polyphenolic compound in vascular wall cells. Albini et al (8) have recently elucidated the anti-angiogenic effects of XN on EC. These authors found that EC's ability to proliferate and invade was effectively inhibited by XN at 5μM. Furthermore, XN also prevented formation of vascular networks by EC in matrigel-coated plates (8).

In order to obtain matured stable vessels, newly formed vascular structures, which are only formed by a thin layer of EC, must be covered by support cells. Therefore, smooth muscle cells proliferation and migration are essential features for the assembly of normal vascularisation. Despite several papers report the inhibitory effects of natural polyphenols in smooth muscle cells, namely by preventing SMC growth and adhesion (15, 16), to our knowledge there are no studies regarding the effects of XN on this type of cells. The vascular effects of polyphenols are relevant for preventing angiogenic vessels formation, which is a pertinent issue for their use as anti-angiogenic agents in the treatment of a huge number of disorders (2-7). Nonetheless, it is also important that these compounds do not affect stabilized blood vessels. The aim of the current study was to identify the effects of XN within the whole angiogenic process. Accordingly, the present study addressed cell viability, apoptosis, invasion and capillary-like structures formation in endothelium and vascular smooth muscle cell cultures and in co-cultures of both cell types.

MATERIALS AND METHODS

Human umbilical vein endothelial cells (HUVEC) were obtained from ScienceCell Research Labs (San Diego, USA). Cells were used between passages 3 and 8 in this study. HUVEC were cultured in M199 medium (Sigma-Aldrich, Portugal) supplemented with 20% fetal bovine serum (FBS) (Invitrogen Life Technologies, Scotland, UK), 1% penicillin/streptomycin (Invitrogen Life Technologies, Scotland, UK), 0.01% heparin (Sigma-Aldrich, Portugal) and 30 μg/mL endothelial cell growth supplement (ECGS) (Sigma-Aldrich, Portugal), and maintained at 37°C in a humidified 5% CO_2 atmosphere. Cells were seeded on plates coated with 0.2% gelatin (Sigma) and allowed to grow. Human fetal aortic smooth muscle (FLTR) cells (SMC) were kindly provided by Dr James Mc Dougall (Fred Hutchinson Cancer Research Center, Seattle, Washington, USA). FLTR cells are immortalized SMC, which retain much of the phenotype of normal adult aortic SMC. These cells exhibit no phenotypic changes after passage 30 (17, 18). Cells were used in passages 50 through 60. SMC were cultured in high glucose Dulbeco's modified Eagle's medium (DMEM). Cells were maintained in 10% FBS and 1% penicillin/streptomycin and cultured at 37°C in a humidified 5% CO_2 atmosphere.

XN (Sigma Aldrich, Lisbon, Portugal) was dissolved in ethanol and then added to cell culture medium at a concentration of 5 μM or 10 μM, established according to its IC_{50} as previously described (19). XN was added to serum-free M199 medium containing endothelial cell growth supplement (ECGS) (HUVEC) and serum-free DMEM (SMC) during 24 h. Control cells were incubated with vehicle (ethanol). Ethanol concentrations were kept below 0.1% in every culture.

HUVEC and SMC were allowed to grow until 70-90% confluence and then incubated with XN or ethanol for 24 h. After the incubation period, cells were washed twice with PBS and subjected to MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as previously described (11).

Cells (1×10^4) plated in glass coverslips were grown for 24 h and then incubated with XN or ethanol for 24 h. TUNEL assay (Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling) was performed using the In Situ Cell Death Detection kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturing instructions (11, 20, 21).

The invasive cell behavior in the presence of XN was quantified *in vitro* using a double-chamber assay by counting the number of cells that invaded a Transwell BD-Matrigel basement membrane matrix inserts (BD-Biosciences, Belgium), according to manufacturer´s instructions. FBS was used as chemoattractant. Results represent the ratio between invading cells in XN treated cultures compared to invasion in control cultures for the same initial amount of cells cultured.

Matrigel assay was performed on growth factor reduced-Matrigel (GFR-Matrigel) (BD Biosciences, Belgium)-coated plates for 24 h as previously described (22).

Briefly, Cells were cultured on GFR-Matrigel coated plates for 24 hours, in medium containing XN or vehicle (ethanol). When cultured on matrigel, endothelial cells assemble into capillary-like structures. The number of cord-like structures was then measured in an inverted microscope. Each cord portion between the ramifications was considered one cord unit. Mean values were obtained by evaluating the whole cultures of each well under the same treatment. Capillary-like structures formation was also evaluated in co-cultures of HUVEC with SMC. SMC were added 6 h after the establishment of cord-like structures by HUVEC. Treatments were performed as described above. A semi-quantitative measurement of cord formation in GFR-Matrigel cultured HUVEC and HUVEC and SMC co-cultures was developed (tube formation index) as previously described (22).

NFκB activity was determined by ELISA assay. Nuclear extracts were prepared from HUVEC and SMC cells using the Nuclear extraction kit (Active Motif, USA). NFκB activity was measured using TransAM NFκB p65/p50 transcription factor assay kit (Active Motif, CA, USA). In brief, nuclear extract samples (5μg) were added to a 96-well plate with immobilized oligonucleotide containing the NFκB consensus site. Sample wells were incubated with NFκB p65 subunit primary antibody, followed by incubation with HRP-conjugated secondary antibody. Quantification was performed at 450 nm and 650 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent, USA).

All experiments were performed in triplicate. Quantifications are expressed as mean (± SEM) of 3 independent experiments and are expressed as percentage of control, which was considered to be 100%. Statistical significance of difference between various groups was evaluated by one-way analysis of variance (ANOVA test) followed by the Bonferroni test. For comparison between two groups, Student's t-test was used. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $p < 0.05$.

RESULTS

Effects of XN in HUVEC

To investigate whether XN exerted any effect on endothelial cells, HUVEC were incubated with 5 or 10 μM XN and cell viability was assessed by MTT assay. Although no change in HUVEC viability was found upon treatment with 5 μM XN, a significant reduction in viable cells was observed after incubating these cells with 10 μM (23.28% ± 7.77%, *$p < 0.05$ vs. control) (Figure 1a). These findings were confirmed by a strong increase in the percentage of apoptotic cells after incubation with 10 μM XN (436.67% ± 52.03, *$p < 0.05$ vs. control) (Figure 1b) as evaluated by TUNEL assay. Again, the number of apoptotic cells was not affected by 5 μM XN (Figure 1b). We next investigated whether XN affected migration and invasion capacity of HUVEC using a double-chamber assay. As illustrated in Figure 1c, XN resulted in a significant decrease in the migratory capacity of HUVEC in a dose-dependent manner (46.67 ± 17.79 and 27.85 ± 7.98 for 5 and 10 μM respectively; *$p < 0.05$ vs. control).

Effects of XN in SMC

After the angiogenic process takes place, the endothelial cell layer of the new vessel stimulates the formation of a basement membrane and the attachment of support cells.

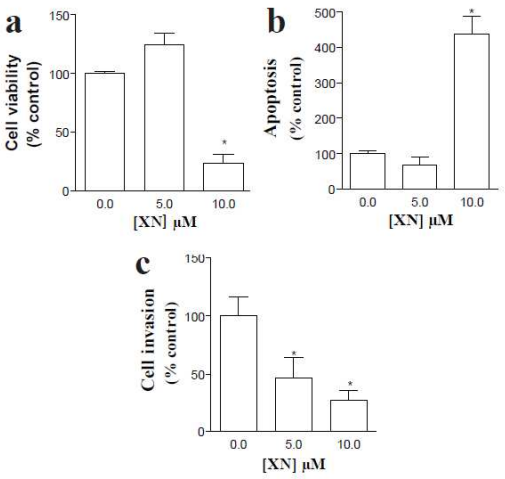

Figure 1. Effects of xanthohumol (XN) in HUVEC viability, apoptosis and invasive capacity. **(a)** Reduced cell viability after incubation with 10 μM XN, as evaluated by MTT assay (*$p<0.05$ vs. Control). XN at 5 μM did not significantly affect HUVEC viability. Results are expressed as percentage of control cells. Bars represent mean (SEM); **(b)** Increased percentage of apoptotic cells after incubation with 10 μM XN (*$p<0.05$ vs Control). No significant results were found upon 5 μM XN treatment relative to controls. Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture; **(c)** Effect of XN in HUVEC invasion. Incubation with XN resulted in decreased cell invasion in a dose-dependent manner (*$p<0.05$ vs. Control). Bars represent the percentage of invading cells relative to the initial amount of cells cultured.

This process is essential for the normal function of the neovessel, since angiogenic vessels, which are only formed by EC, often regress (1, 22). We, thus, evaluated the effects of XN in SMC viability, apoptosis and capacity to invade. A decrease in SMC viability was found after incubation of VSMC with 5 (69.9% ± 0.76%, *p< 0.05 vs. control) and 10 μM XN (51.8% ± 2.76%, *p< 0.05 vs. control) as analyzed by MTT (Figure 2a). According to our findings and in agreement with published work (11, 13), the following studies were performed using XN at 10 μM concentration. Incubation with 10 μM concentration resulted in a significant increase in the percentage of apoptotic cells by TUNEL assays (999.43% ± 113.99%, *p< 0.05 vs. control) (Figure 2b). SMC were then cultured in inserts in serum-free medium and invasive capacity was evaluated in double-chamber assays. FBS was used as chemoattractant in the lower chamber in the presence of XN or ethanol. Treatment with XN resulted in a drastic reduction in SMC invasive ability as compared to controls (26.00% ± 1.30%, *p< 0.05 vs. control) (Figure 2c).

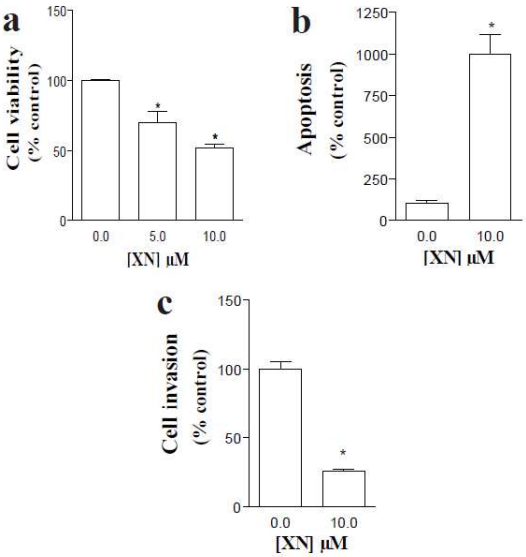

Figure 2. Effects of xanthohumol (XN) in SMC viability, apoptosis and invasive capacity. **(a)** Decreased cell viability after incubation with 5 μM and 10 μM XN as evaluated by MTT assay (*p<0.05 vs. Control). Results are expressed as percentage of control cells. Bars represent mean (SEM); **(b)** XN treatment significantly increased apoptosis as examined by TUNEL assay (*p<0.05 vs. Control). Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained and DAPI-stained nuclei in every culture; **(c)** XN resulted in effective reduction in invasive capacity as quantified in a double-chamber assay (*p<0.05 vs. Control). Bars represent the ratio between invading cells and the initial amount of cells cultured. Assays were repeated three times and performed in duplicate.

Effects of XN in cord-like structures formed by co-cultures of HUVEC and SMC

To form a new blood vessel EC must assemble into vascular capillary structures. HUVEC are able to assemble into highly branched capillary-like structures when cultured on GFR-Matrigel. Therefore, we next examined whether XN was able to affect the formation of these structures. Incubation of HUVEC with ethanol (control, C) during 24 h, led to the formation of highly ramified cord-like structures (Figure 3a). However, the presence of XN in the two concentrations used led to a drastic decrease in the number of these cord-like structures into 56.56% ±13.95% (XN 5 μM) and 53.27% ± 13.25% (XN 10 μM) of control values (*p< 0.05 vs ethanol) (Figure 3a and b). Ramifications were rarely found with loss of differentiated cells in the edges. These findings indicate that even at concentrations as low as 5 μM, XN was able to inhibit vessel assembly, a crucial feature for the angiogenic process.

This prompted us to investigate whether this agent also prevented the assembly of cord structures formed by co-culturing HUVEC together with SMC. We addressed this question by adding SMC after the assembly of HUVEC into capillary-like structures. In the absence of any treatment, HUVEC and SMC co-cultures displayed strings of branched tubule-like structures, which were formed by HUVEC surrounded by SMC. Incubation of these co-cultures with 5 μM XN for 24 h had no effect on the number of ramified structures as compared to ethanol-treated co-cultures (Figure 3b). Remarkably, the number of capillary-like structures was strongly increased by incubating co-cultures with 10 μM XN (188.40% ± 26.37%, *p< 0.05 vs. ethanol) (Figure 3a and b) relative to controls, indicating that XN at this concentration enhanced the assembly of stable vessels.

XN inhibited NFκB activity in both types of cells

NFκB is a transcription factor involved in many cell fates, including cell growth, apoptosis, migration and stimulation of inflammatory factors (1, 23). The broad effects of XN in vascular wall cells promted us to examine whether the activity of this factor was affected by XN in HUVEC and SMC by ELISA. A decrease in NFκB p65 subunit activity was found in HUVEC after incubation with XN in a dose-dependent manner, reaching statistical

significance after treatment with 10 μM XN concentration (Figure 4a) (*p<0.05 vs. ethanol). A tendency towards an association was also found in SMC after incubation with the same concentration of XN (Figure 4b) (p=0.06 for 10μM XN vs. ethanol), indicating that NFκB signalling inactivation is one of the pathways triggered by XN in these two vascular wall cells.

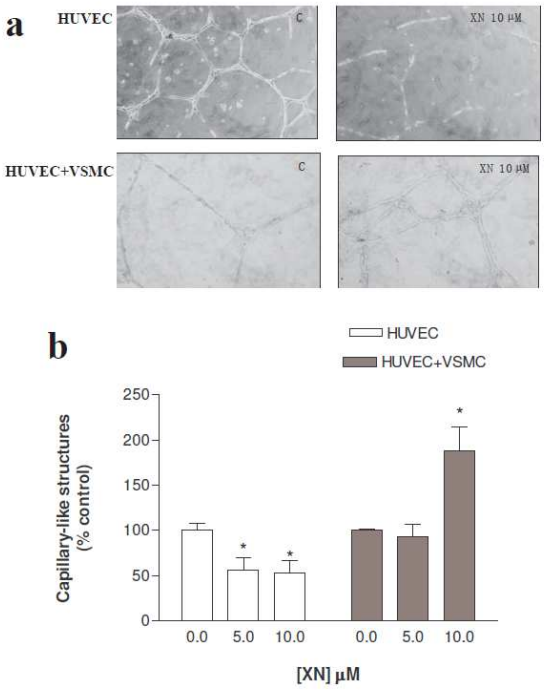

Figure 3. Capillary-like structures assembly was examined in HUVEC cultures or co-cultures of HUVEC and SMC after treatment with xanthohumol (XN). **(a)** In contrast to control cells (C), incubation with 10 μM XN resulted in unconnected structures presenting loosely edges, with many undifferentiated cells. The presence of SMC strengthens these cord structures (HUVEC+SMC). An increased number of these solid structures can be observed in the presence of 10 μM XN. Photos are representative of the whole cultures. Every culture was established in triplicate and visualized under an inverted microscope (x40 magnification); **(b)** Semiquantification of the tube formation index in HUVEC after incubation with XN at 5 or 10 μM (white bars). Reduced number of capillary-like structures formed upon incubation with 5 μM and 10 μM (*p< 0.05). Tubule-like structures formed by HUVEC and SMC co-cultures (dark bars) were enhanced in the presence of 10μM XN (*p< 0.05 vs. Control). Bars correspond to the percentage of the number of tubule-like structures comparatively to controls. Error bars represent SEM between different assays.

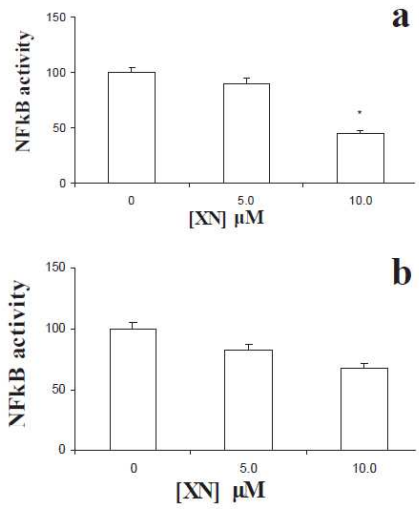

Figure 4. Effects of XN in NFκB p65 subunit activation in HUVEC and SMC. **(a)** A significant reduction in NFκB activity was found in HUVEC treated with XN in comparison to controls (ethanol) (*p< 0.05 vs control); **(b)** A tendency towards significant down-regulation of NFκB activity was found in SMC upon treatment with XN as compared to controls. Equal amounts of protein were loaded. Results are mean (SEM) of three independent experiments performed in triplicate.

DISCUSSION

Angiogenesis involve several modifications both in EC and in SMC (1). Natural polyphenols, including XN, are known to exhibit anti-angiogenic properties (5-9). However, the precise effects on the angiogenic process that are targeted by XN have not been clearly established. Herein, we examined the effects of this polyphenolic compound, in the whole angiogenic course, namely investigating cell viability, migration, invasion and capillary-like structure formation using both EC and SMC.

Incubation with XN at 10 μM resulted in a drastic reduction in the percentage of viable EC as compared to ethanol-treated cells. These results were expected, since anti-proliferative and pro-apoptotic effects of XN have long been described in cancer cells (3, 4, 11), implying that a similar mechanism is probably occurring in EC as well. Corroborating our findings, EC growth and apoptosis were previously reported to be disturbed by XN in tumour angiogenesis (8). These authors pointed out that incubation with XN at 5-15 μM resulted in a slight increase in apoptosis, whereas a high apoptotic rate was

found in the presence of 25 μM XN (8). In the present study, a 10 μM concentration of XN was enough to induce a 4-fold increase in apoptotic rate as compared to ethanol-treated HUVEC. The disparity among the two studies is likely due to the fact that serum-free conditions were used in our experiment, which led to a more significant outcome.

Identical findings in cell viability were obtained in SMC under the same concentrations of XN. SMC constitute the media layer of the blood vessels wall. These cells attach to the basal membrane and promote vessel stabilization. SMC proliferation is a crucial feature for the establishment of mature blood vessels. The absence of SMC in blood vessel wall results in vascular leakage and frequent disruption (1, 24). Furthermore, SMC proliferation is associated with several disorders, including atheroma plaque formation and restenosis (1, 24, 25). XN was able to significantly reduce SMC viability and increase apoptosis in the current study. To our knowledge, this was the first report concerning the effects of XN in SMC. Given the established association between SMC viability and migration and disorders such as restenosis and atherosclerosis, our findings suggest that this polyphenol might be a putative therapeutic agent.

EC invasiveness is another mainstay in angiogenesis. Invasive capacity requires extracellular matrix degradation and involves the activation of EC invasive signalling pathways. Therefore, our finding that XN reduced EC invasion in a dose-dependent manner, indicates the relevance of this compound as an anti-angiogenic agent. Accordingly, XN has been reported to inhibit matrix metalloproteinase-2 release by HUVEC at these concentrations (8). Nevertheless, this reduction in invasive capacity was not restricted to HUVEC. Rather, an effective decrease in matrigel invasion was found in XN-treated SMC in the current study, demonstrating that this compound exerts a huge amount of effects on distinct cells, including vascular wall cells.

Ultimately, EC must assemble into capillary-like structures, in order to form a new blood vessel. We were able to show that XN prevented the formation of these structures on matrigel-coated plates in the two concentrations examined as compared to controls, implying that EC differentiation into cord structures is also affected by this natural polyphenol. Most strikingly, XN was not able to abrogate the assembly of capillary-like structures when HUVEC were co-cultured with SMC. In contrast to the effect found in HUVEC cultures alone, the number of capillary-like structures was not significantly changed by treatment with 5 μM XN in comparison to controls. Interestingly, the number of cord structures doubled in co-cultures incubated with 10 μM XN. Knowing that cord structures on matrigel assay are prone to disruption after 24 h culturing, the increase in cord structures found upon 10 μM XN incubation was attributed to the ability of XN in preventing vessel disruption. SMC bind to extracelular matrix proteins and to EC receptors, resulting in stabilization of blood vessels (1, 26). A series of receptor kinases including transforming growth factor-α, platelet–derived growth factors and angiopoietin-1 signalling pathways become activated by the adhesion of SMC to angiogenic vessels, resulting, thus, in vessel maturation (1, 26). According to our findings, 10 μM XN is likely to activate transduction pathways involved in SMC adhesion, enhancing, therefore, stabilization of blood vessels. This is a novel finding concerning the effects of XN in co-cultures of EC and SMC.

An increasing number of studies regarding the signaling pathways triggered by polyphenols in vascular wall cells have been reported (8, 15, 27, 28). Accordingly, immediate effects of apigenin and quercetin have been attributed to increased nitric oxide synthesis, which improved endothelial dysfunction (27, 28). Furthermore, XN inhibited endothelial NFκB activity, interfering with several intracellular phosphorylation cascades implicated in cell proliferation, migration and anastomosis (1, 12, 23). NFκB is an inflammatory promoter also involved in proliferation and down-regulation of apoptosis (1, 12, 23). The current paper shows that chronic treatment with XN was able to affect several processes within endothelial and smooth muscle cells. These findings led us to examine whether XN had any effect in NFκB activity in both types of cells. A reduced NFκB activity was found in both cells, reaching statistical significance in HUVEC. These findings are in agreement with other studies in the literature (12), and explain the large effects of this polyphenol in angiogenesis. In accordance, Imhof and Aurrand-Lions (29) hypothesized that NFκB was able to induce angiopoietin-2 (Ang2) in endothelial cells, an angiogenic factor that together with VEGF led to angiogenesis stimulation. Therefore, inhibition of NFκB signaling by XN probably results in abrogation of Ang2, explaining the underlying anti-angiogenic effects of this compound. In contrast, the presence of pericytes or SMC resulted in expression of angiopoietin-1, an Ang2 counterpart, preventing angiogenesis, even in the presence of NFκB (29). Accordingly, in co-cultures of HUVEC together with SMC, XN was unable to prevent capillary-

like structure formation. Further studies focused on the inferred pathways induced by XN in these conditions are mandatory. Nonetheless, these findings clearly show that XN effects on vascular wall cells are restricted to angiogenic vessels, providing further evidence for the use of XN as a therapeutic strategy against dysfunctional vessels, whereas not distressing stable ones.

In conclusion, we were able to show that XN, a polyphenol present in diet, exerts a wide range of inhibitory effects in angiogenesis. Namely, XN prevents EC viability, invasion and capillary-like structures formation, while increasing apoptosis of these cells. Additionally, we also showed for the first time that identical effects were described in SMC as well. One probable mechanism for the XN effects in angiogenesis is the reduction of NFκB activity, a well-established angiogenic and inflammatory factor. These broadened effects of XN render this polyphenol a good candidate against disorders widely established in the western world that comprise excessive angiogenesis, such as cardiovascular disease and cancer. Furthermore, the fact that XN also targets SMC as revealed in this study provides new evidence for the use of this agent in pathological situation exhibiting SMC hyperplasia.

ACKNOWLEDGMENTS

The authors would like to thank Dr James McDougall (Fred Hutchinson Cancer Research Center, Seattle, USA) for providing the vascular smooth muscle FLTR cell line. This study was partially funded by FCT (POCI/BM/55556), iBeSa and by "Investigação científica na pré-graduação" program funded by University of Porto.

CONFLICT OF INTERESTS

The authors declare that no conflicting interests exist.

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CAPÍTULO 2

Angiogenesis and inflammation signaling are targets of beer polyphenols on vascular cells

J Cell Biochem. 111:1270-9, 2010

Negrão R, Costa R, Duarte D, Taveira T, Mendanha M, Moura L, Vasques L, Azevedo I, Soares R

ARTICLE
Journal of Cellular Biochemistry 111:1270–1279 (2010)

Journal of Cellular Biochemistry

Angiogenesis and Inflammation Signaling Are Targets of Beer Polyphenols on Vascular Cells

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ABSTRACT

Emerging evidence indicates that chronic inflammation and oxidative stress cluster together with angiogenic imbalance in a wide range of pathologies. In general, natural polyphenols present health-protective properties, which are likely attributed to their effect on oxidative stress and inflammation. Hops used in beer production are a source of polyphenols such as xanthohumol (XN), and its metabolites isoxanthohumol (IXN) and phytoestrogen 8-prenylnaringenin (8PN). Our study aimed to evaluate XN, IXN, and 8PN effects on angiogenesis and inflammation processes. Opposite in vitro effects were observed between 8PN, stimulating endothelial and smooth muscle cell (SMC) growth, motility, invasion and capillary-like structures formation, and XN and IXN, which inhibited them. Mouse matrigel plug and rat skin wound-healing assays confirmed that XN and IXN treatments reduced vessel number as well as serum macrophage enzymatic activity, whereas 8PN increased blood vessels formation in both assays and enzyme activity in the wound-healing assay. A similar profile was found for serum inflammatory interleukin-1β quantification, in the wound-healing assay. Our data indicate that whereas 8PN stimulates angiogenesis, XN and IXN manifested anti-angiogenic and anti-inflammatory effects in identical conditions. These findings suggest that the effects observed for individual compounds on vascular wall cells must be carefully taken into account, as these polyphenols are metabolized after in vivo administration. The modulation of SMC proliferation and migration is also of special relevance, given the role of these cells in many pathological conditions. Furthermore, these results may provide clues for developing useful therapeutic agents against inflammation- and angiogenesis-associated pathologies. J. Cell. Biochem. 111: 1270–1279, 2010.

KEY WORDS: ANGIOGENESIS; INFLAMMATION; ENDOTHELIAL CELLS; SMOOTH MUSCLE CELLS; BLOOD VESSELS; POLYPHENOLS; XANTHOHUMOL; ISOXANTHOHUMOL; 8-PRENYLNARINGENIN; BEER

In adulthood, the angiogenic process is usually localized, as in wound repair and tissue regeneration, and is self-limited in time as happens in ovulation (days), wound healing (weeks), and placentation (months). Pathological angiogenesis, inversely, may develop during years.

The growing life span in western countries has promoted an increase in the proportion of elderly people in the growing population, with consequent increased needs for health care. Cardiovascular diseases (CVD) together with cancer comprise the vast majority of deaths among the elderly people [Hotamisligil, 2006]. Obesity has also emerged as a major public health problem due to its high prevalence and association with increased risk for development of type 2 diabetes mellitus and CVD [Hotamisligil, 2006; Monteiro, 2009; Soares, 2009]. Despite their distinct etiopathogenesis, CVD, cancer, diabetes, and obesity are now being considered angiogenesis-dependent diseases, associated with a chronic inflammation state and oxidative stress that together with hypoxia are the main stimuli of the angiogenic process originating unstable and leaky vessels [Costa et al., 2007; Folkman, 2007]. Although inflammation is an essential process responsible for defending the organism against pathogens, it may also have adverse effects on surrounding tissues, especially when it persists over time [Imhof and Aurrand-Lions, 2006]. Inflammatory cells secrete cytokines and growth factors, which promote angiogenesis, and a strong association between factors involved in inflammation and angiogenesis, playing a role in endothelial cells (EC) biology has been described [Imhof and Aurrand-Lions, 2006; Costa et al., 2007]. Conversely, angiogenesis sustains inflammation by providing oxygen and nutrients for the cell metabolic requirements at inflammatory sites [Costa et al., 2007]. Gaining insights into

Grant sponsor: Fundação para a Ciência e Tecnologia (FCT); Grant number: SFRM/BD/41888/2007; Grant sponsor: European Research Advisory Board (ERAB); Grant number: EA-06 41; Grant sponsor: Institute for Beverages and Health (iBeSa), Portugal; Grant number: P10-08; Grant sponsor: University of Porto Foundation/Santander Totta.

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Received 22 April 2010; Accepted 10 August 2010 • DOI 10.1002/jcb.22850 • © 2010 Wiley-Liss, Inc.
Published online 27 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

angiogenesis and the role of angiogenic factors in several unrelated diseases will enable a better understanding of the potential of angiogenic modulators, as well as the need for preventive and therapeutic strategies both inhibiting and promoting angiogenesis [Folkman, 2007].

Epidemiological and experimental evidence indicates that diets rich in plant-derived foods and several beverages like red-wine, tea, and beer offer a protective effect against several pathologies interfering both in inflammatory and angiogenic processes [Gerhauser, 2005a; Oak et al., 2005; Soares and Azevedo, 2007]. These properties have been mainly attributed to the anti-oxidant and anti-inflammatory effects of polyphenolic compounds [Biesalski, 2007; Stevenson and Hurst, 2007].

Beer is a highly consumed beverage around the world. Hop-derived supplements and beer contain several polyphenols. Xanthohumol (XN), which has received most attention in recent years can be converted to isoxanthohumol (IXN), and to the potent phytoestrogen 8-prenylnaringenin (8PN) during beer production and in vivo metabolism [Nikolic et al., 2005, 2006; Bolca et al., 2007]. Beer polyphenols have generally been described as potent anti-oxidative, anti-inflammatory, and anti-carcinogenic molecules, being, thus, possible contributors to the prevention of pathologies with high incidence and mortality rates in the western world [Stevens and Page, 2004; Gerhauser, 2005a,b; Cho et al., 2008; Magalhaes et al., 2009]. Conversely, the phytoestrogenic properties described for 8PN, namely as an estrogen receptor agonist, enables the putative use of this molecule in pathological conditions such as osteoporosis or menopause-associated complications [Milligan et al., 2002].

Despite the increasing number of studies regarding beer polyphenols, the actions of these compounds in EC and specially vascular SMC are not elucidated. Herein, we investigated the effects of XN, IXN, and 8PN in angiogenesis and inflammation, focusing our attention in both the two types of vascular wall cells—EC and SMC. The possibility of the metabolization of XN into IXN and IXN into 8PN, increases the interest in studying the effects of these compounds using the same experimental conditions. In vivo models able to confirm the angiogenic and inflammatory modulation were also used for the three polyphenols.

MATERIALS AND METHODS

CELL CULTURES

Human umbilical vein endothelial cells (HUVEC) and human aortic smooth muscle cells (HASMC) were purchased in ScienceCell Research Labs (San Diego). HUVECs were cultured in M199 medium (Sigma-Aldrich, Portugal) supplemented with 20% fetal bovine serum (FBS) (Invitrogen Life Technologies, Scotland, UK), 1% penicillin/streptomycin (Invitrogen Life Technologies), 0.01% heparin (Sigma-Aldrich), and 30 μg/ml endothelial cell growth supplement (Sigma-Aldrich), and maintained at 37°C in a humidified 5% carbon dioxide atmosphere. Cells were seeded on plates coated with 0.2% gelatin (Sigma-Aldrich) and allowed to grow. HASMC were cultured in Dulbeco's modified Eagle's medium. HASMC cultures were supplemented with 10% FBS and 1% penicillin/streptomycin and cultured at 37°C in a humidified 5% carbon dioxide atmosphere. Cells were kept between passages 2 and 8 for every experiment. XN, 8PN (Sigma-Aldrich), and IXN (Alexis, Switzerland) were dissolved in ethanol and then added to cell culture medium at a concentration of 0.1–10 μM, established according to the viability assays performed. Polyphenols and vehicle (ethanol) were added to cell cultures in medium supplemented with 2% FBS and 1% penicillin/streptomycin. Control cells were incubated with vehicle (ethanol). Ethanol concentrations were kept below 0.1% in every culture.

CELL VIABILITY

HUVEC and HASMC were allowed to grow until 70–80% confluence and then incubated with 0.01–20 μM XN, IXN, 8PN or ethanol for 24 h. After the incubation period, cells were washed twice with phosphate-buffered saline solution and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [Guerreiro et al., 2007]. Briefly, cells were incubated with MTT solution at a final concentration of 0.5 mg/ml for 3 h and then lysed in dimethylsulfoxide. Absorbance was measured at 540 nm, and the background absorbance, measured at 660 nm, was subtracted. All samples were assayed in duplicate and at least in three independent experiments, and the mean value for each experiment was calculated. The results are given as mean (±SEM) and are expressed as percentage of control, which was considered to be 100%.

CELL APOPTOSIS

HUVEC and HASMC (1×10^4 cells/ml) were grown on glass coverslips and incubated with different concentrations (0.1–10 μM) of tested compounds (XN, IXN, and 8PN) for 24 h. TUNEL assay was performed using the In Situ Cell Death Detection kit (Roche Diagnostics, Switzerland), as reported before [Soares et al., 2004; Rocha et al., 2007]. The percentage of stained cells was evaluated by counting the cells stained with TUNEL (apoptotic cells) divided by the total number of nuclei stained with DAPI (Roche Diagnostics) at a 200× magnification field.

CELL PROLIFERATION

HUVEC and HASMC cultures (1×10^4 cells/ml) were established on glass coverslips following treatment procedures with 0.1–10 μM polyphenols (XN, IXN, and 8PN) for 24 h. Cell proliferation analyses were carried out using cellular incorporation of 5′-bromodeoxyuridine (BrdU), a thymidine analogue. After incubation with BrdU solution at a final concentration of 0.01 mM for 24 h, the number of proliferating cells (positive for BrdU), after immunohistochemistry methods using anti-BrdU-specific antibodies (BrdU In-Situ Detection Kit, BD Biosciences Pharmingen), were evaluated at the microscope, according to the manufacturer's instructions and as previously described [Rocha et al., 2007].

INVASION CAPACITY

The invasive cell behavior in the presence of 0.1–10 μM XN, IXN, and 8PN was quantified in vitro using a double-chamber assay by counting the number of cells that invaded a Transwell BD-Matrigel® basement membrane matrix inserts (BD Biosciences, Belgium),

according to manufacturer's instructions. FBS was used as a chemoattractant. Results represent the ratio between invading cells in polyphenol-treated cultures compared to invasion in control cultures for the same initial amount of cultured cells.

CAPILLARY-LIKE STRUCTURES FORMATION

Cells were cultured on growth factor reduced-Matrigel® (GFR-Matrigel®) (BD Biosciences, Belgium)-coated plates for 24 h as previously described [Soares et al., 2004]. Briefly, HUVEC were cultured on GFR-Matrigel®-coated plates for 24 h, in medium containing 1–10 μM polyphenols or vehicle. When cultured on Matrigel®, EC assemble into capillary-like structures. The number of cord-like structures was then counted at an inverted microscope. Each cord portion between the ramifications was considered one cord unit. Mean values were obtained by evaluating the whole cultures of each well under the same treatment. Treatments were performed as described above. A semi-quantitative measurement of cord formation in GFR-Matrigel® cultured HUVEC was developed as previously described [Soares et al., 2004].

IN VIVO STUDIES

Animal experiments were conducted according to accepted standards of humane animal care (Declaration of Helsinki, European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals).

MATRIGEL PLUG ASSAY

A mixture of Matrigel® and heparin without (negative control, C−) or with recombinant VEGF (positive control, C+) and 10 μM polyphenols (XN, IXN, and 8PN), was subcutaneously inoculated into C57BL/6 mice (purchased at Charles River, Wilmington, MA). The animals were euthanized after 7 days, the matrigel plug was removed, weighed, photographed and the amount of hemoglobin (Hb) in the homogenized plug was measured, as described below. Mice blood was also collected for evaluation of inflammatory factors.

SKIN WOUND-HEALING ASSAY

Wistar rats (Charles River), 8- to 12-week-old were used and kept individually in their cages during the study. After general anesthesia, the rat dorsal skin was shaved and full skin-thickness longitudinal incisions (1.5 cm) were created and the wound edges surgically sutured at 0.5 cm intervals. Polyphenols (XN, IXN, and 8PN) or vehicle (ethanol + water, 1 + 5; C) were administered topically (50 μL of a 50 μM solution), daily as observed in the literature [Malinda et al., 1998; Koczulla et al., 2003; Monteiro et al., 2008]. Rats were examined daily for wound-healing progression. After 7 days wound tissue was collected for histological studies and blood used for inflammatory factors evaluation. Skin wound tissue specimens were fixed in 10% neutral-buffered formalin and paraffin-embedded. Histological and immunohistochemistry analyses were performed in 5-μm tissue sections.

HEMOGLOBIN DETERMINATION

The Hb content of the plug was evaluated after homogenization of the plug in a water–heparin solution, which was then centrifuged at 1,500*g* for 15 min at 20°C. The supernatant (100 μl) was used to measure the Hb content according to the Drabkin's method (Sigma-Aldrich) at 540 nm.

SERUM ANALYSES

IL1β Measurement. Interleukin (IL) 1β was quantified in mice and rats serum by ELISA (IL-1β-EASIA kit, BioSource, Nivelles, Belgium) according to the manufacturer's instructions.

Determination of *N*-Acetylglucosaminidase Activity. The *N*-acetylglucosaminidase (NAG) enzyme is present at high levels in activated macrophages. Inflammation can be evaluated by measuring the levels of the lysosomal NAG enzyme in the serum. Serum was incubated for 10 min at 37°C with 100 μl of p-nitrophenyl-*N*-acetyl-β-D-glucosaminide solution in a 96-well plate. The reaction was stopped by the addition of 0.2 M glycine buffer (pH 10.6) and the substrate hydrolysis was measured at 405 nm.

IMMUNOHISTOCHEMISTRY ANALYSIS

Microvessel density (MVD) was evaluated in each formalin-fixed paraffin-embedded wounded tissue section by immunohistochemistry. Tissue slides were incubated with an anti-von-Willebrand Factor (vWF) antibody (Santa Cruz Biotechnologies, CA). Capillaries were then counted in the three tissue sections, for each animal, and normalized to the total area of the tissue section. Negative controls were carried out by omission of the primary antibody in tissue sections expressing the marker.

STATISTICAL ANALYSES

Every cell experiment was performed at least in three independent experiments. Quantifications are expressed as mean (SEM) and as percentage of control, which was considered to be 100%. Statistical significance of difference between various groups was evaluated by analysis of variance (ANOVA) followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $P < 0.05$.

RESULTS

MICROMOLAR CONCENTRATIONS OF POLYPHENOLS AFFECT HUVEC AND HASMC VIABILITY

Cell cytotoxicity was first analyzed by MTT assay in the two cell cultures upon treatment with 0.01–20 μM of XN, IXN, or 8PN (Fig. 1). Viability of HUVEC was only decreased by the higher concentrations of XN (39.40 ± 3.86% decrease, for 20 μM) and IXN (19.38 ± 5.38% decrease, for 10 μM and 33.59 ± 7.05% decrease, at 20 μM). Conversely, an increase in the number of viable cells was found after incubation with 8PN, in a dose-dependent manner, reaching statistical significance at 20 μM (40.75 ± 9.82% increase). HASMC viability was significantly reduced by XN at higher concentrations as well (39.91 ± 15.82% decrease, for 10 μM and 93.91 ± 0.81% decrease, at 20 μM). No statistical differences were observed when these cells were treated with IXN or 8PN at any concentration.

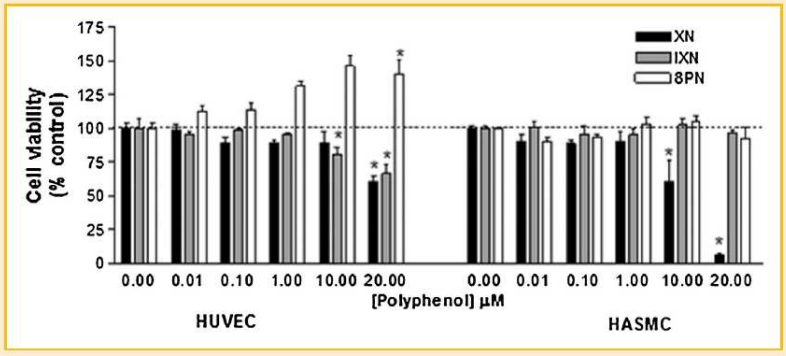

Fig. 1. Effect of polyphenols on HUVEC and HASMC viability. Cell viability after incubation for 24 h with 0.01–20 μM xanthohumol (XN), isoxanthohumol (IXN), or 8-prenylnaringenin (8PN) or vehicle (0.00) was evaluated by MTT assay. Results are means ± SEM of independent experiments ($4 \leq n \leq 7$) and are expressed as percentage of control. $^*P < 0.05$ versus control.

As the decrease in cell viability at 20 μM was too high for some of the tested compounds, we proceeded the study using 10 μM as the higher concentration tested.

XN, IXN, AND 8PN DISTINCTLY AFFECT HUVEC AND HASMC APOPTOSIS, PROLIFERATION, AND INVASION

In order to understand whether the observed cell viability alterations upon polyphenol treatments were due to their actions on apoptosis or cell growth, we next investigated the potential apoptotic and proliferative activity of the three compounds at 0.1, 1, or 10 μM concentration. Incubation of both cell cultures with XN and IXN for 24 h resulted in a significant increase in apoptosis, reaching statistical significance for 10 μM concentration of both compounds in HUVEC cultures (69.88± 21. % increase for XN and 104.51 ± 17.02% increase for IXN) and in HASMC cells (100.82 ± 46.60% increase for XN and 53.55 ± 22.72% increase for IXN) (Fig. 2A). Inversely, treatment with 8PN reduced the percentage of apoptotic cells in both cell types (70.15 ± 9.09% decrease in HUVEC and 50.69 ± 6.69% decrease in HASMC), presenting a greater effect on HUVEC (Fig. 2A).

In contrast, incubation with 0.1–10.0 μM XN or IXN for 24 h significantly decreased cell growth in HUVEC (69.20 ± 5.89% decrease for XN and 65.71 ± 10.79% decrease for IXN treatments, at 10 μM) and in HASMC (82.14 ± 4.67% decrease for XN and 37.37 ± 4.80% decrease for IXN, at 10 μM) as illustrated in Figure 2B. Interestingly, 8PN resulted in a significant alteration of HUVEC proliferation (69.72 ± 18.24% increase at 10 μM), whereas no significant difference was seen in HASMC when compared to vehicle treated cells (Fig. 2B).

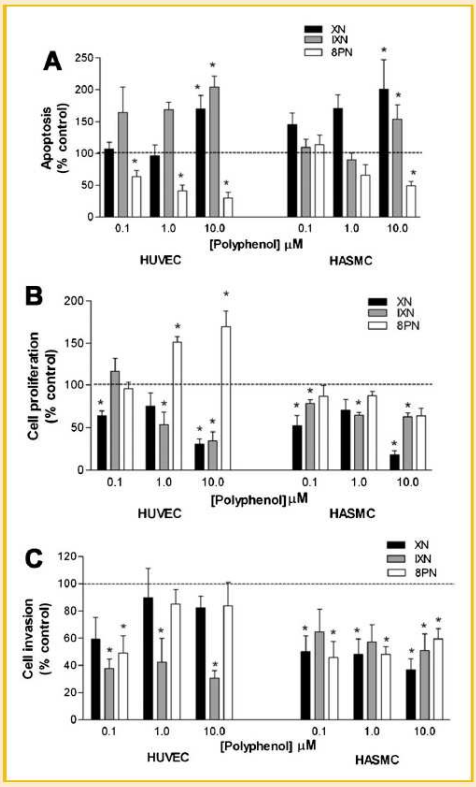

Fig. 2. Effect of polyphenols in HUVEC and HASMC apoptosis, proliferation and invasion. Cells were incubated for 24 h with 0.1–10 μM XN, IXN, or 8PN or vehicle. A: Apoptosis was evaluated by TUNEL assay. Bars represent the percentage of apoptotic cells evaluated by the ratio between TUNEL-stained cells and DAPI-stained nuclei in every culture. B: The percentage of proliferative cells was examined by the ratio between BrdU-stained cells and hematoxylin-stained nuclei in every culture. C: The percentage of invading cells relative to the initial amount of cells cultured using a double-chamber assay. Results are means ± SEM of independent experiments ($4 \leq n \leq 8$) and are expressed as percentage of control. $^*P < 0.05$ versus control.

Cell motility and extracellular matrix invasion are fundamental steps within the angiogenic process. So, we next examined the effects of the three polyphenols on invasion capacity using a double chamber assay. As illustrated in Figure 2C, both XN (63.47 ± 8.30% reduction at 10 μM) and 8PN (40.55 ± 7.48% reduction at 10 μM) significantly reduced HASMC invasive capacity at the three concentrations analyzed. Although IXN showed a tendency towards decreased cell invasion, an effective decrease was only observed after incubation with the highest concentration of IXN (10 μM) (48.96 ± 12.15% reduction at 10 μM). IXN treatments of HUVEC resulted in a significant decrease in the ability to invade (69.72 ± 5.37% reduction for 10 μM) (Fig. 2C).

CAPILLARY-LIKE STRUCTURES FORMATION

To form a new blood vessel, EC must differentiate and reorganize, assembling into vascular capillary structures. HUVEC are able to assemble into highly branched capillary-like structures when cultured on GFR-Matrigel®. Therefore, we next examined whether XN, IXN, or 8PN were able to affect de novo the formation of in vitro capillary-like structures. Incubation of HUVEC cultures on GFR-Matrigel®-coated plates with XN or IXN at the concentration of 10 μM resulted in unconnected structures exhibiting loose edges, with many undifferentiated cells (Fig. 3A). Interestingly, organized and interconnected tubes were obtained after incubation with 10 μM 8PN (Fig. 3A). Quantitative analysis revealed a decrease in the number of capillary-like structures when HUVEC were treated with XN (26.64 ± 4.31% decrease) or IXN (31.33 ± 5.36% decrease), reaching statistical significance for 10μM concentration (Fig. 3B). Remarkably, 8PN-treated cultures exhibited a significant enhancement in the number of capillary-like structures (58.90 ± 4.34% increase) compared to control (Fig. 3B).

Altogether, these findings indicate that XN and IXN exhibit anti-angiogenic effects, whereas 8PN seem to trigger an opposite behavior in both cell cultures.

XN AND IXN DIMINISHED ANGIOGENESIS AND INFLAMMATION, WHEREAS 8PN INCREASED ANGIOGENIC PROCESS IN BOTH MOUSE MATRIGEL PLUG AND RAT WOUND-HEALING ASSAYS

To further examine the effects of the three compounds in angiogenesis and inflammation in a more accurate way, we assessed mouse matrigel plug neovascularization assay. As illustrated in Figure 4A,B, VEGF-containing matrigel (positive controls) presented extensive neovascularization. When matrigel implants in the presence of recombinant VEGF were mixed with either XN or IXN before inoculation, vascular development was strongly inhibited (Fig. 4A,B), with a higher inhibitory response for IXN (92.3 ± 2.32% decrease), identical to the negative controls, in which no VEGF was added. On the other hand, the plugs implanted with 8PN showed a robust angiogenic response identical to positive controls (79.98 ± 15.19%), as highlighted by the red color distributed in the whole plug (Fig. 4B).

Interestingly, analysis of the serum inflammatory enzyme NAG activity in these mice revealed that systemic inflammation decreased in the presence of XN or IXN implanted matrigel (67.40 ± 6.08% decrease for XN and 78.38 ± 2.30% decrease for IXN) (Fig. 4C). Surprisingly, implanted matrigel containing 8PN, which led to angiogenic stimulation similar to positive control, showed a reduction in systemic macrophage activity as revealed by NAG activity assay (69.28 ± 2.25% decrease).

Skin wound healing is a process involving the formation of new extracellular matrix, cell infiltration, and tissue remodeling. Two fundamental physiological conditions are deeply implicated in this process: inflammation and angiogenesis. After a preliminary experiment in the same conditions as described above, with daily monitoring of the wounds and histological evaluation of the inflammatory cytokines and vessel formation (data not shown), we decided to investigate the effect of the studied polyphenols in neovascularization and serum inflammatory markers in rat skin wounds as previously reported [Malinda et al., 1998; Koczulla et al., 2003]. Higher concentrations of polyphenols (50 μM) were used in

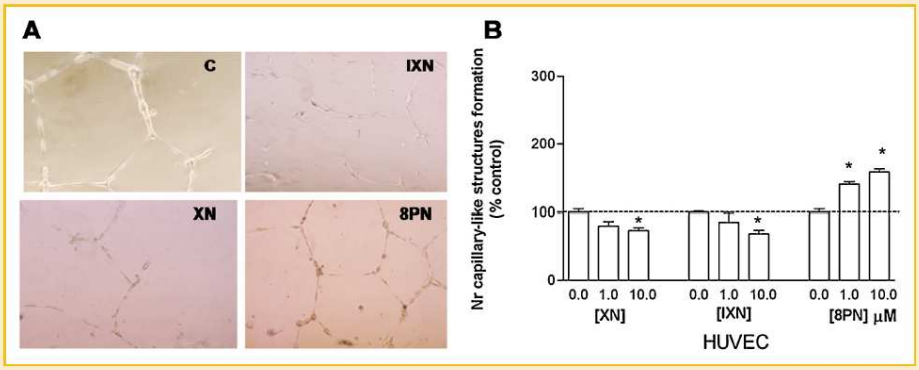

Fig. 3. XN and its metabolites, IXN and 8PN, differently affect the assembly of capillary-like structures. HUVEC were grown on GFR-Matrigel® and incubated with 1 or 10 μM XN, IXN, or 8PN or vehicle (C or 0.0) for 24 h. A: Capillary-like structures formation, after treatment with 10 μM polyphenols, visualized under a phase-contrast microscope. Figures are representative of the whole cultures. Magnification: 200×. B: Semi-quantification of capillary-like structures assembly. Results are means ± SEM of independent experiments ($3 \leq n \leq 5$) and are expressed as percentage of control. *$P < 0.05$ versus control. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

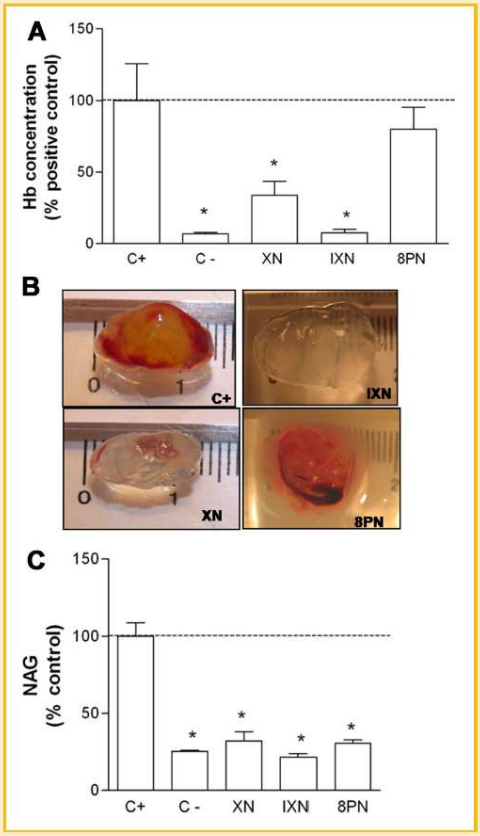

Fig. 4. In vivo evaluation of angiogenesis and inflammation using a mouse matrigel plug assay. A mixture of Matrigel® and heparin without vascular endothelial growth factor (VEGF) (negative control, C−); or of Matrigel®, heparin and VEGF (positive control, C+) and Matrigel®, heparin, VEGF and 10 μM XN, IXN, or 8PN was injected subcutaneously into C57BL/6 mice. A: Quantification of the hemoglobin (Hb) amount in the homogenized plugs by Drabkin's method. B: Representative images of macroscopic visualization of Matrigel plugs. XN and IXN diminished vessel formation in the plug, while 8PN increased it. C: Determination of *N*-acetylglucosaminidase (NAG) activity in mice serum 7 days after plugs implantation. Results are means ± SEM of independent experiments ($5 \leq n \leq 10$) and are expressed as percentage of control. $^*P < 0.05$ versus control. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

this in vivo assay because the compounds were used topically. Externally, healing process seemed completed on day 7 post-injury, with no differences observed in the wound area after a 7-day treatment with XN, IXN, or 8PN relative to vehicle. However, the corresponding histological sections used for immunohistochemical MVD quantification in the remodeled tissue revealed that upon treatments with XN and IXN the width of the granulation tissue formed at the incision site was thinner when compared to the corresponding area in controls (Fig. 5A). In contrast, 8PN treatment led to increased thickness of granulation tissue in certain areas (Fig. 5A). This apparent increase in granulation tissue observed upon 8PN treatment was accompanied by an increasing number of blood vessels in the area, contrary to what was observed after XN and IXN treatments, as accomplished by immunohistochemistry analyses (Fig. 5B). XN and IXN topic administration resulted in a decrease in MVD in the vicinity of the incision area (42.46 ± 9.71% decrease for XN and 50.46 ± 6.95% decrease for IXN) (Fig. 5C). Inversely, treatment with 8PN substantially increased the number of vessels assembled when compared to control (114.36 ± 2.42% increase). In addition, the activity of NAG enzyme in these rats serum was higher than in controls, revealing an increased systemic inflammatory status in rats treated with 8PN (28.67 ± 4.66% increase), whereas a significant lower inflammation for treatments with XN and IXN were observed (44.25 ± 6.20% decrease for XN and 22.72 ± 2.04% decrease for IXN) (Fig. 6A). These latter findings were further corroborated by an identical profile obtained for IL1β determination in rat sera (Fig. 6B). Higher concentration of tested polyphenols (50 μM) in this in vivo assay was used, when comparing polyphenol concentrations used in in vitro assays for EC and SMC treatments (10 μM). As this is an in vivo assay bioavailability and metabolization of these compounds must be taken into account, which justifies the application of higher doses in the skin treatments. Other authors observed no toxic effects with 100 μM ingestion of XN [Monteiro et al., 2008].

DISCUSSION

The present study investigated the effect of three beer polyphenolic compounds in angiogenesis and inflammation. We were able to demonstrate that overall, XN and IXN exerted anti-angiogenic effects both in cell cultures as well as in the in vivo angiogenic established models. In contrast, 8PN had the opposite effect, enhancing angiogenesis by acting on several angiogenic steps. These findings were in general accompanied by systemic inflammatory actions in mouse matrigel plug and rat wound-healing assays.

Our in vitro findings enabled us to identify which steps of the angiogenic process were targeted by each polyphenol. XN and IXN presented broad anti-angiogenic actions. Cell growth was compromised by XN and IXN in both cell cultures. Other authors have described similar growth effects in tumor cell lines [Colgate et al., 2007; Dell'Eva et al., 2007] and in EC [Bertl et al., 2004; Albini et al., 2006]. In addition, both compounds activated programmed cell death mechanisms in both vascular wall cells studied (HUVEC and HASMC) as well. XN and IXN also affected HUVEC and HASMC invasion ability and prevented the formation of capillary-like structures, implying their already established action on EC differentiation into cord structures [Bertl et al., 2004; Albini et al., 2006; Negrão et al., 2007]. Furthermore, the fact that these polyphenols also target SMC proliferation and migration, as revealed in this study, provides new evidence for the use of this agent in pathological situations exhibiting SMC hyperplasia, such as atherosclerosis or restenosis.

Inversely, 8PN exhibited anti-apoptotic effects in both cell cultures, but strikingly it stimulated HUVEC while it did not change HASMC proliferation. These results are not in accordance with the

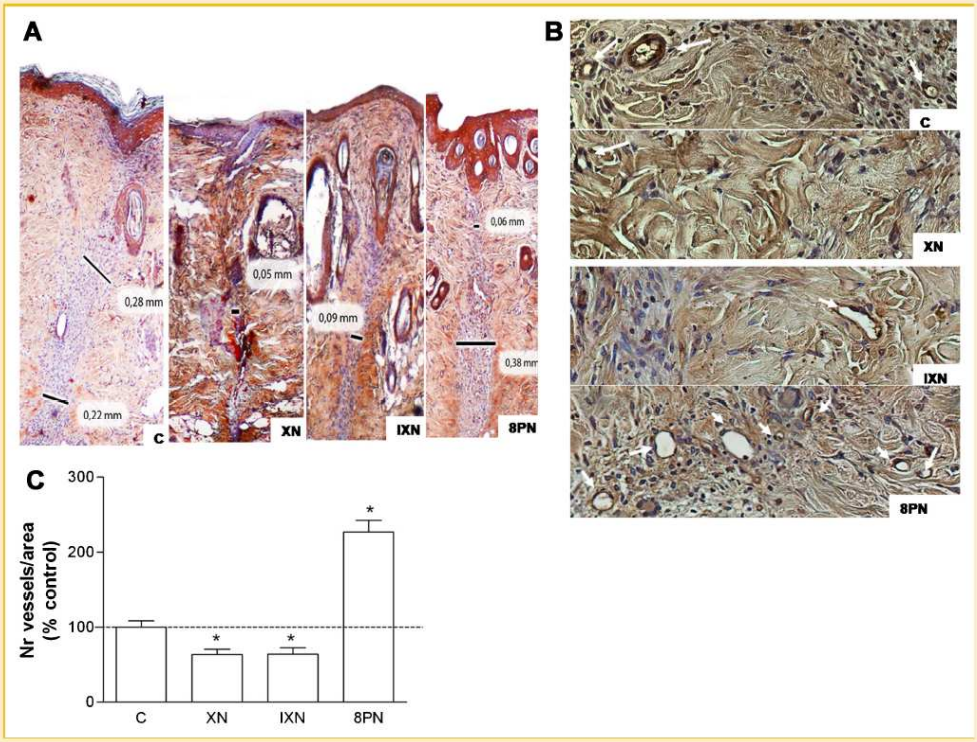

Fig. 5. In vivo skin wound-healing assay. Longitudinal incisions were created on the dorsal surface of the rats and 50 μM XN, IXN, 8PN or vehicle (C) were administered topically, daily. After 7 days, wounded tissue was collected for angiogenesis evaluation. A: Hematoxylin-stained micrographs of wound tissue sections, highlighting different thickness (black bar) of granulation tissue fulfilling the incision with different treatments (magnification: 40×). B: Wound tissue section micrographs from controls or rats treated with polyphenols, using von-Willebrand Factor (vWF) for evaluation of blood vessels (immunostaining, magnification: 200×). Arrows indicate blood vessels. C: Quantification of blood vessels present in three tissue sections, for each animal, and normalized to the total area of the tissue section. Results are means ± SEM of independent experiments ($4 \leq n \leq 7$) and are expressed as percentage of control. $^{*}P < 0.05$ versus control. [Color figure can be viewed in the online issue, which is available at www.wileyonlinelibrary.com.]

previous reports in the literature [Pepper et al., 2004], attributing in vitro anti-angiogenic effects for 8PN in EC. Brunelli et al. [2007] suggested a biphasic action on cell growth for 8PN, showing estrogenic properties and increasing proliferation in hormone responsive cells at concentrations below 10 μM, while inhibiting proliferation at higher concentrations. Estrogens are established angiogenic promoters [Soares et al., 2003, 2004], rendering this molecule a putative pro-angiogenic agent. Furthermore, despite invasiveness capacity was not highlighted by the double chamber assay used, the number of in vitro cord structures increased significantly when HUVEC were treated with 8PN, supporting its pro-angiogenic effects (Fig. 3). Nevertheless, the mechanisms by which 8PN acts remain unknown.

Interestingly enough, XN and IXN significantly prevented VEGF-induced formation of de novo microvessels on the matrigel plug assay (Fig. 4), confirming that these two agents may inactivate VEGF signaling pathways. Although some studies have already highlighted the anti-angiogenic effect of XN, to our knowledge, this is the first report on the anti-angiogenic effects of IXN in vivo.

Chronic inflammation and angiogenesis are two joint partners. Inflammatory mediators produced by immune cells target EC to produce angiogenic factors, but those cells can directly release high amounts of distinct angiogenic factors. On the other hand, angiogenesis sustains inflammation, by providing oxygen and nutrients for the metabolic needs of the cells present at the inflammatory site as well as by enabling extravasation of immune cells [Costa et al., 2007]. Transgenic mice overexpressing VEGF show enhanced angiogenesis and immune responses [Xia et al., 2003], highlighting the existence of a complex coordination between blood vessels and immune cells [Costa et al., 2007]. Accordingly, mice implanted with XN and IXN-containing matrigel plugs presented decreased NAG macrophage enzyme activity in serum. These findings imply that the polyphenols retained inside the matrigel plug may exert systemic anti-inflammatory effects.

Unexpectedly, serum macrophage activity was also reduced by 8PN (Fig. 4C). In fact, matrigel plug assay is a well-established model to evaluate angiogenesis within the matrigel, but it is also a relatively artificial one. 8PN may directly or indirectly interfere with

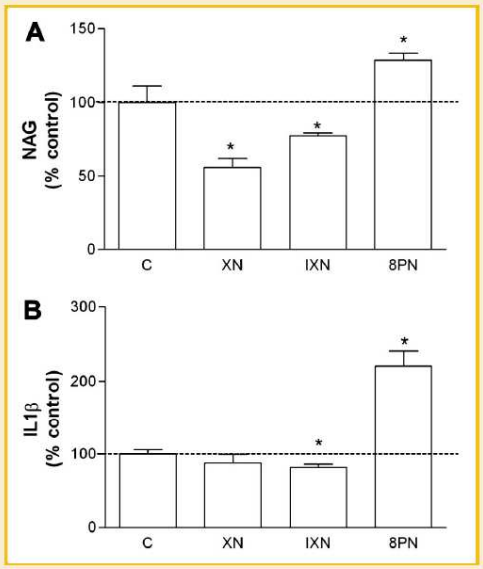

Fig. 6. In vivo rat skin wound-healing assay. Longitudinal incisions were created on the dorsal surface of the rats and 50 μM XN, IXN, 8PN or vehicle (C) were administered topically, daily. After 7 days, blood was collected for inflammation evaluation. A: Serum *N*-acetylglucosaminidase (NAG) activity. B: IL1β levels in rats serum. Results are means ± SEM of independent experiments ($4 \leq n \leq 7$) and are expressed as percentage of control. $^*P < 0.05$ versus control.

the release of specific stromal cell or matrix-derived factors which prevent inflammation.

A more physiological and accurate model for angiogenesis and inflammation evaluation is the skin wound-healing assay. No visible differences between the treatments in the time or external aspect of the healing process were found. But the histology of the injured area was effectively different. While XN and IXN efficiently diminished the development of the inflammatory and angiogenic processes in the skin incision, 8PN strongly stimulated both processes. The anti-inflammatory properties of XN are already extensively described [Cho et al., 2008; Monteiro et al., 2008; Magalhaes et al., 2009]. NFκB, a central regulator of the inflammatory process, is one of the known repressed pathways by this compound as well as IL1β production [Monteiro et al., 2008]. Again, we did not find any previous reports regarding the IXN effects in inflammation. Interestingly, we observed reduced thickness in the granulation tissue within the incision area upon topic treatment with XN and IXN. This is primarily attributed to their local effects in modulating angiogenesis and inflammation. A very recent study described the existence of a H_2O_2 gradient, just preceding the movement of the neutrophils towards the wound [Niethammer et al., 2009]. Initial H_2O_2 gradient expansion may be prevented by local application of these polyphenols. 8PN, on the other hand, enhanced vessel formation and increased inflammatory systemic markers (NAG activity and IL1β expression) in an apparent contradiction to previously described in vivo inhibition of angiogenesis by 8PN, in the chorioallantoic membrane model, where 8PN seemed to affect both vessel length and caliber [Pepper et al., 2004]. Yet, small capillaries were still present and less organized in the same study, consistently with our results that point towards a pro-angiogenic profile of 8PN.

Particular attention must be given to these three polyphenols, as they displayed opposite effects on vascular wall cells. Concerning their bioavailability, in hop cones, XN is the most abundant prenylated chalcone but in hop-derived products, generally IXN predominates over XN and 8PN [Gerhauser, 2005a]. Although polyphenols intestinal absorption may be rather limited, recent studies indicate that XN can be metabolized to IXN in the stomach [Nikolic et al., 2005]. Further, intestinal IXN may be converted in 8PN by intestinal bacteria [Possemiers et al., 2008] and by human liver microsomes [Nikolic et al., 2005]. Therefore, upon oral consumption of beer, or even XN, a mixture of the three compounds will be present in the human organism. The final outcome is likely dependent on the tissue concentration of the three molecules, and depends on the type of ingested beer, the nutritional context, the dose of ingested compound and the individual metabolization capacity of these polyphenols. Moreover, the doses studied here using topical administration or Matrigel injection with polyphenols are higher than reached dietary doses. Nonetheless, the pharmacological application of these promising compounds may also be of great interest. Serum concentrations of approximately 0.1 μM 8PN were observed after a single pharmacological dose of this polyphenol in post-menapausal women [Rad et al., 2006]. The concentrations employed may be achieved in specific tissues, by selective uptake, or repeated administration of 8PN [Brunelli et al., 2007], being possible for 8PN to be completely absorbed even at high concentrations in animals and probably also in humans [Pepper et al., 2004]. XN bioavailability is very low [Stevens and Page, 2004] and its metabolites were detected in plasma in the maximum concentration of 0.180 μM after oral administration to rats (50 mg/kg). In a very recent study, Bolca et al. [2010] found nM levels of these polyphenols in women serum after 5 days ingestion of these compounds. The most abundant polyphenols in human diet are not necessarily the most bioavailable ones or even the ones that have the most interesting effects. Even at very low concentrations, polyphenols may remain in blood for long enough to accumulate in target sites and to be able to exert their effects. The effects observed in rat wound-healing experiments led us to propose that further investigation on topic administration of these polyphenolic compounds should be done, as they might be a useful strategy for treating skin lesions, which involve often angiogenic and inflammatory impairment. The first anti-angiogenic drugs have only recently been approved for therapeutic use [Carmeliet, 2005] but due to emergent knowledge emphasizing the close partnership between inflammation and angiogenesis in several diseases, the development of therapeutic approaches against both angiogenesis and chronic inflammation may provide more effective therapies [Costa et al., 2007]. XN is able to inhibit tumor growth by targeting NFκB and Akt pathways and angiogenesis [Albini et al., 2006], has anti-inflammatory effects [Gerhauser, 2005b; Albini et al., 2006] and in vitro activity against Bcr-Abl+ cells, retaining cytotoxicity in

imatinib-resistant cells [Monteghirfo et al., 2008]. These characteristics render this molecule a promising drug. Food industry is also interested in the health benefits of these compounds, especially XN, and the production of a XN-enriched beer has gained interest in the brewing industry [Magalhaes et al., 2009]. According to the current study, IXN is also promising. Conversely, stimulation of angiogenesis can be very useful in the treatment of several pathological conditions such as tissue repair and ischemic conditions like ischemic stroke and heart and limb ischemia, rendering 8PN an interesting molecule.

In conclusion, these XN, IXN, and 8PN in vitro results outlined several angiogenic steps targeted by these polyphenols that were also confirmed by in vivo assays. Furthermore, the fact that these compounds also target SMC proliferation and migration, as revealed in this study, provides new evidence for the use of this agent in pathologies associated with altered SMC growth. Nevertheless, cellular and molecular mechanism need to be elucidated, since the mechanism of these polyphenols is still unclear.

ACKNOWLEDGMENTS

This study was partially funded by FCT (SFRM/BD/41888/2007); ERAB (European Advisory Board; EA0641), iBeSa (Institute for Beverages and Health, Portugal; P10-08), and University of Porto Fundation/Santander Totta.

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CAPÍTULO 3

Xanthohumol-supplemented beer modulates angiogenesis and inflammation in a skin wound healing model. Involvement of local adipocytes

J Cell Biochem 113: 100-9, 2012

Negrão R, Costa R, Duarte D, Taveira T, Coelho P, Guimarães JT, Guardão L, Azevedo I, Soares R

ARTICLE
Journal of Cellular Biochemistry 113:100–109 (2012)

Journal of Cellular Biochemistry

Xanthohumol-Supplemented Beer Modulates Angiogenesis and Inflammation in a Skin Wound Healing Model. Involvement of Local Adipocytes

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ABSTRACT

Angiogenesis and inflammation are two intermingled processes that play a role in wound healing. Nevertheless, whenever exacerbated, these processes result in nonhealing wounds. Xanthohumol (XN), a beer-derived polyphenol, inhibits these processes in many physiopathological situations. This study aimed at examining whether XN ingestion affects wound healing. Wistar rats drinking water, 5% ethanol, stout beer (SB) or stout beer supplemented with 10 mg/L XN (Suppl SB) for 4 weeks, were subjected to a 1.5 cm full skin-thickness longitudinal incision, and further maintained under the same beverage conditions for another week. No differences in beverage consumption or body weight were found throughout the study but food intake decreased in every group relative to controls. Consumption of Suppl SB resulted in decreased serum VEGF levels (18.42%), *N*-acetylglucosaminidase activity (27.77%), IL1β concentration (9.07%), and NO released (77.06%), accompanied by a reduced redox state as observed by increased GSH/GSSG ratio (to 198.80%). Also, the number of blood vessels within the wound granulation tissue seems to reduce in animals drinking Suppl SB (23.08%). Interestingly, SB and primarily Suppl SB showed a tendency to increase adipocyte number (to 194.26% and 156.68%, respectively) and reduce adipocyte size (4.60% and 24.64%, respectively) within the granuloma. Liver function and metabolism did not change among the animal groups as analyzed by plasma biochemical parameters, indicating no beverage toxicity. This study shows that XN intake in its natural beer context reduced inflammation, oxidative stress, and angiogenesis, ameliorating the wound healing process, suggesting that this polyphenol may exert beneficial effect as a nutritional supplement. J. Cell. Biochem. 113: 100–109, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: XANTHOHUMOL; BEER; WOUND HEALING; ANGIOGENESIS; INFLAMMATION; BLOOD VESSELS; POLYPHENOLS

Angiogenesis, the formation of new capillaries from pre-existent blood vessels, is a complex and highly regulated process that requires interaction between different types of cells, inflammatory cytokines, growth factors, and the extracellular matrix. Angiogenesis is a major contributor to tissue vascularization with primordial importance both in physiology and disease, as blood vessels are responsible for the transport of oxygen, nutrients, and other molecules to tissues and for removing metabolic waste [Costa et al., 2007].

Under physiological conditions, angiogenesis is highly regulated by the balance between pro- and anti-angiogenic factors, and in adulthood it occurs only in self-limited processes as for example in wound repair and tissue regeneration [Gollisch et al., 2009]. Pathological angiogenesis, inversely, may develop during years [Folkman, 2007] in conditions such as in cancer, cardiovascular disease, diabetes, obesity, and other pathologies associated with chronic inflammation.

In mammals, wound healing is a result of three overlapping processes: Hemostasis and inflammation, granulation tissue formation, re-epithelialization and remodelling, which enable wound closure and the restoration of a functional barrier [Tan et al., 2007]. Wound healing normally develops without

Grant sponsor: Fundação para a Ciência e Tecnologia (FCT) SFRM/BD/41888/2007 PTDC/SAU-OSM/102083/2008 PEst-OE/SAU/UI0038/2011; Grant sponsor: Institute for Beverages and Health (iBeSa), Portugal P10-08.
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Received 7 June 2011; Accepted 17 August 2011 • DOI 10.1002/jcb.23332 • © 2011 Wiley Periodicals, Inc.
Published online 24 August 2011 in Wiley Online Library (wileyonlinelibrary.com).

complications, but the resolution of inflammation is critical. Indeed, several conditions like ageing, obesity, and many other disorders can compromise the normal resolution of the inflammation. This can result in chronic wound and ulcer development, leading to tissue damage [Pond, 2003]. Hence, reduction of the inflammatory phase is the aim of several therapeutic measures in order to resolve chronic wounds.

Macrophages are the most abundant cells 5 days upon wound injury, producing cytokines and growth factors required for wound healing, promoting collagen production, angiogenesis, and re-epithelization with the formation of granulation tissue, the hallmark of an establishing healing response [Mahdavian Delavary et al., 2011]. Wound angiogenesis starts right at the beginning of the healing process and is amplified by inflammation and hypoxia, two driving forces for wound angiogenesis, leading to the production of VEGF, the most potent pro-angiogenic factor. VEGF, in turn, promotes blood vessel permeability enabling inflammatory factors and nutrients release necessary for the healing process, and also the survival and proliferation of endothelial cells (EC), guiding the newly formed blood vessels, that appear in the wound bed by 3–5 days after injury. EC, vascular smooth muscle cells (VSMC), macrophages, fibroblast, and extracellular matrix present in the wound area secrete several angiogenic factors, growth factors, and proteases that support the angiogenic process. At later stages of the healing process these regulators gradually return to normal level, which enhance new blood vessels stability [Chung et al., 2010]. When insufficient angiogenic stimulation occurs, it compromises the neovascularization and the normal regeneration of the wounded tissue, as new blood vessels are fundamental for the formation of the provisional matrix and for the delivering of oxygen and nutrients to growing tissue and removing metabolic waste products [Eming et al., 2007]. However, excessive pro-angiogenic stimulation also may result in poor perfusion as abnormal and no functional capillaries are formed. Both processes can result in chronic wound and ulcer development [Shoab et al., 1999].

Epidemiological and experimental evidence indicates that diets rich in plant-derived food protect from several pathologies, associated with inflammatory and angiogenic processes [Gerhauser, 2005; Oak et al., 2005; Soares and Azevedo, 2007]. These properties have mainly been attributed to the anti-oxidant and anti-inflammatory effects of polyphenolic compounds [Biesalski, 2007; Stevenson and Hurst, 2007].

Beer consumption has increased worldwide, especially among the young generations. Beer contains more than 800 compounds, many of which are polyphenols that contribute to its flavour characteristics. Many of these polyphenols have been described as potent anti-oxidative, anti-inflammatory and anti-carcinogenic molecules, being, thus, possible contributors to the prevention of pathologies with high incidence and mortality rates in the western world [Stevens and Page, 2004; Gerhauser, 2005; Cho et al., 2008; Magalhaes et al., 2009].

Xanthohumol (XN) is a polyphenol almost exclusively present in hops, used in beer production. Due to the described health properties attributed to XN, as anti-cancer, anti-invasive, anti-angiogenic, anti-inflammatory, and anti-oxidant [Gerhauser, 2005; Albini et al., 2006; Guerreiro et al., 2007; Monteiro et al., 2008; Negrao et al., 2010], production of a polyphenol-enriched beer has been considered an interesting challenge to the brewing industry, particularly, because most beer production processing results in beers with a very low content in XN (0.002–1.2 mg/L) [Gerhauser, 2005].

In 2004, two new beverages were introduced in the German market, XAN Hefeweissbeer® and XAN Wellness®, with elevated levels of XN (1.4 mg/L and 4 mg/L respectively) [Winkelmann, 2004; Magalhaes et al., 2009]. Other following XN-enriched products are in development and trial, namely muesli bars, chocolate, yoghurt, bread, pastries, and biscuits (Xan products, TA-XAN AG, Company SCHULTZEPLUS, Germany) [Xan, 2011]. Nevertheless, the use of these food additives must be carefully taken, as very few studies about the safety of these additives were performed until now.

Previous work of our group demonstrated that different polyphenols present in beer exert different effects on angiogenesis and inflammation, resulting in differences in the wound healing process when these compounds were administered topically in the wound area [Negrao et al., 2010]. Namely, whereas 8-prenylnaringenin (8PN) stimulates angiogenesis, XN and isoxanthohumol (IXN) manifested anti-angiogenic and anti-inflammatory effects in identical conditions [Negrao et al., 2010].

In beer, these polyphenols are not isolated, being mixed with several other compounds in a complex matrix that may alter their final biological effect. Moreover, the administration route (e.g., ingestion vs. topical application) of polyphenols might lead to different actions in the body. The effects of the ingestion of XN were already studied by several groups [Albini et al., 2006; Monteiro et al., 2008; Bolca et al., 2010; Dorn et al., 2010]. However, the effects of beer and XN-supplemented beer ingestion on inflammation and angiogenesis were not clearly examined.

Given its dependence on angiogenesis and inflammation, wound healing is a good model for studying the effects of polyphenols in these two processes. In this work, we aimed to determine whether prolonged stout beer (SB) and XN supplemented stout beer (10 mg/L) intake could influence skin wound healing process, through angiogenesis and inflammation modulation. Furthermore, since adipose tissue is known to play a major role in oxidative stress and in the inflammatory process, we did also characterize the presence and morphology of adipocytes within skin damage tissue.

MATERIALS AND METHODS

IN VIVO STUDIES

All research animals were obtained, maintained, and used in experiments conducted according to accepted standards of humane animal care (Declaration of Helsinki, European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals). A veterinary accompanied all steps involving animal care and manipulation. All the authors involved in animal studies in the present study received accreditation from the Portuguese Veterinarian Administration as a competent person for animal experimentation (investigator–coordinator).

BEER INFORMATION

The beer used in this study was Superbock® SB (UNICER, Porto, Portugal), with the forward polyphenols composition: Polyphenols: 255 ± 13 mg/L; XN: Traces (μM). The final concentration in the supplemented stout beer was 10 mg/L. In order to ensure batch-to-batch consistency in XN levels in the baseline beer, we used SB from the same batch along all the experiment, assuring consistency regarding this point along the experiment.

SKIN WOUND-HEALING ASSAY

Wistar rats (Charles River, Wilmington, MA,), 6-week-old, were maintained under standard temperature and light conditions (20–22°C, 12-h light/dark cycle). The animals were divided into four groups, of six animals each, that were subjected to different treatments: (1) water (control group, C), (2) 5% ethanol solution in water (Et), (3) Superbock® SB, and (4) Superbock® SB supplemented with XN 10 mg/L (Suppl SB). The beverages were supplied to the rats ad libitum in dark bottles. All animals were maintained on standard ad libitum rodent chow. Body weight gain was monitored twice weekly. Beverages and animal pellet food were renewed and intake was assessed every 2–3 days. After 4 weeks of treatment, the animals were anesthetized, dorsal skin of the rat was shaved and cleaned. Full skin-thickness longitudinal incisions (1.5 cm) were created on the dorsal surface of the rat and the wound edges closed with surgical sutures at 0.5 cm intervals. The animals were kept in individual cages and continued beverage treatment for another week. Rats were examined daily for wound healing progression. After 7 days the animals were anesthetized and blood was drawn from the left ventricle to heparin-containing tubes for glutathione determination in plasma. Serum was also obtained for inflammatory factors and biochemical parameters evaluation. Wound tissue was removed, fixed in 10% neutral-buffered formalin, paraffin-embedded, and sectioned for histological and immunohistochemical analyses.

EVALUATION OF PLASMA BIOCHEMICAL PARAMETERS

Plasma biochemical markers were measured in the Central Laboratory, Department of Clinical Pathology, Hospital São João, using conventional methods with an Olympus AU5400® automated clinical chemistry analyzer. (Beckman-Coulter®, Izasa, Porto, Portugal). Parameters evaluated included hepatic function markers such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) and markers of metabolic status including glucose, uric acid, triglycerides, total cholesterol, very low density lipoproteins cholesterol (VLDL), low density lipoproteins cholesterol (LDL), and high density lipoproteins cholesterol (HDL). LDL was calculated according to Friedewald's equation [Friedewald et al., 1972]: LDL-cholesterol = total cholesterol − HDL-cholesterol − (triglycerides/5). VLDL = TRG/5.

GSH AND GSSG DETERMINATION IN RAT PLASMA

Chromatographic detection of GSH/GSSG was performed as described in Glutathione (GSH/GSSG) HPLC Kit (Immundiagnostik, Germany) using a reverse-phase HPLC VWR-Hitachi Elite LaChrom System (VWR, Germany) and plasma samples. The system consisted of a pump model L-2130, an autosampler L-27200, a L-2300 Column Oven, a BDS-Hypersil C18 analytical column (10 cm × 4.6 mm i.d.; 3-μm particle size) (Thermo Scientific) with no guard column and an FL detector model L-2485. After running the samples, the chromatograms were analyzed using Agilent EZChrom Elite 3.3.2 software (Agilent Technologies).

IMMUNOHISTOCHEMISTRY ANALYSIS

The microvessel density was evaluated in each formalin-fixed paraffin-embedded wounded tissue section. Immunostaining for von-Willebrand Factor (vWF) diluted 1:100 (Millipore, MA) was preceded by tissue slide digestion with pepsin freshly prepared solution (0.5% in 5 mM HCl) at 37°C for 20 min. Negative controls were carried out by omission of the primary antibody in tissue sections expressing the marker. To ensure specific immunostaining by vWF, tissue slides of paraffin-embedded B16 mellanoma tumors were immunostained against vWF. Microvessel density was then evaluated in three tissue sections, for each animal, and normalised to the total area of the tissue section.

ELISA ASSAYS

VEGF was quantified in rats serum by ELISA (Rat VEGF immunoassay kit, R&D Systems, Abingdon, UK) according to the manufacturer's instructions.

Interleukin (IL) 1β was quantified in rats serum by ELISA (IL-1β-EASIA kit, BioSource, Nivelles, Belgium) according to the manufacturer's instructions.

DETERMINATION OF N-ACETYLGLUCOSAMINIDASE ACTIVITY

The *N*-acetylglucosaminidase (NAG) enzyme is present at high levels in activated macrophages. Inflammation can be evaluated by measuring the levels of the lysosomal NAG enzyme in the serum. Serum was incubated for 10 min at 37°C with 100 μl of *p*-nitrophenyl-*N*-acetyl-beta-D-glucosaminide solution in a 96-well plate. The reaction was stopped by the addition of 0.2 M glycine buffer (pH 10.6) and the subtrate hydrolysis was measured at 405 nm.

NO DETERMINATION

NO level was determined as the concentration of nitrate plus nitrite in the serum by colorimetric assay. Serum was incubated with equal volume of Griess Reagent in a 96-well microtiter plate, for 15 min at room temperature. Measurement was performed in a spectrophotometer plate reader at 550 nm. Data were expressed as NO concentration (μM).

ADIPOCYTE SIZE AND NUMBER

Hematoxylin- and eosin-stained 5 μm-thick paraffin-embedded sections of wound healing areas were observed in a blind study and adipocyte size measurement and quantification were performed as described. Briefly, microscopic pictures covering the whole analyzed area were taken. Using Adobe Photoshop CS5, a compound image was created with all the acquired pictures. All adipocytes were then outlined and adipocyte number and area measurements were computed automatically.

STATISTICAL ANALYSES

Every assay was performed at least in three independent experiments. Quantifications are expressed as mean ± SEM and/or as percentage of control, which was considered to be 100%. Statistical significance of difference between various groups was evaluated by analysis of variance (ANOVA) followed by the Bonferroni test. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $P \leq 0.05$.

RESULTS

FOOD AND BEVERAGE INTAKE AND BODY WEIGHT MEASUREMENT

Every group ingested the same amount of beverage per day. However, the higher caloric intake among the groups drinking 5% ethanol-containing beverages was compensated by the ingestion of significantly smaller amounts of food by Et (7.23 ± 0.23 g/day/100 g body weight), SB (6.65 ± 0.25 g/day/100 g body weight), and Suppl SB (6.35 ± 0.24 g/day/100 g body weight) treated animals when compared to control group (8.30 ± 0.24 g/day/100 g body weight) (Fig. 1A,B).

We next examined whether the different treatments exerted any influence on rat body weight. Body weight gradually increased in every studied group throughout the 5 weeks of treatment, exhibiting an identical rate of weight gain for each group (Fig. 1C). Accordingly, at the end of experiment (5 weeks), body weight did not differ between the four groups of treated animals (Fig. 1C).

BIOCHEMICAL PARAMETERS AND HEPATIC ENZYME ACTIVITY EVALUATION

We then analyzed hepatic and biochemical serum parameters as indicators of liver damage and function, and of metabolism, after different beverage treatments. The hepatic enzyme activities of ALT, AST, gamma-glutamyltransferase (GGT), and ALP exhibited similar values among the four different treated animal groups, indicating that there was no hepatic toxicity with the ingested doses of these beverages, during 5 weeks (Table I).

In order to evaluate the metabolic profile of the studied animals, we also quantified biochemical parameters including glucose, uric acid, triglycerides, cholesterol, VLDL, LDL, and HDL. Careful analysis of Table I seemed to indicate a similar decrease in plasma triglycerides in both SB and Suppl SB, two tested beers with very different quantities of bioactive components, comparatively to C and Et groups. However, statistical analysis of the results did not confirm that difference. No significant alterations were observed for all other biochemical parameters, confirming a normal hepatic cell function regarding carbohydrate and lipid metabolism (Table I).

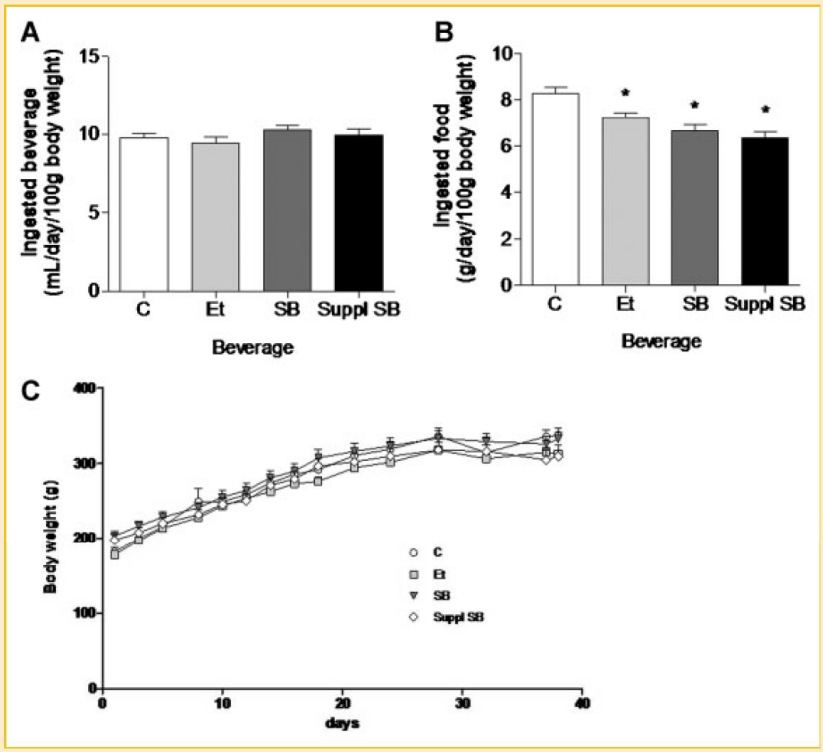

Fig. 1. Mean values for (A) beverage and (B) food intake by Wistar rats subjected to distinct treatments during 5 weeks. C: Rat body weight measurement during the five-week treatment with the different beverages intake. C, control group; Et, 5% ethanol solution in water; SB, Superbock® stout beer; Suppl SB, xanthohumol-supplemented Superbock® stout beer. Results are presented as means ± SEM (n = 7) *$P \leq 0.05$ versus C.

TABLE I. Analysis of Plasma Biochemical Markers in Wistar Rats After 5-Week Consumption of Water (C, Control), 5% Ethanol (Et), Stout Beer (SB), or XN-Supplemented Stout Beer (Suppl SB)

	C	Et	SB	Suppl SB
AST (U/L)	103.4 ± 13.04	80.3 ± 8.78	69.0 ± 4.28	75.0 ± 7.0
ALT (U/L)	26.0 ± 0.7	26.0 ± 1.79	23.8 ± 1.9	21.67 ± 2.36
AST/ALT	3.8 ± 0.7	3.2 ± 0.3	3.1 ± 0.4	3.1 ± 0.2
ALP (U/L)	116.7 ± 9.7	91.3 ± 7.1	144.8 ± 13.7	108.2 ± 14.0
GGT(U/L)	2.0 ± 0.2	1.3 ± 0.3	2.0 ± 0.2	2.8 ± 0.2
Glucose (mg/dl)	147.0 ± 7.0	189.0 ± 36.0	135.0 ± 6.0	160.0 ± 9.0
Uric acid (mg/dl)	1.09 ± 0.06	0.91 ± 0.12	0.73 ± 0.07	0.74 ± 0.14
Triglycerides (mg/dL)	90.0 ± 7.0	87.0 ± 8.0	64.0 ± 8.0	68.0 ± 9.0
Cholesterol (mg/dl)	43.0 ± 2.0	41.0 ± 2.0	41.0 ± 2.0	47.0 ± 3.0
VLDL (mg/dl)	23.0 ± 3.0	22.0 ± 3.0	18.0 ± 4.0	18.0 ± 4.0
LDL (mg/dl)	12.0 ± 1.0	11.0 ± 1.0	11.0 ± 1.0	12.0 ± 1.0
HDL (mg/dl)	23.0 ± 1.0	26.0 ± 2.0	23.0 ± 1.026.0	25.0 ± 2.0

Evaluated parameters included hepatic function markers, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), and alkaline phosphatase (ALP) activities and markers of metabolic status including glucose, uric acid, triglycerides, total cholesterol, very low density lipoprotein cholesterol (VLDL), low density lipoprotein cholesterol (LDL), and high density lipoprotein cholesterol (HDL). Results are presented as means ± SEM of independent experiments ($4 \leq n \leq 6$).

SB AND SUPPL SB BEVERAGE TREATMENTS IMPROVED ANTI-OXIDANT DEFENSES

To characterize the general redox state after 5 weeks of treatment with the study beverages, we evaluated endogenous anti-oxidant defenses. As reduced glutathione (GSH) plays a critical role in the maintenance of the thiol redox status in cells, the determination of GSH or the GSH/oxidized glutathione (GSSG) ratio is a useful marker of oxidative stress. The effect of treatments on plasma glutathione levels is illustrated in Figure 2. Chronic consumption of both SB and Suppl SB resulted in an increase of plasma GSH levels (to 124.79 ± 7.60% and 152.69 ± 11.41%, respectively) as compared to C (100.00 ± 15.04%) and Et (97.21 ± 18.37%) groups. Nevertheless, a statistically significant difference was only observed in the group that ingested Suppl SB. No differences were found between C and Et groups. In contrast, all the beverages significantly reduced serum GSSG levels to 68.33 ± 11.12% (Et), 51.84 ± 3.88% (SB), and 56.47 ± 4.85% (Suppl SB) relatively to control group. Accordingly, the GSH/GSSG ratio in rat plasma increased slightly in Et treated animals (to 134.31 ± 16.65%), and significantly in SB (to 163.82 ± 10.02%) and Suppl SB (to 198.80 ± 8.44%) groups. This latter showed a significant increase in comparison both to C and Et rat groups (Fig. 2). Altogether, these findings indicate that XN-enriched beer is able to increase systemic anti-oxidant defences in skin-injured Wistar rats.

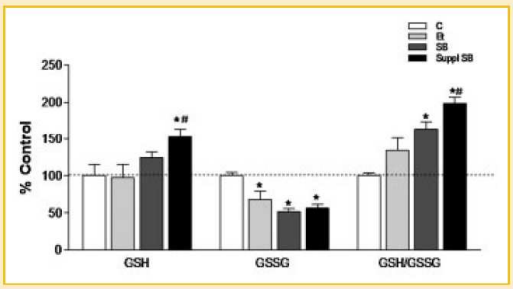

Fig. 2. Chromatographic detection of GSH and GSSG in plasma of animals treated with C, water control group; Et, 5% ethanol solution in water; SB, Superbock® stout beer; Suppl SB, xanthohumol-supplemented Superbock® stout beer. Results are means ± SEM ($4 \leq n \leq 6$) and are expressed as percentage of control. *$P \leq 0.05$ versus C; #$P \leq 0.05$ versus Et.

POLYPHENOL-RICH BEVERAGES DIMINISHED LOCAL AND SYSTEMIC ANGIOGENESIS AND INFLAMMATORY MARKERS

Skin wound healing is a process involving the formation of new extracellular matrix, cell infiltration, and tissue remodeling. Two fundamental physiological phenomena are deeply implicated in this process: Inflammation and angiogenesis. On day 7 post-injury, macroscopic evaluation of the external surface of the healing area, in the dorsal skin of the animals, showed no differences in the wound area after a 7-day treatment with any beverage, comparatively to the control group. However, the corresponding histological sections revealed that the granulation tissue of the incision area of animals treated with Suppl SB seemed more homogeneous, thinner, and with less inflammatory infiltration than that of control animals (Fig. 3C,D).

The ingestion of 5% ethanol was accompanied by a slight increase in the number of blood vessels in the wounded area (21.10 ± 2.19 vessels/mm^2) in comparison to controls (16.68 ± 1.51 vessels/mm^2). Inversely, SB (15.88 ± 1.94 vessels/mm^2) and Suppl SB (12.83 ± 1.00 vessels/mm^2) ingestion led to a tendency to microvessel density reduction (Fig. 3A,B) surrounding the wounded area, although none of these observed effects reached statistical significance relative to controls. Interestingly, consumption of XN-enriched beer resulted in a significant decrease in the number of blood vessels within the wounds when compared to ethanol treated mice (#$P < 0.05$ vs. Et).

An identical profile was found for serum VEGF quantification (Fig. 4A). Ingestion of 5% ethanol increased VEGF in the serum (60.74 ± 2.94 pg/ml) comparatively to control group (52.43 ± 0.80 pg/ml) ($P \leq 0.05$ vs. C). In contrast, consumption of SB and Suppl SB significantly reduced VEGF levels in rats serum (47.51 ± 1.71 pg/ml and 42.77 ± 0.30 pg/ml, respectively). Remarkably, VEGF was significantly decreased in Suppl SB as compared to C, Et, and SB.

The activity of pro-inflammatory markers in rats serum followed the same outline. NAG activity was higher in Et group (150.18 ± 8.32%), revealing an increased systemic inflammatory

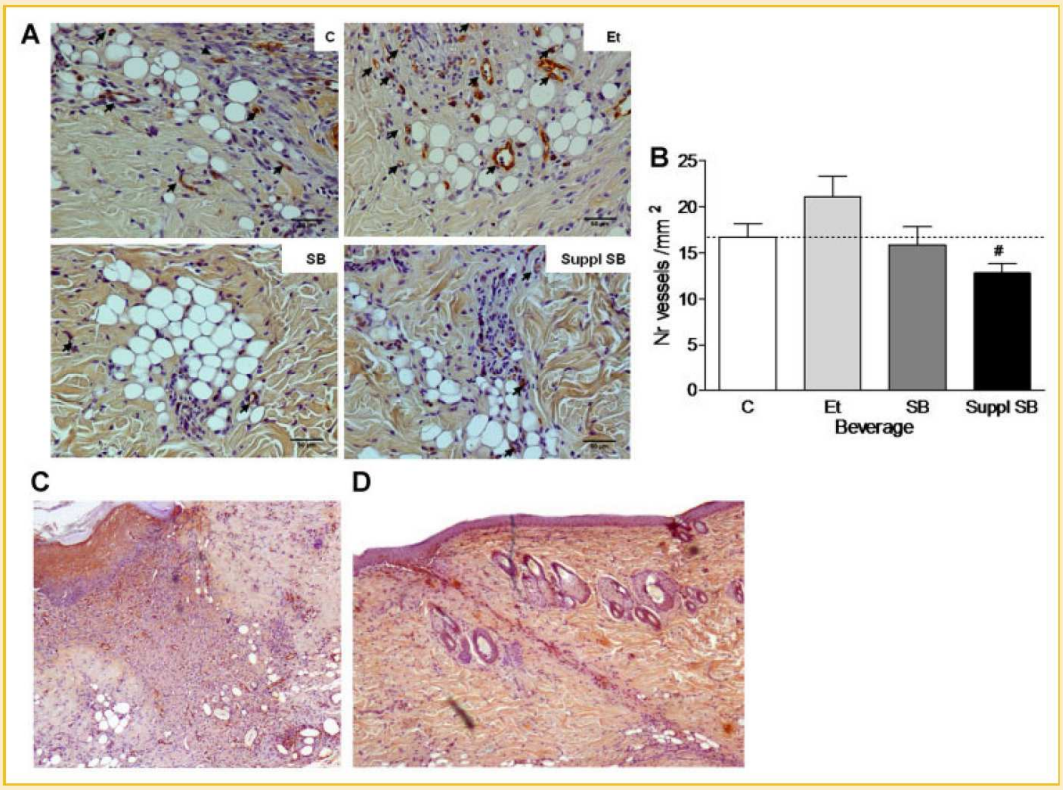

Fig. 3. Skin wound-healing assay. After 4 weeks of beverage supplied to the rats ad libitum in dark bottles, longitudinal incisions were created on the dorsal surface of the rats and beverages were supplied for more 7 days. Water control group (C); 5% ethanol solution in water (Et); Superbock® stout beer (SB); and xanthohumol supplemented stout beer (Suppl SB). Then, wound tissue was collected for angiogenesis evaluation. A, wound tissue section micrographs, using anti-von-Willebrand Factor (vWF) for evaluation of blood vessels (immunostaining, magnification: 200×). Black arrows indicate blood vessels. B, quantification of blood vessels present in three tissue sections, for each animal, and normalized to the total area of the tissue section. Results are presented as means ± SEM ($4 \leq n \leq 6$), #$P < 0.05$ vs. Et. C: Control (C) and (D) Suppl SB, immuno-stained micrographs of wound tissue sections, highlighting different thickness of granulation tissue fulfilling the incision with different treatments (magnification: 40×).

status significantly different from control group (100.00 ± 16.94%) and diminished to values similar to those obtained with control groups in rats that ingested SB (106.04 ± 10.04%), decreasing even more for Suppl SB (to 72.23 ± 8.62%) (Fig. 4B). These latter findings were corroborated by an identical profile obtained for IL1β determination (Fig. 4C). Et increased IL1β levels in serum to 138.13 ± 2.00% and Suppl SB diminished its levels to 90.93 ± 1.53%.

NO determinations resulted in a highly significant NO reduction for both SB and Suppl SB treatments to 35.23 ± 1.84% and to 22.94 ± 2.68%, respectively. Serum NO amount in Et group (106.11 ± 7.55%) was similar to that observed in C group (100.00 ± 13.12%).

SB AND SUPPL SB TEND TO INCREASE ADIPOCYTE NUMBER AND DIMINISH ITS SIZE IN THE WOUND SITE AREA

A closer observation of the skin damaged tissue revealed that the presence of adipocytes within granulation tissue was distinct among the different groups (Fig. 3A). These findings, together with the recent established role of adipocytes in inflammation, prompted us to determine the size and number of these cells in wound healing process after SB or Suppl SB consumption.

Determination of adipocyte area revealed a tendency to a progressive decrease of the adipocyte size in SB (476.0 ± 21.50 μm^2) and Suppl SB (376.0 ± 45.88 μm^2) when compared to C (499.0 ± 64.01 μm^2) and to Et (515.5 ± 23.55 μm^2) treated animals. This reduction in adipocyte size seemed higher for Suppl SB, although this difference did not reach statistical significance (Fig. 5A). Histological analysis also revealed that the number of adipocytes in the wound-healing area tended to increase in SB (76.25 ± 18.12 adipocyte /mm^2) and Suppl SB (61.50 ± 11.50 adi-adipocyte /mm^2) when compared to C (39.25 ± 6.25 adipocyte / mm^2) and Et treated (37.43 ± 4.95 adipocyte /mm^2) groups, although again not reaching statistical significance (Fig. 5B).

DISCUSSION

Wound healing is a physiological process necessary for repair and regeneration of injured skin tissue. This process greatly depends on the crosstalk between inflammation, oxidative stress, and

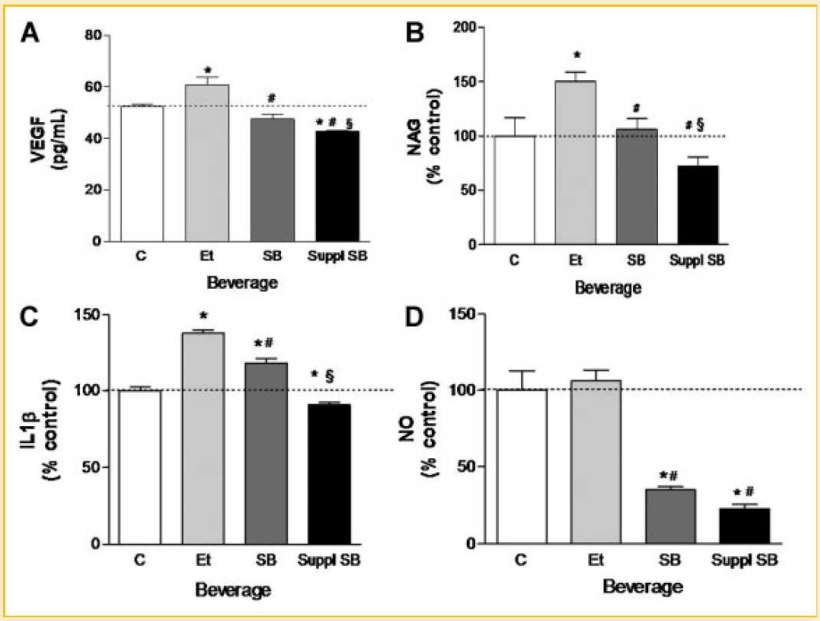

Fig. 4. Evaluation of angiogenesis and inflammatory markers in skin injured Wistar rats, after 4 weeks of beverage supplied to the rats ad libitum in dark bottles. C, Water control group; Et, 5% ethanol solution in water; SB, Superbock® stout beer; Suppl SB, xanthohumol-supplemented Superbock® stout beer. Longitudinal incisions were created on the dorsal surface of the rats and beverages were supplied for more 7 days. Blood was then collected for systemic angiogenesis and inflammation markers evaluation. A: VEGF levels; (B) N-acetylglucosaminidase (NAG) activity; (C) IL1β levels; (D) NO levels. Results are presented as means ± SEM ($4 \leq n \leq 6$) and are expressed as percentage of control. $^{*}P \leq 0.05$ versus C; $\#P \leq 0.05$ versus Et; $\S P \leq 0.05$ versus SB.

angiogenesis [Soares, 2009]. Discovering novel promoters of optimal wound healing, as well as identifying possible nutritional intervention factors able to ameliorate the process becomes a mandatory issue, as compromise of the normal resolution of the inflammation and angiogenic processes, can result in chronic wound and ulcer development, leading to tissue damage.

The current study demonstrates that consumption of SB and particularly XN-supplemented stout beer (Suppl SB) seemed to improve wound healing resolution. We were able to show that Suppl SB wound bed seemed better re-epithelizated than the correspondent area in control group. Also, the number of blood vessels tends to decrease in rat wounds consuming Suppl SB, and this was accompanied by reduced levels of VEGF, oxidative stress, and inflammatory markers in blood serum. Altogether, these findings led us to assume that wound healing enhancement was due to a reduction in the inflammatory phase and consequently a more controlled angiogenic process.

Inflammatory cells appear very rapidly in a wound after injury and remain until 3–5 days, as reported by Kagawa and collaborators [Kagawa et al., 2009]. Accordingly, our results demonstrate that at day 7, SB and Suppl SB diminished the levels of inflammatory markers, namely IL-1β, NAG activity, and NO. On contrary, at this time point, rats treated with Et exhibited increased systemic IL-1β and NAG activity, indicating the occurrence of macrophage activation.

Although with different etiology, most chronic wounds result from a deficient progression from the inflammatory to the granulation tissue formation. Abnormal macrophage infiltration and prolonged inflammation has been described in chronic and diabetic wounds [Loots et al., 1998; Rodero and Khosrotehrani, 2010]. So, the decrease in systemic inflammatory markers observed after SB and Suppl SB consumption may result in a better controlled inflammatory response, in the presence of polyphenols, which were even able to overcome the pro-inflammatory profile induced by the ethanol present in the tested beverages. A better regulation of the inflammatory phase could improve wounds healing characterized by an excessive inflammatory profile, through a better transition to the granulation phase. Patients with chronic venous ulcers have sustained higher production of VEGF in the wound, which explains the vascular permeability and increased transudation of the wound area, resulting in disruptive new vessels [Shoab et al., 1999]. The decreased VEGF levels, observed for Suppl SB treatment, together with improved inflammatory conditions, could promote the stabilization of the new blood vessels, resulting in a better perfusion of the wounded tissue, helping the resolution of this kind of chronic wounds. Diabetic wounds also exhibit delayed wound closure but in contrast, VEGF levels are lower, leading to poor angiogenesis. Qiao demonstrated that the number of EC in diabetic rat wounds is identical to that found in nondiabetic wounds, but they were unable to form functional vessels [Qiao et al., 2011]. Then, chronic wounds may differ accordingly to established pathologies. In some situations wound resolution would improve by diminishing angiogenic stimulation and in others by enhancing angiogenesis.

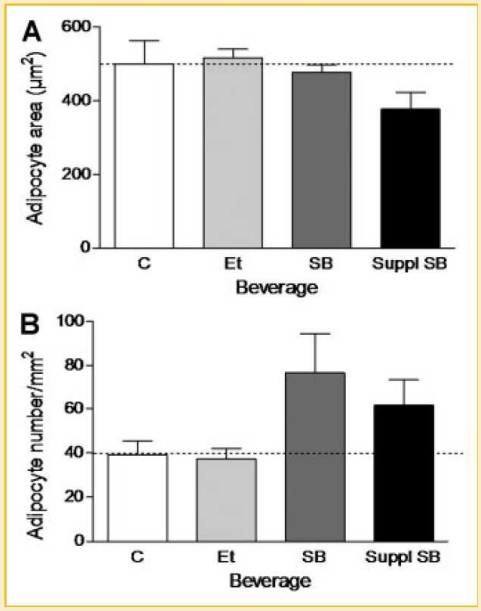

Fig. 5. Adipocytes (A) size and (B) number observed under identity occultation in hematoxylin & eosin (HE)-stained 5 µm-thick sections of skin wound healing area. C, control (water-drinking animals); Et, 5% ethanol solution in water; SB, Superbock® stout beer; Suppl SB, xanthohumol-supplemented Superbock® stout beer. Results are represented as means ± SEM ($4 \leq n \leq 6$).

But not always an increase in angiogenesis is related to better perfusion of the wound area, as the new blood vessels need to have conditions to stabilize and function properly. As polyphenols, and more specifically XN, can act simultaneously in different molecular targets, involving both inflammation and angiogenesis, the resulting regulation of the interplay of these two processes can improve chronic and also normal wound healing resolution, as XN can finely modulate inflammation and angiogenesis, accordingly to the progression of wound environment.

The study was accomplished by providing beverages to rats during a total of 5 weeks, 4 weeks before performing the skin incisions and 1 after the incisions were done. This study design allowed us to better understand the potential health benefits of regular ingestion of polyphenol-rich beverages in their natural context and its potential preventive effect. Beer ingestion in our rat experiment corresponded to a moderate alcohol intake by humans, approximately to 0.9 L beer/day by a man of 60 kg [Reagan-Shaw et al., 2008]. The establishment of the chronic treatment in our experimental model gains particular relevance, considering that polyphenols accumulate in macrophages and other cells or tissues at relatively high concentrations [Kawai et al., 2008; Bolca et al., 2010], when comparing with plasma concentrations. It has also been suggested that polyphenols may exert biological effects through specific targets at much lower concentrations than those initially suspected. Indeed, low amounts of polyphenols may specifically interact with proteins or particular lipid bilayer components [Fraga et al., 2010] and this may also explain the observed in vivo effects despite the low bioavailability described for polyphenols.

In agreement with the literature, biochemical analyses of serum parameters revealed no significant differences between the groups treated with the different beverages, indicating a normal liver function and lipid and carbohydrate metabolism in treated animals. In line with our results, Dorn et al. [2010] described no adverse toxic effects of 1,000 mg XN /kg body weight/day after oral ingestion in mice during 3 weeks. Other authors also observed no toxic effects with 100µM ingestion of XN [Monteiro et al., 2008]. XN has been described to alter lipid and carbohydrate metabolism, lowering levels of plasma glucose and triglycerides [Gasowski et al., 2004; Nozawa, 2005]. Although our results seemed to indicate a decrease in serum triglycerides in SB and Suppl SB, these results were not statistically different.

During the last decades, the beneficial role of polyphenols was almost exclusively attributed to their anti-oxidant capacity, related to the reduction of excessive production of reactive oxygen species, which would induce an oxidative stress state [Holst and Williamson, 2008]. Nowadays, it is believed that polyphenol mechanisms of action in vivo are much more complex and these compounds may even exert their effect by acting as pro-oxidants, at low concentrations, preparing cells to respond in more severe stress conditions [Tosetti et al., 2009]. Ethanol by itself diminished GSSG levels in serum but did not change GSH, resulting in a slight increase, although not statistically significant, of the GSH/GSSG ratio. Other authors suggest that moderate ethanol consumption can stimulate anti-oxidative defences protecting from oxidation produced by ethanol consumption. These authors reported an increase in glutathione reductase activity, necessary for the utilization of GSSG formed and regenerate the GSH, maintaining the GSH/GSSG ratio, accordingly to our results [Assuncao et al., 2009]. Other authors [Roig et al., 1999] observed similar effects in what concern GSH/GSSH ratios for ethanol. Our results demonstrated that XN, in the presence of ethanol, substantially and significantly, increased GSH and diminished GSSG, resulting in a great improvement of GSH/GSSG ratio, suggesting not only an increase of anti-oxidant-defences but probably, a decrease in oxidant levels due to its inherent anti-oxidant capacity. We found that consumption of both SB and Suppl SB resulted in a significantly higher GSH/GSSG ratio in serum than the one obtained in control rats. Although not statistically significant the increase in GSH and GSH/GSSG seems to be consistently higher for Suppl SB comparatively with SB. Also, the difference of GSH between C or Et groups and Suppl SB was statistically significant but not when comparing C or Et with SB. In what concerns GSSG levels, both SB and Suppl SB effects were similar.

GSH is used to eliminate peroxides, maintain thiol/disulfide redox state of proteins, and maintain the redox state of ascorbate and (indirectly) vitamin E in their reduced and functional forms [Jones, 2006]. The apparently higher level of GSH after Suppl SB ingestion may be due to the higher levels of XN, a known anti-oxidant polyphenol. This anti-oxidative capacity may be considered one of the potential contributors to wound healing resolution by XN. Altavilla et al. [2001] proposed that diabetes is associated with elevated oxidative stress and inflammation, and that by reducing

lipid peroxidation in wounds of diabetic mice, it would be possible to reverse edema and stimulate the healing process. This would correspond to the reversion of the negative effects of diabetes in wound healing. In a recent paper [Strathmann et al., 2010], however, XN is described as a rapid inducer of thiol depletion and a producer of superoxide radical at mitochondria, inducing disruption of mitochondrial integrity and apoptosis in cancer cells, but at much higher concentrations (1.6–50 μM) than those obtained in vivo, as far as it has been described [Gerhauser, 2005].

Several studies have tried to evaluate the effect of ethanol consumption on angiogenesis. Morrow et al. [2008] demonstrated that moderate consumption of alcohol enhances endothelial pro-angiogenic activity in vitro through a novel Notch-angiopoeitin-1/Tie2 signaling pathway. Tan et al. [2007] reported that moderate ethanol consumption (1% ethanol), associated with alcoholic beverage consumption in humans, equivalent to two drinks per day induced the expression of VEGF and angiogenesis in mice melanoma. Other groups did also report an increase in angiogenesis in different models both in vivo and in vitro [Gu et al., 2005; Bora et al., 2006] in response to ethanol ingestion. On the other hand, Radek et al. [2008] found that acute ethanol exposure impairs angiogenesis probably by changes in the response to VEGF and hypoxia by endothelial cells, in a wound healing model. Although not statistically significant, our results suggest that chronic ingestion of 5% ethanol increases the number of vessels formed in the wound area, after 7 days of the injury, when compared to control (water ingestion) as already described by other groups (see above). Strikingly, SB and Suppl SB reversed ethanol effect. Consistently, we observed an increase of systemic inflammatory markers as well as VEGF upon consumption of ethanol, which was again reversed by SB and Suppl SB.

In recent years the way we see adipose tissue has changed from a simple lipid and energy storage to a multifunctional complex organ with autocrine, paracrine, and endocrine functions. A remarkable finding of the present work that corroborates this idea was the appearance of distinct adipose tissue patterns within the granuloma tissue upon different treatments. SB and particularly Suppl SB consumption led to decreased adipocyte size and increased adipocyte number in wounded skin corresponding to subcutaneous adipose tissue. The precise function of these adipocytes in the granulation tissue area remains unknown. Interestingly, adipocyte hypertrophy has been associated with inflammatory dysfunction [Monteiro et al., 2006; Monteiro, 2009]. It has been described that adipose tissue from obese rats impaired wound contraction associated with an increased inflammatory phase [Nascimento and Costa, 2006]. Although necessary for wounds to heal, inflammatory cytokines are present in excess in nonhealing wounds. Adipocytes are known to communicate with endothelial and inflammatory cells in a paracrine manner, through the production of several cytokines that modulate blood vessel remodeling and assembly, thus promoting the healing process. Therefore, the fact that Suppl SB results in reduced adipocyte size may imply a beneficial effect of this beverage on inflammation. Furthermore, these results are in total agreement with the decreased inflammatory markers and reduced redox state observed in the serum of these rats. These findings suggest that XN is able to affect adipose tissue homeostasis, increasing adipocyte hyperplasia and reducing adipocyte hypertrophy, probably by affecting inflammatory and vascular components. Nevertheless, definitely this assumption deserves further investigation.

In summary, polyphenols are involved in complex signaling networks between many cell types, including inflammatory, endothelial, and adipocyte cells. The present study revealed that chronic ingestion of SB and XN-supplemented stout beer are able to control the complex interactions between inflammation and angiogenic processes in skin wound healing, both locally and systemically, resulting in improved wound healing resolution. These effects suggest that this polyphenol may exert beneficial effect as a nutritional supplement.

The interest in natural compounds, such as the polyphenols that are useful for disease prevention or treatment is increasing. Nevertheless, concern about potential toxic effects associated to different doses, and individual metabolism, must be taken into account before their implementation as food additives and potential therapeutic agents.

ACKNOWLEDGMENTS

The authors thank Paulo Magalhães for the production of xanthohumol and suggestions regarding beer supplementation. This study was partially funded by FCT (SFRM/BD/41888/2007 and PTDC/SAU-OSM/102083/2008 and PEst-OE/SAU/UI0038/2011); iBeSa (Institute for Beverages and Health, Portugal; P10-08).

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CAPÍTULO 4

Different effects of catechin on angiogenesis and inflammation depending on VEGF levels

J Nutr Biochem (in press)

Negrão R, Costa R, Duarte D, Taveira T, Azevedo I, Soares R

Different effects of catechin on angiogenesis and inflammation depending on VEGF levels

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Although physiological and pathological angiogenesis develop through similar processes, during pathological angiogenesis, pro-angiogenic factors are exacerbated. Polyphenols have been considered therapeutic tools for conditions exhibiting enhanced angiogenesis. However, the possibility that these compounds may also prevent vascularization in physiological situations is a major drawback for their use. The purpose of the current study was to investigate the effects of 0.1-100 μM catechin on endothelial cells (EC) and vascular smooth muscle cells (VSMC), regarding angiogenic and inflammatory processes. Catechin modulation of angiogenesis and inflammation was also evaluated *in vivo*, using different models of angiogenesis: one physiological (skin-wound healing assay) and another one resembling pathological angiogenesis, exhibiting higher VEGF stimulation (matrigel plug assay). The *in vitro* results showed that 100 μM catechin increased viability (to 165.58% and to 165.34%) and decreased apoptosis (53.45% and 92.65%) and proliferation (33.19% and 23.36%) of EC and VSMC, respectively. Catechin affected migration and invasion, tending to increase both in EC and decreasing them in VSMC, however it did not change sprouting angiogenesis. Nevertheless, catechin diminished *in vitro* inflammatory modulators such as TNFα (58.66% for HUVEC and 85.46% for HASMC) and NFκB (38.43% for VSMC). The *in vivo* results demonstrated that catechin did not change angiogenesis and inflammation in skin-wound healing model and substantially decreased these processes in matrigel plug assay. Altogether, the current study showed that catechin has different effects in angiogenesis and inflammation depending on VEGF levels. The absence of adverse effects in mature vasculature favours catechin potential use against pathological situations where angiogenesis is stimulated.

Keywords: angiogenesis; inflammation; blood vessels; endothelial cells; smooth muscle cells; catechin.

Introduction

Angiogenesis refers to the growth and remodelling process of an existent vasculature to form a new branching network, characteristic of mature blood vessels. In the presence of a pro-angiogenic stimulus, local endothelial cells (EC) change their shape, degrade and invade the extracellular matrix. During this process EC proliferate, forming tubular structures that coalesce with other newly forming vessels. These new structures are ultimately covered by pericytes or vascular smooth muscle cells (VSMC), giving rise to mature and stable blood vessels that enable adequate blood flow and prevent further sprouting. A decrease in pericyte coverage of blood vessels has been associated with increased vessel permeability and tumour metastasis [1, 2]. The interplay between the two vascular wall cells, EC and VSMC, is now getting more attention.

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Under physiological conditions, angiogenesis is highly regulated by the balance between a huge number of pro- and anti-angiogenic factors, acting synergistically to maintain functionality of blood vessels [3]. In the adult, angiogenesis maintains physiological homeostasis and tissue integrity during wound healing, inflammation, endometrial growth in menstrual cycle and following ischemia [3, 4]. Despite angiogenesis is mandatory for the repair of damaged tissue, when deregulated it can induce tissue damage in inflammatory disorders and other conditions like diabetes, cardiovascular disease and cancer [5, 6].

Vascular endothelial growth factor (VEGF) is the most potent pro-angiogenic factor known. Skin wound repair requires the formation of new blood vessels, VEGF being a key player for this physiological process. Although physiological and pathological angiogenesis develop through many similar processes, pathological angiogenesis as occurs in tumours, due to angiogenic stimulus like hypoxia and inflammation-related events, does not regress. In contrast, it persists stimulated by tumour-secreted factors [1]. In tumour angiogenesis

VEGF functions not only as a paracrine mediator as in physiological angiogenesis, but also as an autocrine factor [7], playing crucial roles both in angiogenesis and inflammation.

Inflammation has a central role in fighting pathogens and in wound healing. It is a complex biological process involving several cell types and inflammatory mediators. When not properly controlled, it can result in adverse effects on surrounding tissues and in chronic inflammation. Several evidences demonstrate that inflammation and angiogenesis depend on each other [8]. EC-immune cell cross-talk results in the regulation of blood vessel formation and functionality, by immune cells and the microenvironment [1, 9].

It is increasingly recognized that several fruits, vegetables and some beverages like tea, wine and beer possess health promoting effects that have been attributed to polyphenol compounds, ubiquitously found in the plant kingdom. Indeed, apart from their physiological roles in plants, these compounds are important components in the human diet. Several biological activities of polyphenols have been described. They seem to have beneficial effect on pathologies like cancer, cardiovascular diseases, and diabetes and in the prevention of the establishment of neurodegenerative diseases, obesity and ageing [10-14]. Experimental studies have been focused on the anti-inflammatory, anti-oxidant and anti-cancer activities of plant polyphenols, showing that they may also modulate cell signalling [15, 16].

Tea and wine are two of the most popular consumed polyphenol-rich beverages worldwide. Of all polyphenols present in nature, flavonoids comprise the most abundant group and, within this group, flavanols are the most frequent ones. Catechin (Cat) and epicatechin are the most common flavanols in fruits. Catechin is especially abundant in fruits such as cocoa, grape, apple, apricot and cherry and in beverages such as tea and red wine [17].

The anti-angiogenic effect of plant-derived polyphenols has been a matter of study in the last decade. Namely, inside the flavanol group, epigallocatechin-3-gallate (EGCG), the most abundant flavanol in green tea, has been extensively studied, concerning anticancer properties and angiogenesis modulation.

However, studies regarding the effects of catechin in angiogenesis and particularly in both EC and VSMC, are almost inexistent to present date.

Taking these findings together, the purpose of the current study was to investigate the effects of this abundant polyphenol, catechin, on several steps of the angiogenic process in both EC and VSMC. The *in vivo* modulation of angiogenesis and inflammation by this polyphenol was also evaluated, using two different models of angiogenesis: a physiological one (skin-wound healing assay) and another one resembling pathological angiogenesis, exhibiting higher VEGF stimulation (matrigel plug assay).

Materials and Methods

Cell cultures

Human umbilical vein endothelial cells (HUVEC) were obtained from ScienceCell Research Labs (San Diego, USA). Cells were used between passages 3 and 8 and cultured in M199 medium (Sigma-Aldrich, Portugal) supplemented with 20% fetal bovine serum (FBS) (Invitrogen Life Technologies, Scotland, UK), 1% penicillin/streptomycin (Invitrogen Life Technologies, Scotland, UK), 0.01% heparin (Sigma-Aldrich, Portugal) and 30µg/mL endothelial cell growth supplement (Sigma-Aldrich, Portugal) in plates coated with 0.2% gelatin (Sigma-Aldrich, Portugal). They were maintained at 37°C in a humidified 5% carbon dioxide atmosphere. Human aortic smooth muscle cells (HASMC) were obtained from ScienceCell Research Labs (San Diego, EUA), kept between passages 2 and 8 and cultured in Dulbecco's modified Eagle's medium, supplemented with 10% FBS and 1% penicillin/streptomycin and cultured at 37°C in a humidified 5% carbon dioxide atmosphere. Catechin (Sigma-Aldrich, Portugal), was dissolved in ethanol and then added to cell culture medium at a concentration of 0.1-100 µM, established according to the viability assays performed. Catechin and vehicle (ethanol) were added to cell cultures in medium supplemented with 2% FBS and 1% penicillin/streptomycin. Control cells were incubated with vehicle (ethanol). Ethanol concentrations were kept below 0.1% in every culture.

Cell viability

HUVEC and HASMC were allowed to grow until 70-80% confluence and then incubated with 0.01-100 µM catechin or ethanol for 24 h. After the incubation period, cells were washed twice with phosphate-buffered saline solution and subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described [18]. Briefly, cells were incubated with MTT solution at a final concentration of 0.5 mg/mL for 3 h and then lysed in dimethylsulfoxide. Optical density was measured at 540 nm, and the background absorbance measured at 660 nm was subtracted. All samples were assayed at least in three independent experiments in duplicate, and the mean value for each experiment was calculated. The results are given as mean ± SEM and are expressed as percentage of control, which was considered to be 100%.

Cell apoptosis

HUVEC and HASMC ($1x10^4$ cells/mL) were grown on glass coverslips and incubated with different concentrations (1-100 µM) of catechin for 24 h. TUNEL assay was performed using the In Situ Cell Death Detection kit (Roche Diagnostics, Switzerland), as reported before [18, 19]. The percentage of stained cells was evaluated by counting the cells stained with

TUNEL (apoptotic cells) divided by the total number of nuclei stained with DAPI (Roche Diagnostics, Switzerland) at a x200 magnification field.

Cell proliferation
HUVEC and HASMC cultures (1x10⁴ cells/mL) were established on glass coverslips and were allowed to grow until 70-80% confluence following treatment procedures with 1-100 μM catechin for 24 h. Cell proliferation analyses were carried out using cellular incorporation of 5'-bromodeoxyuridine (BrdU), a thymidine analogue. After incubation with BrdU solution at a final concentration of 0.01 mM for 24 h, the number of proliferating cells (positive for BrdU), after immunohistochemistry methods using anti-BrdU-specific antibodies (BrdU In-Situ Detection Kit, BD Biosciences Pharmingen, USA), was evaluated at the microscope, according to the manufacturer's instructions.

Migration assay
HUVEC and HASMC cultures (1x10⁴ cells/mL) were established on glass coverslips and allowed to form confluent monolayers. A scratch was applied through the monolayer using a sterile 200 μL pipette tip. Cells were washed and treated with 100 μM catechin or 0.1% ethanol, for 24 h. Cell migration was evaluated by counting the number of cells that migrated through the injured area.

Invasion capacity
The invasive cell behaviour in the presence of 1-100 μM catechin for 24 h was quantified *in vitro* using a double-chamber assay by counting the number of cells that invaded a Transwell BD-Matrigel® basement membrane matrix inserts (BD-Biosciences, Belgium), according to manufacturer´s instructions. FBS was used as a chemoattractant. Results represent the ratio between invading cells in polyphenol-treated cultures compared to invasion in control cultures for the same initial amount of cultured cells.

Capillary-like structures formation
Cells were cultured on growth factor reduced-Matrigel®-coated plates (GFR-Matrigel®, BD Biosciences, Belgium) for 24 h as previously described [19]. Briefly, HUVEC were cultured on GFR-Matrigel®-coated plates for 24 h, in medium containing 1-100 μM catechin or vehicle. When cultured on Matrigel®, EC assemble into capillary-like structures. The number of cord-like structures was then counted on an inverted microscope. Each cord portion between the ramifications was considered one cord unit. Mean values were obtained by evaluating the whole cultures of each well under the same treatment. Treatments were performed as described above. A semi-quantitative measurement of cord formation in GFR-Matrigel® cultured HUVEC was developed as previously described [19].

Aortic ring assay
Aortic ring assay was performed as an *ex vivo* assay to evaluate microvessel outgrowth. In brief, thoracic aorta were removed from normal Wistar rats and washed in PBS. After removing fibro-adipose tissue, aortas were cross-sectioned, washed in cell media and placed on matrigel-coated 24-well plates, embedded on matrigel (BD Biosciences, USA) and cultured in adequate cell medium for 24 h. Catechin or ethanol were then added to cell cultures in medium supplemented with 2% FBS. Aortic rings were visualized on an inverted microscope and photographed 4 days after incubations.

Tumour necrosis factor-alpha quantification
Tumour necrosis factor-alpha (TNFα) was quantified in 200 μL culture medium in contact with HUVEC and HASMC after 24 h of cell treatment with 50-100 μM catechin or 0.1% ethanol, by ELISA (TNF-α/ TNFSF1A Immunoassay, R&D Systems, Abingdon, United Kingdom) according to the manufacturer's instructions. Quantification was performed at 450 nm and 550 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent, USA).

Nuclear Factor-Kappa B activity assay
Nuclear Factor-Kappa B (NFκB) activity was determined by ELISA assay. HUVEC and HASMC cells were treated with 50-100 μM catechin or 0.1% ethanol for 24 h. Nuclear extracts were prepared from the Nuclear extraction kit (Active Motif, USA). NFκB activity was measured using TransAM NFκB p65/p50 transcription factor assay kit (Active Motif, CA, USA). In brief, nuclear extract samples (5 μg) were added to biotinylated oligonucleotide containing the NFκB consensus site. This mixture was then added to a streptavidin-coated 96-well plate. Sample wells were incubated with NFκB p65 subunit primary antibody, followed by incubation with HRP-conjugated secondary antibody. Quantification was performed at 450 nm and 650 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent, USA).

Nitric oxide determination
Nitric oxide (NO) level was determined as the concentration of nitrate plus nitrite in the extracellular medium in contact with HUVEC and HASMC after 24 h of cell treatment with 50-100 μM catechin or 0.1% ethanol and also in animal serum by colorimetric assay. Serum was incubated with equal volume of Griess Reagent in a 96-well microtiter plate, for 15 min at room temperature. Measurement was performed in a spectrophotometer plate reader at 550 nm. Data were expressed as NO concentration (μM).

In vivo *studies*
Animal experiments were conducted according to accepted standards of humane animal care (Declaration of Helsinki, European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals). All the authors involved in animal studies in the present study received accreditation from the Portuguese Veterinarian Administration as a competent person for animal experimentation (investigator-coordinator) since 2009.

Skin wound-healing assay
Wistar rats (Charles River, Wilmington, MA, USA), 8-12-weeks-old were used and kept individually in their cages during the study. After general anaesthesia, dorsal skin of the rat was shaved and cleaned. Full skin-thickness longitudinal incisions (1.5 cm) were created on the dorsal surface of the rat and the wound edges closed with surgical sutures at 0.5 cm intervals. Catechin or vehicle (ethanol + water, 1+5; C) were administered topically (50 µL of a 50 µM solution), daily. Rats were examined daily for wound healing progression. After 7 days, wounded tissue was collected for histology studies and blood was also used for evaluation of inflammatory factors. Skin wound tissue specimens were then collected, fixed in 10% neutral-buffered formalin and embedded in paraffin. Histological and immunohistochemistry analyses were performed in 5 µm tissue sections.

Immunohistochemistry analysis
Microvessel density was evaluated in each formalin-fixed paraffin-embedded wounded tissue section. Tissue slides were incubated with an anti-von-Willebrand Factor (vWF) antibody (Millipore MA, USA). Capillaries were then counted in the three tissue sections, for each animal, and normalised to the total area of the tissue section. Negative controls were carried out by omission of the primary antibody in tissue sections expressing the marker.

Determination of N-acetylglucosaminidase activity
The N-acetylglucosaminidase (NAG) enzyme is present at high levels in activated macrophages. Inflammation can be evaluated by measuring the levels of the lysosomal NAG enzyme in the serum. Serum was incubated for 10 min at 37°C with 100 µL of p-nitrophenyl-N-acetyl-beta-D-glucosaminide solution in a 96-well plate. The reaction was stopped by the addition of 0.2 M glycine buffer (pH 10.6) and the substrate hydrolysis was measured at 405 nm.

Interleukin-1β measurement
Interleukin (IL)-1β was quantified in mice and rats serum by ELISA (IL-1β-EASIA kit, BioSource, Nivelles, Belgium) according to the manufacturer's instructions.

Matrigel plug assay
A mixture of Matrigel® and heparin without (negative control, C-) or with recombinant VEGF (positive control, C+) and 100 µM catechin, was subcutaneously inoculated into C57BL/6 mice (purchased at Charles River, Wilmington, MA, USA). The animals were euthanized after 7 days, the matrigel plug was removed, weighed, photographed and the amount of haemoglobin (Hb) in the homogenised plug was measured, as described below. Mice blood was also collected for evaluation of inflammatory factors.

Haemoglobin determination
The Hb content of the plug was evaluated after homogenisation of the plug in a water-heparin solution, which was then centrifuged at 1500 x *g* for 15 min at 20°C. The supernatant (100 µL) was used to measure the Hb content according to the Drabkin's method (Sigma-Aldrich, Portugal) at 540 nm.

Statistical analyses
Every cell experiment was performed at least in three independent experiments. Quantifications are expressed as mean ± SEM and as percentage of control, which was considered to be 100%. Statistical significance of difference between various groups was evaluated by analysis of variance (ANOVA) followed by Bonferroni's test. For comparison between two groups, Student's *t*-test was used. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $p \leq 0.05$.

Results

Micromolar concentrations of catechin increased viability and diminished apoptosis and proliferation in HUVEC and HASMC
Viability was assessed by MTT assay in the two cell types upon treatment with 0.01-100 µM of Cat at 80% confluent cultures (Figure 1A). Catechin (10 and 100 µM) increased the viability of HUVEC and HASMC (increase to 165.58 ± 5.04% and to 165.34 ± 31.12% of control to 100 µM).
To understand whether these effects of catechin were related to apoptosis or cell growth, we next investigated the apoptotic and proliferative potential activity of this polyphenol. Incubation of either cell culture with 0.1-100 µM catechin for 24 h resulted in a significant decrease in apoptosis, with statistical significance upon 1 µM Cat in HUVEC and 10 µM Cat in HASMC (53.45 ± 12.88% and 92.7 ± 4.85% for HUVEC and HASMC, respectively). This effect was consistently higher in HASMC (Figure 1B). In addition, incubation with 1.0-100.0 µM Cat for 24 h also decreased cell proliferation in HUVEC (33.19 ± 13.56% decrease) and HASMC (23.36 ± 8.39% decrease) for 100 µM as assessed by BrdU assay and illustrated in Figure 1C.

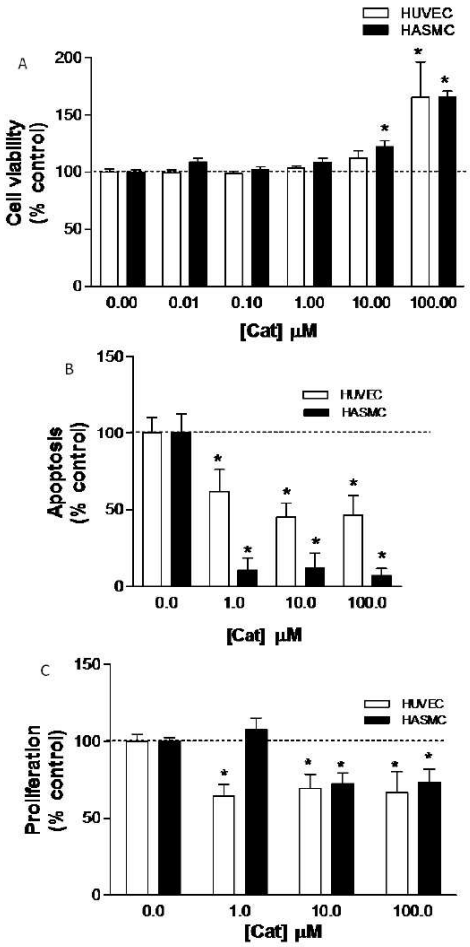

Figure 1 Effect of catechin (Cat) on (A) viability, (B) apoptosis and (C) proliferation of HUVEC and HASMC. Cells were incubated for 24 h with 0.01-100 µM Cat or vehicle (0 µM). Cell viability was evaluated by MTT assay, apoptosis was evaluated by TUNEL and the percentage of proliferative cells was examined by the ratio between BrdU-stained cells and hematoxylin-stained nuclei in every culture. Results are means ± SEM of independent experiments (4≤n≤8) and are expressed as percentage of control. *p≤0.05 vs. control.

Catechin increased migration of HUVEC but diminished HASMC migration and invasion capacity

Cell motility and extracellular matrix invasion are fundamental steps within the angiogenic process. Therefore, we next investigated the effects of catechin on migration and invasion capacity using the injury and double chamber assays, respectively.

As illustrated in Figure 2A, treatment with 100 µM catechin for 24 h increased the migration capacity of HUVEC cells in culture, while reducing HASMC migration in the same conditions. Interestingly, treatment with catechin significantly diminished invasion capacity in both cell cultures at 1 µM (39.66 ± 6.29% for HUVEC and 56.62 ± 14.41% for HASMC decrease) as analysed by transwell assays (Figure 2B). Nevertheless, as catechin concentration increased, there was a tendency to increase invasion capacity of HUVEC (to 135.43 ± 47.59% at 100 µM) although not reaching statistical significance. On the contrary, invasiveness of HASMC treated with catechin tends to remain lower than invasiveness of control group, but, once again, without statistically significance (15.91 ± 23.94% decrease relative to control for 100 µM) (Figure 2B).

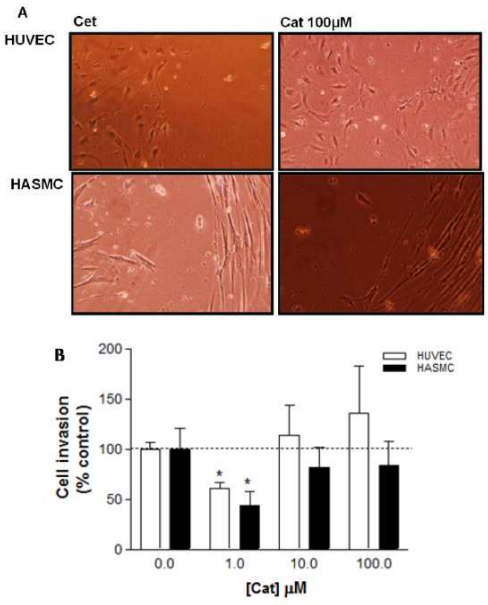

Figure 2 Effect of catechin (Cat) in HUVEC and HASMC (A) migration and (B) invasion. Cells were incubated for 24 h with 1-100 µM Cat or vehicle (Cet or 0 µM). Cell migration was visualized by injury assay in confluent cell cultures. Pictures are representative of independent studies. Invasion was measured using a double-chamber assay. Results are means ± SEM of independent experiments (4≤n≤8) and are expressed as percentage of control. *p≤0.05 vs. control.

Formation of capillary-like structures was not affected by catechin treatment

EC cells must differentiate and reorganize, assembling into vascular capillary structures, in order to form a new blood vessel. HUVEC are able to assemble into highly branched capillary-like structures when cultured on GFR-Matrigel®.

Therefore, we tested whether catechin treatments were able to alter the formation of *in vitro* vascular-like structures. Well organized interconnected tubular structures were observed in every culture (Figure 3A). Quantitative analysis revealed that no significant difference in the number of structures formed was found in any catechin-treated culture comparatively to

control group (vehicle, ethanol 0.1% or 0 μM), except for 10 μM catechin that was able to increase the number of capillary-like structure formation to 159.70 ± 17.83% (Figure 3B).

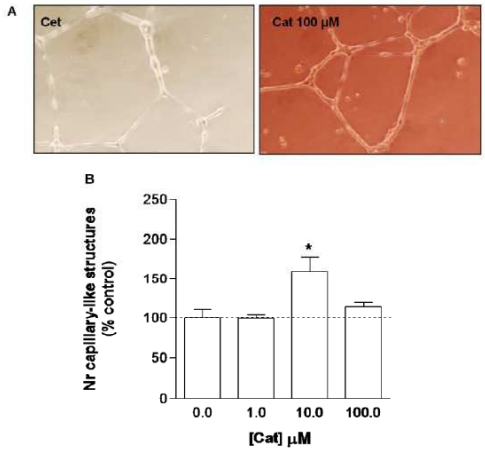

Figure 3 Formation of capillary-like structures. HUVEC were grown on GFR-Matrigel® and incubated with 1-100 μM catechin (Cat) or vehicle (Cet or 0 μM) for 24 h. (A) Capillary-like structures formation, after treatment with 100 μM Cat, visualized under a phase-contrast microscope. Figures are representative of the whole cultures. Magnification: x200. (B) Semi-quantification of capillary-like structures assembly. Results are means ± SEM of independent experiments (3≤n≤5) and are expressed as percentage of control. *p≤0.05 vs. control.

Catechin did not change vessel outgrowth in aortic ring assay

Knowing that angiogenesis involves not only EC but also other cell types such as VSMC present in the surrounding environment, we next confirmed the ex vivo effect of catechin on angiogenic sprouting, by incubating rat aortic rings with 100 μM catechin or vehicle (ethanol 0.1%). Catechin treatment resulted in a small network of angiogenic vessels surrounding the aortic rings very similar to that observed in ethanol-treated group (control) (Figure 4). This finding suggests that catechin does not affect vessel formation as already observed in in vitro endothelial assembly into capillary-like structures (see Figure 3A).

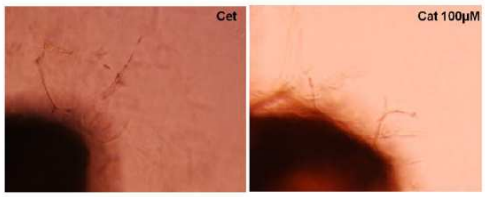

Figure 4 Aortic ring assay showing vascular structures formed from the aorta after 100 μM catechin (Cat) or ethanol treatment (Cet) (Magnification: x200).

Catechin modulated inflammatory markers in HUVEC and HASMC in a different manner

Given the strong crosstalk between angiogenesis and inflammation, together with the fact that most natural polyphenols are often anti-inflammatory agents as well, we next analysed the effects of catechin on inflammatory markers. The pro-inflammatory effects of TNFα are primarily due to its ability to activate NFκB that plays a central role in the inflammatory process, regulating the expression of several inflammatory and angiogenic-related genes. Thus, TNFα seems to be a good candidate to investigate catechin's effect on this crosstalk. The results revealed a reduction in the TNFα levels after 100 μM catechin treatment of HUVEC (58.66 ± 16.20% reduction) and an even more pronounced reduction in HASMC (85.46 ± 9.95% reduction) as can be observed in Figure 5A.

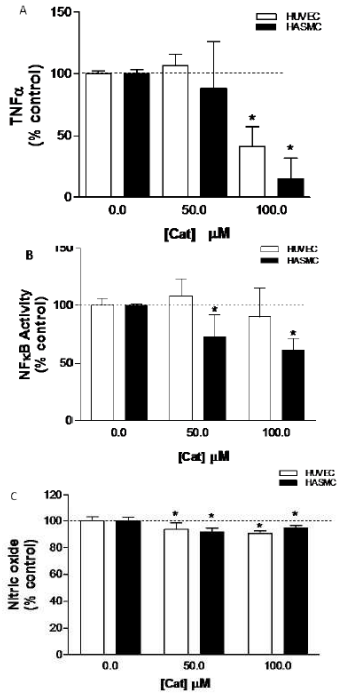

Figure 5 Catechin effect on inflammation. HUVEC and HASMC were treated with vehicle (0 μM) or 50-100μM catechin (Cat) during 24 h. Inflammatory markers were then evaluated in extracellular medium. (A) TNFα was measured by ELISA and quantification was performed at 450nm and 550nm. (B) NFκB activity was determined using TransAM NFκB p65/p50 transcription factor assay kit and quantification was performed at 450 nm and 650 nm. (C) NO level was determined as the concentration of nitrate plus nitrite by Griess reaction and measurements performed at 550 nm. Results are means ± SEM of independent experiments (3≤n≤6) and are expressed as percentage of control. *p≤0.05 vs. control.

NFκB activity was only decreased in HASMC, reaching maximal inhibition with 100 μM catechin (38.43 ± 6.01% inhibition) comparatively with control group (Figure 5B). NFκB was slightly decreased in HUVEC, though not reaching statistical difference relative to control (Figure 5B). In addition, exposure of either HUVEC or HASMC to 50-100 μM catechin slightly decreased extracellular NO levels (decreases of 9.63 ± 0.84% and 5.43 ± 0.81% for 100 μM, for HUVEC and HASMC, respectively) as can be seen in Figure 5C.

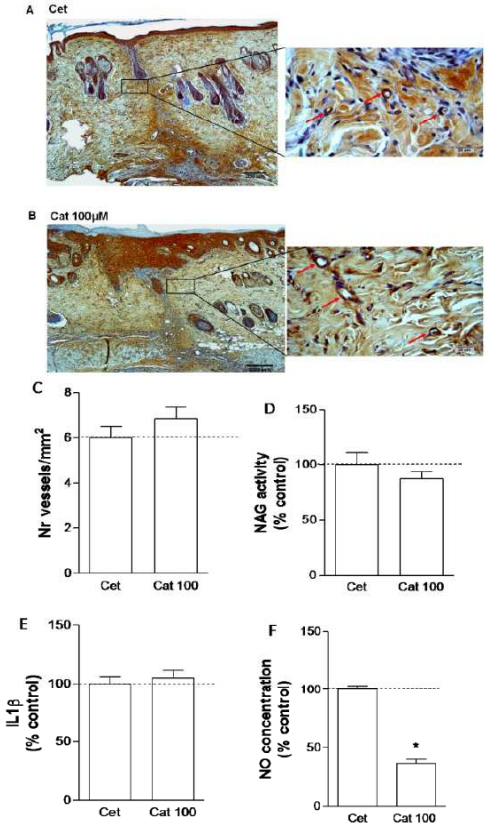

Figure 6 *In vivo* skin wound-healing assay. Longitudinal incisions were created on the dorsal surface of the rats and vehicle (Cet) or 100 μM catechin (Cat) were administered topically, daily. After 7 days, wounded tissue was collected for angiogenesis evaluation. Micrographs of wound tissue sections highlighting thickness and appearance of granulation tissue, fulfilling the incision with different treatments (magnification: x40): (A) control animals and (B) rats treated with catechin. Amplification of wound area (magnification: x200): tissue section micrograph using von-Willebrand factor for evaluation of blood vessels. Red arrows indicate blood vessels. (C) Quantification of blood vessels present in three tissue sections, for each animal, and normalized to the total area of the tissue section. (D) N-acetylglucosaminidase (NAG) activity, (E) IL-1β levels and (F) NO determination in rats serum. Results are means ± SEM of independent experiments (4≤n≤7) and are expressed as percentage of control. *p≤0.05 vs. control.

Catechin did not affect angiogenesis or inflammation in skin wound healing assay

Skin wound healing is a process involving the formation of new extracellular matrix, cell infiltration and tissue remodelling. Inflammation and angiogenesis are two physiological conditions crucial to this process.

We used this experimental model, with no VEGF stimulation, to investigate if the *in vivo* effect of catechin in neovascularization and inflammation changes according to VEGF stimulation. The healing process was completed on day 7 post injury, and no differences were observed in the wound areas topically treated with catechin compared to vehicle.

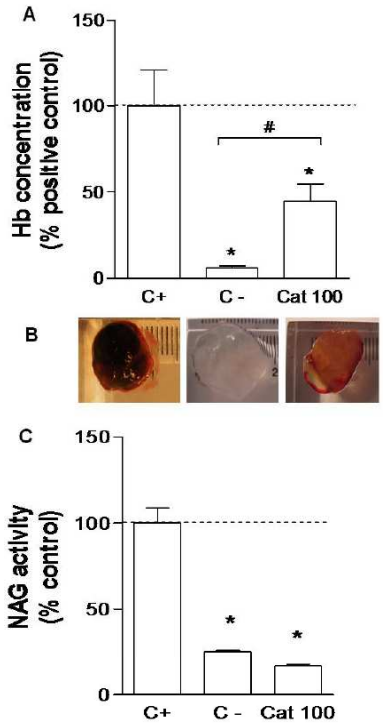

Figure 6 *In vivo* evaluation of angiogenesis and inflammation by Matrigel plug assay. A mixture of Matrigel® and heparin without vascular endothelial growth factor (VEGF) (negative control, C-); or of Matrigel®, heparin and VEGF (positive control, C+) and Matrigel®, heparin, VEGF and 100 μM catechin (Cat) was injected subcutaneously into C57BL/6 mice. (A) Quantification of the haemoglobin (Hb) amount in the homogenized plugs by Drabkin's method. (B) Representative images of macroscopic visualization of Matrigel plugs. (C) Determination of N-acetylglucosaminidase (NAG) activity in mice serum 7 days after plugs implantation. Results are means ± SEM of independent experiments (5≤n≤10) and are expressed as percentage of control. *p≤0.05 vs. positive control, #p≤0.05 vs. negative control.

The histological sections used for quantification of blood vessels in the tissue revealed that treatment with catechin did not affect the formation of granulation tissue at the incision (Figures 6A and 6B). Indeed,

topical catechin did not change microvessel density in the vicinity of the incision area (6.85 ± 0.52 vessels/mm²) comparatively to control group (6.02 ± 0.49 vessels/mm², respectively) (Figure 6C).
In agreement with these findings, the activity of NAG enzyme in rats' serum was also similar in the two treatments, revealing an identical systemic inflammatory status in rats treated with 100μM catechin (87.71 ± 6.32 %) or ethanol 0.1% (100.00 ± 11.24%) as illustrated in Figure 6D. These findings were corroborated by an identical profile obtained for IL-1β levels in rat serum (Figure 6E). On the other hand, rat serum NO decreased significantly after topical 100 μM catechin administration when compared with controls (63.47 ± 3.65%) as observed in Figure 6F.

Catechin diminished VEGF stimulated angiogenesis and inflammation in matrigel plug assay

The other *in vivo* model used to evaluate the modulation of angiogenic process by catechin was the matrigel plug neovascularization assay. As illustrated in Figure 7A and 7B, VEGF-containing matrigel (positive control, C+) presented extensive neovascularization. Matrigel implants containing recombinant VEGF and catechin resulted in a strong inhibition of vascular development, as quantified by haemoglobin plug content (Figure 7A and B). The plugs implanted with catechin showed an angiogenic response between both positive and negative controls (55.30 ± 10.03%), as highlighted by the red colour distributed in the whole plug (Figure 7B).
Accordingly, analysis of the inflammatory enzyme NAG activity in the serum of mice implanted with matrigel plugs revealed that systemic inflammation decreased in the presence of catechin containing plug (decrease of 83.48 ± 1.04% of control) (See Figure 7C).

Discussion

The present study investigated the effect of the flavanol catechin in several steps of the angiogenic process, like proliferation, migration, invasion and capillary differentiation capacity, simultaneously in HUVEC and HASMC cultures. *In vivo* models were also used to test catechin effect on physiological angiogenesis (skin-wound healing assay) and pathological angiogenesis, presenting higher VEGF stimulation (matrigel plug assay).
We were able to demonstrate in sub-confluent cell cultures that 100 μM catechin increased HUVEC and HASMC viability. This was probably due to a decrease in apoptosis. Proliferation of both cell cultures was also reduced upon 10 μM catechin incubation in both cell cultures, though to a less extent. These apparently controversial findings may be explained by the fact that proliferation was examined in 70% confluent cultures, which is required for cell growth assays. However, the lack of cell-cell interaction may prevent cells from proliferating, eventually leading to growth reduction. These results suggest that catechin stabilizes HUVEC and HASMC in the absence of a prominent pro-angiogenic environment. A deeper look into the results reveals that most of the effects were observed at 1 μM catechin. The physiological relevance of these results depends on catechin bioavailability after consumption of nutritional sources. Catechin plasma concentration after human ingestion of 200 mL of red wine is about 2 μM [20] and total polyphenol intake is about 1g/day. So, as catechin is abundant in many fruits and vegetables it can reach serum concentrations higher than 2 μM. Nevertheless, polyphenols may accumulate in cells, tissues and membranes at higher concentrations, since catechins can bind to lipid bilayers through relatively strong interactions [21, 22]. So, catechin concentrations used in this study include physiological concentrations and also higher catechin doses that may be relevant for therapeutic applications.
Our results further showed that catechin increased HUVEC migration, and tended to augment invasion capacity at higher concentrations, although never reaching statistical significance. Strikingly, at 1 μM catechin HUVEC invasion capacity was significantly decreased. Opposite effects within this range of catechin concentrations upon blood vessel function have been observed by other authors [23]. Strikingly, HASMC migration and invasion capacity tended to decrease with the same treatment. Given the role of VSMC proliferation and migration towards the blood vessel intima layer in the formation of atheroma plaque, the results obtained with HASMC indicate that catechin may protect from cardiovascular diseases. The reduced migration and invasion of VSMC in the presence of green tea polyphenols have already been described by several authors [24-26].
The assembly of capillary-like structures by HUVEC was not affected by catechin incubation at most concentrations. These findings were confirmed using aortic ring assay, a more accurate method to examine the complex interactions occurring between EC and VSMC within a blood vessel wall [27]. In agreement, 100 μM catechin resulted in no changes in the vascular network surrounding the aortic rings, comparing to control. These results may indicate that the relations between EC and VSMC, important in blood vessel stabilization, may not be the principal target of catechin effect on blood vessels. In contrast to our results, it was recently described that catechin inhibits new vessel sprouting from rat aortic rings, strongly supporting the efficacy of catechin in suppressing neovascularization. Nevertheless, these studies were performed in stimulated cultures [28], leading to the assumption that the effect of catechin in neovascularization might depend on whether angiogenesis stimuli are present or not, rather than on the presence of interplay between EC and VSMC.

A state of chronic inflammation is one of the major angiogenic stimulators [6, 8]. TNFα is a key inflammatory cytokine involved in systemic inflammation and is stimulated in the acute phase reaction, being produced mainly by macrophages. Treatment of both HUVEC and HASMC with 100 μM catechin resulted in a very significant decrease in TNFα secretion to the extracellular medium. TNFα signalling is mediated in part by NFκB, which plays a central role in the regulation of genes associated with inflammation, cell survival and proliferation. Treating HUVEC with 50-100μM catechin did not significantly change NFκB activity. In turn, catechin reduced NFκB activity in HASMC. The decrease in TNFα and NFκB activity, even in normal cell environment (in the absence of inflammatory or angiogenesis stimuli), may be interesting regarding resolution of inflammation and angiogenesis in physiological conditions. Other authors described similar effects for polyphenol-treated cells, but generally this effect is only observed in stimulated cells [29]. Accordingly, Kurbitz et al described that epicatechin-3-gallate inhibited TNFα-induced activation of NFκB and consequently inhibited secretion of pro-inflammatory factors and invasion of pancreatic ductal adenocarcinoma cells [30]. It is, hence, possible that the results on invasive capacity already described upon HUVEC and HASMC treatment may involve NFκB activity.

Nitric oxide (NO) is produced in large amounts by inducible nitric oxide synthase (iNOS) after immunological stimuli, which is upregulated by TNFα and NFκB in inflammation. In agreement, chronic, overexpression of NO is a feature of inflammatory conditions such as diabetes, cardiovascular diseases and various carcinomas. In this work NO levels suffered a small but significant reduction after HUVEC and HASMC treatment with catechin, supporting TNFα and NFκB putative role previously observed. These findings are corroborated by other studies performed in different models which did also report inhibition of NO by this compound [28, 29, 31]. Nevertheless, we do not know whether this reduction of NO levels can reach physiological relevance.

Altogether, the *in vitro* results showed that catechin increased viability but decreased apoptosis of HUVEC and HASMC. It did also seem to alter migration and invasion in a distinct manner, increasing it in HUVEC and decreasing it in HASMC. Catechin did not change *in vitro* sprouting and did not seem to interfere with EC-VSMC interplay. It diminished *in vitro* inflammatory modulators, affecting both EC and VSMC, but generally manifesting a more pronounced effect in HASMC.

Comparing our *in vitro* results to what *has* been recently described for catechin [28] led us to hypothesize that catechin effects in angiogenesis depended on the presence or absence of angiogenic stimuli. To test this, two different *in vivo* models were performed. Using the physiological skin-wound healing model, we could demonstrate that after topical administration of catechin for 7 days, the number of vessels formed in the injured area were similar to control group, confirming that it did not affect the angiogenic process, according to results obtained for capillary-like structures and aortic-ring assay. Additionally, inflammatory marker levels as NAG and IL-1β, were also not changed in catechin-treated animals. This may be explained by the fact that the inflammatory phase of wound-healing process that starts just upon injury induction, should have already been resolved by day 7.

Only a decrease in NO serum levels could be observed after 7 days of treatment. These findings are in agreement with the ones described by Sanae et al, in which administration of catechins in inflammatory sites led to vessel contraction activity and inactivation of NO [23], indicating a beneficial effect of this polyphenol.

Matrigel-plug assay is a highly stimulated angiogenic model (comprising abundant VEGF stimulus). Catechin was able to substantially prevent the recruitment of new blood vessels within the plug, and also diminished macrophages activation, supporting the anti-angiogenic and anti-inflammatory effects described by others for catechin [28] and for other polyphenols [18, 32, 33].

Altogether, the current study was able to show that catechin presents distinct effects in blood vessel wall cells, depending on the presence or absence of angiogenesis and inflammation stimuli, such as VEGF. The absence of adverse effects in mature vasculature by catechin emphasizes its potential use against pathological situations where angiogenesis is stimulated. Molecular studies are now mandatory in order to elucidate the modulation of angiogenesis by catechin in physiological as well as in pathological conditions.

Acknowledgments

This study was partially funded by Fundação para a Ciência e Tecnologia, Portugal (FCT) (SFRM/BD/41888/2007; PTDC/SAU-OSM/102083/2008 and Pest-OE/SAU/UI0038/2011) and iBeSa (Institute for Beverages and Health, Portugal).

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CAPÍTULO 5

Isoxanthohumol modulates angiogenesis and inflammation
via VEGFR2, Erk and angiopoietins-Tie2 signaling pathways
(submitted)

Negrão R, Duarte D, Costa R, Soares R

Isoxanthohumol modulates angiogenesis and inflammation via VEGFR2, Erk and angiopoietins-Tie2 signaling pathways

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Abstract

Angiogenesis and accompanying biological processes (e.g. inflammation) are becoming distinguished players in the pathogenesis of many heterogeneous diseases, such as cancer, diabetes or cardiovascular disease. Therefore, it is crucial to study new compounds that are able to modulate these events and to understand their cellular and molecular targets. Isoxanthohumol (IXN) is a polyphenol with recognized anti-oxidant and anti-inflammatory properties. Recently, IXN has also appeared as an interesting anti-angiogenic molecule. The aim of this study was to evaluate the effects of IXN on different *in vivo* angiogenic steps and to describe the underlying molecular mechanisms in endothelial (EC) and vascular smooth muscle cells (VSMC).

The developmental retinal angiogenesis of mice pups was used to analyze the angiogenic profile of IXN during different time points. To elucidate the effect of IXN on fundamental angiogenic and inflammatory signaling pathways, we performed retina immunofluorescence and protein quantification by western blotting on human umbilical vein endothelial cells (HUVEC) and human aortic smooth muscle cells (HASMC).

IXN inhibited sprouting angiogenesis, arteriolar and venular differentiation and vascular coverage by mural cells. Some of these effects were, however, overcome at later time points, probably due to complex vascular-stromal interactions and impaired vascular pruning. IXN also decreased inflammatory signals, namely TNFα and NFκB, and angiogenic signals, including VEGFR-2, angiopoietins 1 and 2, Tie-2, Akt and especially, Erk.

Taken together, these results grant IXN interesting properties on *in vivo* vascular proliferation and stabilization and on modulation of the EC-VSMC-inflammatory crosstalk, which may be used as the basis of future research with this molecule on angiogenesis and inflammation-related diseases.

Keywords: Angiogenesis; Inflammation; Blood vessels; Polyphenols; Isoxanthohumol; Tie2; Angiopoietins, VEGFR2; Erk1/2 ; Retinal angiogenesis.

Introduction

Polyphenols are constituents of food and drinks derived from plants, commonly consumed by humans. The consumption of polyphenol-rich diets seems to prevent several pathologies like cardiovascular disease, diabetes and cancer [Dell'Agli et al., 2004; Gerhauser, 2005; Kawai et al., 2008; Sabu et al., 2002].

Flavonoids are one of the most promising and studied polyphenol groups. Xanthohumol (XN), a prenylated chalcone, is a flavonoid present in hop cones, the female inflorescences of the hop plant (*Humulus lupulus* L.), largely used in the brewing industry as a preservative and flavoring agent to add bitterness and aroma to beer [Stevens and Page, 2004]. XN is the most abundant prenyl flavonoid present in hops, representing 82–89% of its total content [Stevens et al., 1999b]. During beer production most of the XN is transformed into the flavanone, isoxanthohumol (IXN) [Stevens et al., 1999a].

This explains why commercial beers, the major dietary source of prenylated flavonoids, are generally characterized by low levels of XN (0.002-1.2 mg/L) and higher levels of IXN (0.04-3.44 mg/L) [Gerhauser, 2005]. It has also been demonstrated that XN can be converted to IXN through acid-catalyzed cyclization in the stomach, during digestion [Nikolic et al., 2005]. Although very little is known about the human metabolism of these natural products, women administration of prenyl flavonoids in the form of a capsule containing 2.04 mg XN, 1.20 mg IXN, and 0.10 mg 8-prenylnaringenin (8PN), 3 times a day, during 5 days, resulted in serum concentrations of 4.99 nmol/L (XN), 14.86 nmol/L (IXN) and 2.20 nmol/L (8PN), demonstrating that IXN is the most abundant prenyl flavonoid in the human body after ingestion of these polyphenols [Bolca et al., 2010]. Also, after administration of 48 mmol/kg body weight of XN to rats, IXN, free or conjugated, was present at much higher concentration in blood (5.91 µmol/L) than XN itself (0.76 µmol/L) [Hanske et al., 2010]. IXN has also demonstrated interesting biological properties. It inhibits *in vivo* angiogenesis, alters folic acid transport through human trophoblasts and intestinal Caco-2 cells, inhibits transforming growth factor beta (TGFβ)

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signaling and pro-inflammatory gene expression and modulates adipogenesis [Keating et al., 2008; Lemos et al., 2007; Negrão et al., 2010; Serwe et al., 2011; Yang et al., 2007]. Therefore, although more data on the bioavailability of XN, IXN and other bioactive prenyl flavonoids are needed to judge their efficacy, IXN seems to be a very interesting compound, whose biological properties deserve to be investigated.

The circulatory system enables the transport of nutrients and chemicals necessary for cellular functions from central organs to the extremities of the body. It is formed during the first stages of development, being essential for the development and survival of almost all tissues. As with the development of any tissue, the development of retina requires the formation of a complex vascular system. In non-primate species, the retina is avascular at birth and neovascularization occurs during the first weeks postnatally in a highly reproducible spatial and temporal fashion [Dorrell and Friedlander, 2006]. Mice retinal vessels are formed by sprouting of capillaries from pre-existent vessels in a process termed angiogenesis. So, the retina offers several advantages as a model for studying vascular development, allowing the study of molecular events during developmental angiogenesis in a natural context [Uemura et al., 2006].

Angiogenesis is a strictly regulated process, involving the interplay between both anti- and pro-angiogenic factors. This process involves sprouting, growth and bridge formation from already pre-existent blood vessels. Guidance of endothelial cells (EC) and mural cells, remodeling and pruning, as well as maturation of nascent blood vessels are also fundamental for the formation of a functional vasculature [Conway et al., 2001].

Vascular endothelial growth factor (VEGF) and its receptors (VEGFR) are fundamental in the initial phase of the angiogenic process. EC are guided into avascular areas by several pro-angiogenic molecules, VEGF being the most important one [Ferrara, 2009; Gerhardt et al., 2003]. VEGF stimulates EC mitogenesis and vascular permeability through VEGFR2 and is fundamental for EC survival [Chung et al., 2010]. Angiopoietins-Tie2 pathways are essential for angiogenic regulation in initial and also in later stages of vessel formation, during vessel remodeling and maturation by coverage with mural cells. Tie2 is a receptor tyrosine kinase, almost exclusively expressed in EC. Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) compete for binding to Tie2 and interplay with VEGF, regulating vessel sprouting or stability, depending on the context [Eklund and Olsen, 2006]. Ang1 regulates both vascular quiescence and angiogenesis. Binding to Tie2, it regulates the formation of vascular basement membrane leading to vascular stabilization, by promoting interactions between EC, surrounding cells and extracellular matrix. It also inhibits vascular permeability and inflammation [Davis et al., 1996; Zhang et al., 2011]. In the presence of VEGF, Ang2 promotes EC proliferation and migration [Oh et al., 1999]. However, in the absence of VEGF, it unsettles blood vessels and stimulates vessel regression [Saharinen et al., 2010].

Identifying the cellular pathways involved in IXN modulation of angiogenesis and inflammation, and their interplay, will provide fundamental information to explore IXN potential in the development of multiple target therapies for angiogenesis and inflammation related pathologies. In addition, better nutritional recommendations could also be achieved.

The aim of this study was to investigate IXN effects on vessel sprouting, growing and maturation and on the interactions between EC, vascular smooth muscle cells (VSMC) and inflammatory cells, using a model of *in vivo* angiogenesis. The implicated cellular signaling pathways were also investigated.

Here, we show, for the first time, that IXN diminished vessel sprouting and stabilization, probably by decreasing VEGFR2 expression on EC and by downregulating Ang-Tie2 pathways. IXN also reduced inflammatory markers, as NFκB and TNFα, in both EC and VSMC types, modulated Akt and, more effectively, Erk signalling pathways.

Materials and methods

In vivo *assay*

All research animals were obtained, maintained and used in experiments according to accepted standards of humane animal care (Declaration of Helsinki, European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals). A veterinarian accompanied every step involving animal care and manipulation. All the authors involved in animal studies in the present study received accreditation from the Portuguese Veterinarian Administration as a competent person for animal experimentation (investigator- coordinator).

Analysis of retinal angiogenesis

C57BL/6 mice pups were injected intraperitoneally with 25 µl of 50 µM IXN or 0.01 M phosphate buffered saline (PBS), daily, from post-natal day (P)0 until P3, P4, P6 or P14. At the ages of P4, P5, P7 and P15, pups were euthanized with pentobarbital 6 mg/ml and eyes were enucleated and fixed in 4% *p*-formaldehyde overnight. For whole mount staining, retinas were dissected out, washed 3 x 5 minutes in PBS, permeabilized in 4% BSA, 0.5% Triton-X100, 0.01 M PBS overnight at 4ºC and incubated with primary and secondary antibodies diluted in 4% BSA-PBS for 1 hour at room temperature (RT); washes of 3 x 5 minutes and 5 x 10 minutes with 0.01% Tween-20 in 0.01 M PBS were performed between and after incubations. Retinas were flattened with radial relaxing incisions and mounted using ProlongGold antifade mounting media (Invitrogen, CA, USA). For retina sectioning, eyes were included in 30% sucrose overnight and the cornea and lens were dissected out; the tissue was then included in OCT and 8 µm sections were obtained using a cryostat. Tissue sections were kept in a humidified chamber overnight at RT, washed

in 0.01 M PBS, stained and mounted as described above. The following antibodies and dilutions were used: FITC-conjugated Griffonia simplicifolia (Bandeiraea) isolectin B4 (1:50, Sigma-Aldrich, MO, USA), mouse anti-alpha smooth muscle actin, clone ASM-1 (1:200, Millipore, CA, USA), rabbit anti-VEGFR2 (1:200, Cell Signaling Technology, MA, USA), and goat anti-mouse Texas Red (1:500, Santa Cruz Biotechnology, CA, USA) and donkey anti-rabbit Alexa-568 (1:500, Invitrogen, CA, USA) conjugated secondary antibodies. Cell nuclei were labeled with DAPI (Roche Diagnostics, Basel, Switzerland). Finally, samples were imaged on a Nikon Eclipse 50i fluorescence microscope with NIS-Elements BR 3.00 software (Nikon, Japan) and on a Zeiss Imager Z1 microscope in ApoTome mode for optical sectioning, with AxionVision 40 v4.8.2.0 software (Carl Zeiss MicroImaging, Germany) and selected fields were captured, merged using Adobe Photoshop CS3 and analysed using ImageJ 1.43m (NIH, USA) and VesSeg Tool (V0.1.4, University of Lübeck, Germany) softwares.

Quantitative analysis of retinal vasculature

The following parameters were evaluated and quantified: vasculature length per retinal radius (ratio between two parallel radial distances measured from the center of the retina to the tip of the vascular network and to the edge of the retinal leaf; average from at least 4 grouped measurements per retina), arteriolar and venular length per retinal radius (arteriolar and venular phenotypes were easily identified and a similar measurement to the later was done considering arteriolar or venular lengths instead of total vasculature length; average from at least 2 grouped measurements per retina), arteriolar diameter (radially oriented arterioles were identified and diameters of the total length of the vessel measured; the measurement was normalized to the respective retinal radius; average from numerous diameters of 2 vessels per retina), capillary-free space width (sequential distances from the main arteriole to the lateral parallel capillaries were measured; average from numerous widths accompanying 2 vessels per retina), branch point per 100 μm^2 (an area of capillary plexus between 2 main vessels was selected and branch points were identified and counted; counts were normalized to the respective total area; average from counts of 2 areas per retina), vessel density (for P5 retinas the same area used for branch point counting was converted in a binary black and white image using ImageJ and the percentage of white vessels was calculated; for P15 retinas, fields of view of the second layer were selected and vessels were segmented and converted in a binary image by running the hysteresis thresholding of VesSeg Tool and the percentage of white vessels was calculated; average from densities of 2 areas per retina), tip cells per 100 µm vessel length (tip cells were defined, at the angiogenic front, as bulging cells with filopodial ramifications and counted; the measurement was normalized to the vascular length where the tip cells seated in; average of numerous measurements of one field per retina), filopodia per 100 µm vessel length (defined as thin dendritic-shaped protrusions emerging from tip EC at the angiogenic front; counts were normalized to the corresponding vascular front length; average of numerous measurements of one field per retina), filopodia length (several measurements were done in a selected field of view; average of numerous measurements of one field per retina), and alpha-smooth muscle actin (α-SMA) intensity plot profile (using ImageJ, a selection line was drawn along main arterioles stained with α-SMA antibody and a plot profile of intensity versus distance was generated; statistical analysis was done to compare intensities between 100 µm distance intervals; average of 5 vessels per group).

Cell cultures

Human umbilical vein endothelial cells (HUVEC) were purchased from ScienceCell Research Labs (San Diego, USA), used between passages 3 and 8, and cultured in 0.2% gelatin-coated plates (Sigma-Aldrich, Portugal), using M199 medium (Sigma-Aldrich, Portugal) supplemented with 20% fetal bovine serum (FBS) (Invitrogen Life Technologies, Scotland, UK), 1% penicillin/streptomycin (Invitrogen Life Technologies, Scotland, UK), 0.01% heparin (Sigma-Aldrich, Portugal) and 30 µg/mL endothelial cell growth supplement (Sigma-Aldrich, Portugal), and maintained at 37° C in a humidified 5% carbon dioxide atmosphere. Human aortic smooth muscle cells (HASMC) were obtained from ScienceCell Research Labs (San Diego, EUA). Cells were kept between passages 2 and 8 in every experiment, cultured in Dulbeco's modified Eagle's medium supplemented with 10% FBS and 1% penicillin/streptomycin and cultured at 37° C in a humidified 5% carbon dioxide atmosphere.

IXN (Alexis, Switzerland) was dissolved in ethanol and then added to cell culture medium at a concentration of 0.1-10 µM, during 24 h, according to the viability assay previously reported [Negrão et al., 2010]. IXN and/or vehicle (ethanol) were added to cell cultures in medium supplemented with 2% FBS and 1% penicillin/streptomycin. Control cells were incubated with vehicle (ethanol). Ethanol concentrations were kept below 0.1% in every culture.

Nitric oxide determination

Nitric oxide (NO) level was determined as the concentration of nitrate plus nitrite in the extracellular medium in contact with HUVEC and HASMC after 24 h of cell treatment with 0.1-10 µM IXN, by colorimetric assay. Extracellular medium was incubated with equal volume of Griess Reagent in a 96-well microtiter plate, for 15 min, at room temperature. Measurement was performed in a spectrophotometer plat reader at 550 nm. Data were expressed as NO concentration (µM).

Protein Extraction and Preparation

Intracellular proteins were isolated from HUVEC and HASMC lysates, after 24h of cell treatment with 0.1-10 µM isoxanthohumol, using the Tripure® isolation reagent, according to manufacturer's instructions (Roche Diagnostics, Basel, Switzerland) and kept at -80ºC until use.

Proteins present in extracellular medium in contact with HUVEC and HASMC, after 24 h of cell treatment with 0.1-10 µM IXN, were extracted with ethanol 100%, dissolved in 200 µL SDS 1% and kept at -80ºC until use.

Western blotting assay

Proteins were isolated from HUVEC and HASMC lysates using Tripure (Roche Diagnostics, Basel, Switzerland) and quantified using a spectrophotometer (Jenway, 6405 UV/vis, Essex, UK). Equal amounts of protein were subjected to 8% or 12% SDS-PAGE with a 5% stacking gel. After electrophoresis, proteins were blotted into a Hybond nitrocellulose membrane (Amersham, Arlington, USA), using a mini-transblot electrophoretic transfer cell (Amersham Biosciences, USA). Immunodetection for VEGFR2, pAkt and pErk1/2 (Cell Signaling, MA, USA), and for Tie2 and β-actin (Santa Cruz Biotechnology), was accomplished with enhanced chemiluminescence (ECL kit, Amersham Biosciences, USA).

The relative intensity of each protein blotting analysis was measured using a computerized software program (Biorad, CA, USA) and normalized with β-actin bands to compare the expression of proteins in the different treatment groups. Experiments were repeated at least three times.

Tumor necrosis factor-alpha quantification

Tumor necrosis factor alpha (TNFα) was quantified in 200 µL of protein extract, obtained from culture medium in contact with HUVEC and HASMC after 24 h of cell treatment with 0.1-10 µM IXN or 0.1% ethanol, by ELISA (TNFα/ TNFSF1A Immunoassay, R&D Systems, Abingdon, United Kingdom) according to the manufacturer's instructions. Quantification was performed at 450 nm and 550 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent, USA).

Nuclear factor kappa-B activity assay

Nuclear factor kappa-B (NFκB) activity was quantified in 200 µL of nuclear protein extract obtained from HUVEC and HASMC after 24h of cell treatment with 0.1-10 µM IXN or 0.1% ethanol, by ELISA. Nuclear extracts were prepared from the nuclear extraction kit (Active Motif, USA). NFκB activity was measured using TransAM NFκB p65/p50 transcription factor assay kit (Active Motif, CA, USA). In brief, nuclear extract samples (5 µg) were added to biotinylated oligonucleotide containing the NFκB consensus site. This mixture was then added to a streptavidin-coated 96-well plate. Sample wells were incubated with NFκB p65 subunit primary antibody, followed by incubation with HRP-conjugated secondary antibody. Quantification was performed at 450 nm and 650 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent, USA).

Angiopoietin-1 and Angiopoietin-2 quantification

Angiopoietin-1 (Ang1) and Angiopoietin 2 (Ang2) were quantified in 200 µL of protein extract obtained from culture medium in contact with HUVEC and HASMC after 24h of cell treatment with 0.1-10 µM IXN or 0.1% ethanol, by ELISA (DANG10 and DANG20 Immunoassay, R&D Systems, Abingdon, United Kingdom), according to the manufacturer's instructions. Quantifications were performed at 450 nm and 550 nm using a plate reader (Thermo Electron Corporation, Multiskan Ascent, USA).

Statistical analyses

Quantifications are expressed as mean ± SEM and/or as percentage of control, which was considered to be 100%. Statistical significance of difference between various groups was evaluated by analysis of variance (ANOVA) followed by the Bonferroni test. For comparison between two groups, Student's *t*-test was used. A difference between experimental groups was considered significant with a confidence interval of 95%, whenever $p \leq 0.05$ (GraphPad Prism, version 5.03).

Results

To study the effects of IXN on *in vivo* vessel sprouting, growing and maturation, and the interactions between EC, VSMC and inflammatory cells, the mouse retinal vasculature was used as a model and IXN tested at different time points. There is a strong pro-angiogenic stimulus in developmental angiogenesis of the retina and, therefore, an ideal environment to study the modulatory effect of a designated compound.

Isoxanthohumol inhibits in vivo *sprouting angiogenesis*

Developmental sprouting angiogenesis was evaluated by measuring the vasculature length and normalizing it to the corresponding retinal radius. The maximum time-point considered was post-natal day (P)7 due to the formation of downward vessels that branch from the superficial vascular layer from this day on.

No differences were observed at P4 between treated and non-treated mice (Figure 1). However, IXN inhibited sprouting angiogenesis on both P5 (0.6891 ± 0.01136 vs. 0.5484 ± 0.008334; C vs. IXN; $p<0.0001$) and P7 (0.9035 ± 0.006005 vs. 0.7916 ± 0.01456; C vs. IXN; $p<0.0001$) (Figure 1).

Isoxanthohumol has no effect on sprouting angiogenesis at P15

In order to evaluate the effect of IXN on retinal angiogenesis over time, a latter post-natal day, P15, was considered.

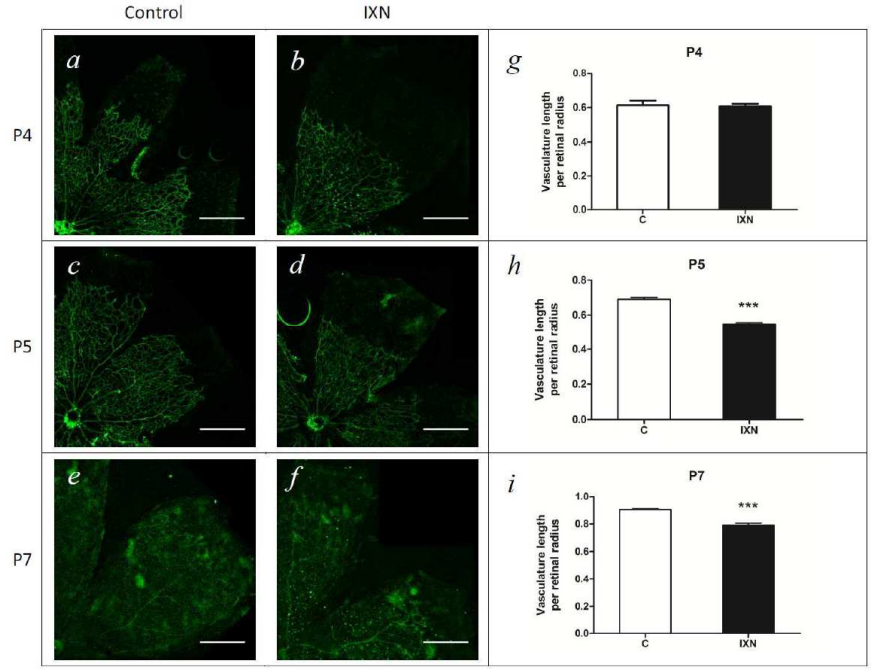

Figure 1- Retinal angiogenesis at different post-natal time points (P4, P5, P7). (a-f) Representative quarters of retinas stained with FITC-conjugated isolectin B4. At P5 and P7, IXN treatment seems to decrease vascular growth. Scale bars, 500 µm. (g-i) Quantitative analysis of vessel spreading in the two study groups by measuring the ratio between vessel length and retina radial length. As suggested by the corresponding images, IXN has no significant effect on vessel sprouting at P4 and significantly decreases it at P5 and P7 time points. ***p<0.0001 (Student's *t* test). Error bars represent SEM.

At this time-point the superficial and profound vascular layers of the retina are fully completed and the vessels start to sprout downwards (from the superficial layer) and upwards (from the profound layer) to form an intermediate and final vascular layer. Therefore, this 2nd layer and branching process have been observed, using optical sectioning and OCT retina cross-sections, respectively.

Vessel density in the intermediate layer was compared between groups and no differences were evident (11.07 ± 0.1923 vs. 10.94 ± 0.05465; C vs. IXN; p=0.5558) (Figure 2). Furthermore, in OCT cross-sections the vessel migration appeared to be normal in both control and IXN-treated mice (Figure 2).

Isoxanthohumol alters arteriolar-venular patterning, vessel morphology and survival

Next, IXN effect on different features of vessel differentiation and remodeling of P5 retinas was addressed. During angiogenesis, vessels differentiate and assume a specific arteriolar or venular commitment. This phenotype is well distinguishable by observing the main vessels that emerge from the central retinal artery. Usually, arterioles are straighter and have a more stable diameter along the vessel length, presenting a surrounding capillary-free area that informs on EC survival and proliferation and branch into a diffuse network of capillaries only near to the growing vascular front. On the other hand, venules vary frequently in direction and diameter, have neighboring capillaries that coalesce near the main vessel and are usually shorter [Stalmans et al., 2002]. The effect of IXN on this phenotypic commitment was analyzed by measuring the arteriolar and venular length and normalizing them to the vasculature length, to avoid bias. The arteriolar length (0.6749 ± 0.02618 vs. 0.5720 ± 0.02474; C vs. IXN; p=0.0105) and particularly, the venular length (0.6254 ± 0.01921 vs. 0.4761 ± 0.01731; C vs. IXN; 0.0001) were significantly decreased by IXN (Figure 3). This evidences another modulatory role of IXN on angiogenesis, apart from the anti-proliferative effect already described.

Then, it was investigated if IXN affected the survival and, to a lesser extent, the proliferation of EC in the capillary-free zone surrounding the main vessels, an area with high remodeling activity at early stages [Fruttiger, 2007], by measuring the distance between the vessel and the capillary bed. A significant decrease of the capillary-free space width in the IXN group was found (44.79 ± 1.756 vs. 35.84 ± 1.999; C vs. IXN; p=0.0035), compatible with an anti-apoptotic effect of the compound (Figure 3).

The arteriole diameter was also measured, as it gives important information on vessel coverage and vasomotor state. IXN reduced substantially the average arterioles diameter (12.96 ± 0.3438 vs. 10.94 ± 0.2866; C vs. IXN; p=0.0003) (Figure 3).

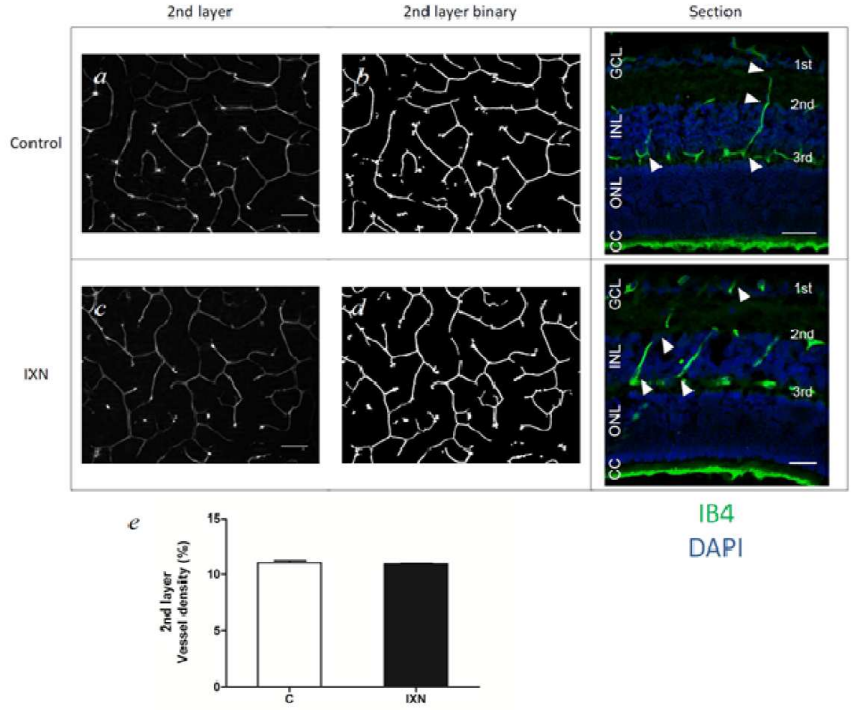

Figure 2- Retinal angiogenesis in P15 mice. At P15, no differences in vascularization of the pre-established 1st layer were observed between groups (data not shown). (a, c) Representative fields of view of 2nd layer vessels; vessels were stained with IB4. (b, d) Corresponding binary images, used for vessel density comparison; (scale bars, 50 µm). (e) Vessel density comparison indicates that IXN treatment does not alter vessel sprouting in the 2nd layer at P15 (p=0.5558; error bars represent SEM). Right panels show OCT cross-sections of P15 retinas; in both groups, vessels migrate perpendicularly (arrowheads) from the 1st and 3rd layers to form the intermediate vascular plexus; IB4 and DAPI were used for vessel and nuclei staining, respectively (CC, choriocapillaries; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; 1st, superficial or 1st layer; 2nd, intermediate or 2nd layer; 3rd, deep or 3rd layer; scale bars, 25 µm).

Vessel density and branching were also assessed by selecting representative areas of the capillary network. Interestingly, no differences in density (0.4150 ± 0.01069 vs. 0.4144 ± 0.01245; C vs. IXN; p=0.9713) or branching (13.98 ± 0.4840 vs. 12.74 ± 0.9929; C vs. IXN; p=0.2755) were detected between IXN and the control group (Figure 3).

Tip cell migration are not affected by isoxanthohumol

Taking the vascular front as a good area to analyze the migratory profile of tip cells (the cells in the edge, which lead the way of growing vessels) and their motile vascular protrusions, filopodia, IXN interference with these specialized angiogenic processes has been evaluated. Mice treated with IXN had reduced numbers of tip cells (4.645 ± 0.6769 vs. 3.221 ± 0.4103; C vs. IXN; p=0.0889) and filopodia (28.85 ± 4.811 vs. 18.84 ± 2.617; C vs. IXN; p=0.0841) but the effect was not sufficiently strong to reach statistical significance (Figure 4). Furthermore, the treatment had no effect on filopodia length (21.88 ± 1.001 vs. 20.88 ± 1.096; C vs. IXN; p=0.5092) (Figure 4). Microglia or resident tissue macrophages have been implicated in vascular anastomosis [Fantin et al., 2010]. In control samples, as expected, we found migroglia embracing two vascular tip cells next to each other. Distinctly, in IXN-treated mice retina, preserved vessel merging but a lack of macrophages co-localizing with the fusion sites were observed (Figure 5).

Isoxanthohumol impairs vessel coverage by αSMA-positive cells

It was also investigated if mural cells (i.e. perycites and smooth muscle cells) were affected by IXN, as suggested by the observed decreased diameter of arterioles upon treatment with this polyphenol. As depicted in Figure 6, αSMA staining revealed strongly diminished vessel coverage at P5 and P7 time-points for IXN treatments. A quantitative analysis of αSMA staining at P5 was performed by measuring the intensity of αSMA labeling along 500 µm of vessel length and normalizing it to the first 100 µm. In control animals a tendency towards decrease of αSMA intensity along vessel length was observed. Nevertheless, the decrease was statistically significant only for 401-500 µm interval. Under IXN treatment a decrease in αSMA stain intensity was also observed, but with statistical significance for all intervals of vessel length when comparing to the reference interval (0-100 µm) (Figure 7).

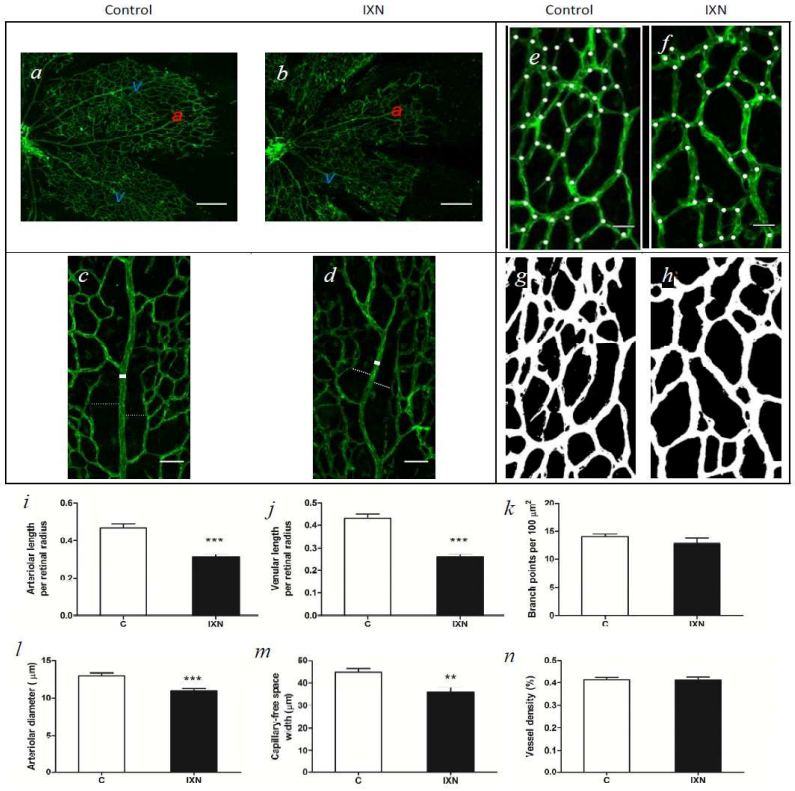

Figure 3- IXN influences vascular differentiation and diameter and endothelial cell survival at P5. (a, b, i) Arteriolar length is significantly decreased by IXN treatment. Note that the vessels with arterial phenotype (red letter a) can only be differentiated until a certain length, where the capillary plexus emerges near the vascular front. (a, b, j) Venular length is significantly decreased by IXN treatment. Similar to arterioles, the vessels with venular phenotype (blue letter v) are discontinued by a broad capillary network. (c, d, l) Arteriolar diameter is significantly decreased by IXN treatment. Representative captures of vessel trajectories show thinner arterioles in the IXN-treated group (diameter identified by white collars). (c, d, m) Capillary-free space width is significantly decreased by IXN treatment. The distance (broken lines) between the main vessels and the surrounding capillaries informs on endothelial cell survival/proliferation in that area and therefore on vascular pruning. (e, f, k) Vessel branching is not affected by IXN treatment. White dots identify branch points in representative fields of capillary networks. (g, h, n) Vessel density was not affected by IXN treatment. The same fields used to analyze vessel branching were converted into binary images to facilitate vessel density assessment by measuring the percentage of white vessels. (a-f) Vessels are labeled with isolectin B4. Scale bars: (a-d) 50 µm, (e, f) 25 µm. Statistical analysis: (i, j) ***p<0.0001, (k) p=0.2755, (l) ***p=0.0003, (m)**p=0.0035, (n) p=0.9713 (Student's *t* test). Error bars represent SEM

Interestingly, when comparing αSMA intensity for each length interval in IXN treatments with the respective interval of control animals, it was verified that the difference was significant for the intermediate intervals of length, 201-300 µm and 301-400 µm (Figure 7). As observed before with sprouting angiogenesis, at P4 (Figure 6) and P15 (Figure 8) the differences were not so evident, and again, a recovery process seemed to take place.

Isoxanthohumol modifies VEGFR2 expression pattern

After staining P5 retinas with an anti-VEGFR-2 antibody, it was observed that the receptor distribution was mainly restricted to the neural retina (Figure 9). Interestingly, one could almost deduce the localization of the vessels by identifying label-free areas. The surrounding expression of the receptor nearly seemed to lead the way to new capillaries. This observation was confirmed by double-staining the retinas with IB4 and anti-VEGFR-2. IXN modified the expression pattern of VEGFR-2 and instead of the control's striped distribution following stable vessels (Figure 9) a "cheese pattern" was observed, full of free-label holes that pointed to a VEGFR-2 matrix encircling immature capillaries (Figure 9). Remarkably, this was not evident after the vascular front, where no vessels had yet appeared (Figure 9). Noteworthy, no differences in the overall signal intensity were observed between the groups.

Isoxanthohumol decreases inflammatory markers release by HUVEC and HASMC

In order to better understand the IXN modulation of angiogenesis sprouting and vessel coverage, nuclear inflammatory and angiogenic molecular pathways that may be involved in the regulation of these processes and in EC-VSMC crosstalk were studied using *in vitro* experiments with IXN-treated HUVEC and HASMC.

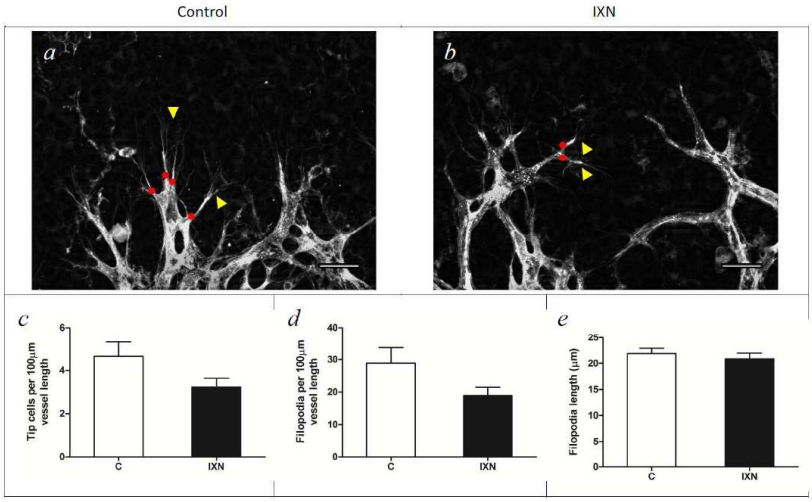

Figure 4- IXN did not affect endothelial cell migration of the vascular front at P5. (a, b) Representative captures of isolectin B4 immunofluorescence showing the vascular front, where endothelial tip cell migration actively takes place. Tip cells (red dots) are separated at the base from stalk cells and have cellular protrusions, called filopodia (yellow arrowheads). Migration of this region can be ascertained by evaluating the number of tip cells, filopodia and filopodia length and by normalizing it to the corresponding vascular front length. Upon IXN treatment no serious defects on sprouting angiogenesis are apparent; nevertheless, in the IXN group the characteristic vertical and unidirectional orientation of tip cells and filopodia seem to be more random, and horizontal, backwards driven filopodia are more frequent. Scale bars, 25 µm. (c) IXN decreased the number of tip cells but the effect did not reach statistical significance (p=0.0889). (d) Filopodia counts were decreased in IXN group but again the effect was not statistically significant (p=0.0841). (e) IXN treatment did not affect filopodia length (p=0.5092). (c-e) Student's *t* test. Error bars represent SEM.

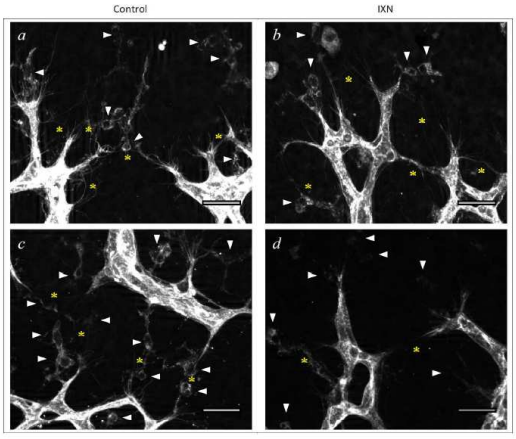

Figure 5- Vessel fusion mediated by microglia at P5. (a, c) Isolectin B4 staining reveals actively remodeling regions of the sprouting vessels. Microglia or tissue macrophages (arrowheads) neighbor tip cells and dock at the vessel fusion sites (asterisk), establishing their role as 'bridge cells'. (b, d) IXN appears to impair the interplay between microglia and fusing filopodia; the association of tissue macrophages to fusion sites was not so frequent, yet vessel merging still occurred. Scale bars, 25 µm.

Besides regulating permeability and blood flow, NO has been described as a pro-angiogenic factor [Ziche and Morbidelli, 2009]. After treatment of HUVEC and HASMC with 0.01-10 µM IXN, no differences were observed in NO levels measured in the extracellular medium contacting with both cells (Figure 10 A).

TNFα is an inflammatory cytokine, involved in systemic inflammation, which activates NFκB, which in turn regulates the expression of several inflammatory and angiogenic-related genes. IXN reduced TNFα levels in HASMC (18.37 ± 3.10% and 26.53 ± 2.34% for 5.0 and 10 µM IXN, respectively) and in HUVEC (39.66 ± 5.81% for 10 µM IXN), as illustrated in Figure 10B. Accordingly, NFκB activity revealed exactly the same profile. NFκB activity was decreased in HASMC (23.53 ± 1.00% for 10 µM IXN) and reached maximal inhibition in HUVEC (42.19 ± 4.54% for 10 µM IXN) (Figure 10 C).

Isoxanthohumol reduces vascular endothelial growth factor receptor-2 expression

In order to elucidate the molecular mechanisms that stand behind the described morphological findings of IXN in in vivo angiogenesis, the VEGFR2 expression was then examined by Western blotting. VEGF binds specifically to 2 membrane receptors VEGFR1 and VEGFR2, presenting tyrosine kinase activity. VEGFR2 is expressed in EC and is the predominant receptor in angiogenic signaling. It appears to mediate almost all of the known cellular responses to VEGF [Ferrara et al., 2003]. As illustrated in Figure 11 B the expression of VEGFR2 was detected in HUVEC cultures. Furthermore, a tendency towards decreased expression was found when HUVEC were incubated with increasing concentrations of IXN, even at the lowest concentration tested (0.1 µM) (Figure 11 A and 11 B).

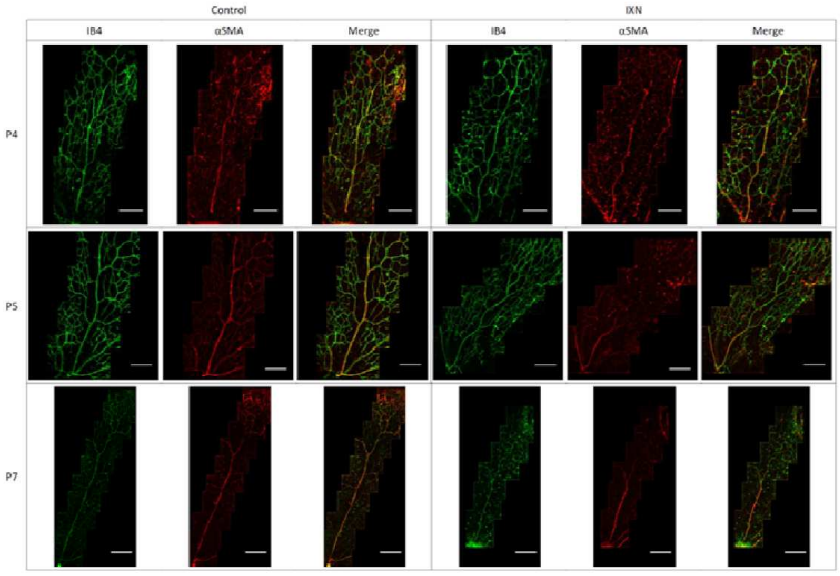

Figure 6- IXN impaired vessel coverage by mural cells at different time points (P4, P5 and P7). Isolectin B4 and αSMA labeling was used to respectively reveal vessels and mural cells (pericytes and smooth muscle cells) and to demonstrate vasculature development and stabilization at sequential post-natal time points. Consecutive snaps of complete vessel lengths were merged and visualized. At P4 (first row), in the IXN group, the αSMA immunofluorescence intensity was lower and smaller branches were not labeled; scale bars, 150 µm. At P5 (second row), the disruptive effect of IXN on vessel coverage was more profound; αSMA staining was not able to reach the expected length and there was a serious defect on branch coating; scale bars, 150 µm. At P7 (third row), the recruitment of mural cells around the final segment of the main vessel was impaired, upon IXN treatment; scale bars, 250 µm.

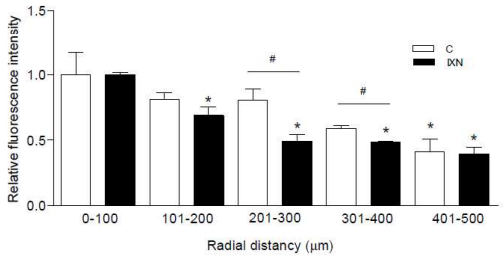

Figure 7- αSMA immunofluorescence intensity rapidly decreased along the vessel under IXN treatment. The intensity of αSMA labeling from five vessels of different P5 retinas from each group was plotted in an intensity x distance graph. Five distance intervals of 100 µm were considered and the first interval (0-100 µm) was defined as 100% of fluorescence intensity to normalize the analysis. IXN decreased αSMA vessel coverage for all the considered distances when compared to the correspondent first interval and when compared to the correspondent intervals of the control at 201-300 and 301-400 µm intervals. *p≤0.05 vs. reference interval (ANOVA followed by Bonferroni test). #p≤0.05 vs. correspondent control interval (Student's *t* test). Error bars represent SEM.

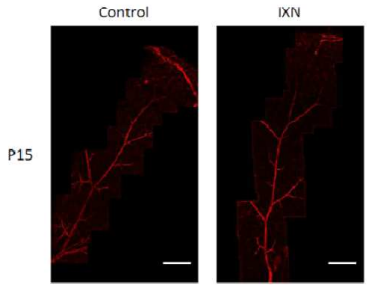

Figure 8- IXN poorly affected vessel coverage at P15. Both images show mature vessels from the established superficial layer of P15 retinas, coated with αSMA-positive mural cells. Despite of lower αSMA intensity at the tip of the vessels from the IXN group, the defect was not as serious as previously shown and a sequential recovery of mural cells recruitment seemed to occur from P5 to P15. Scale bars, 250 µm.

A significant decrease in the expression of the receptor was observed after incubation with 10 µM IXN (55.16 ± 17.17%) when compared to control group (Figure 11 A).

Isoxanthohumol decreases Akt and Erk expression, two established downstream molecules of VEGFR2 signaling

To further evaluate the effects of IXN on VEGF signaling, we next examined the expression of the phosphorylated forms of Akt and Erk, two established downstream molecules known to be implicated in cell survival and proliferation, growth and differentiation. The expression of p-Akt, and therefore Akt activation, was significantly reduced (47.39% of control) only in HUVEC, and after treatment with the highest concentration of IXN (10 µM) (see Figure 12 A and B).

In contrast, p-Erk expression was affected in HUVEC and also in HASMC, after incubation with IXN (Figure 13 A and B). A gradual and significant decrease of the phosphorylated-molecule was observed upon 0.1-10 µM IXN treatment, reaching 52.17 % and 68.65 % decrease, respectively for HUVEC and HASMC (see Figure 13 A).

R. Negrão et al. / Isoxanthohumol modulates angiogenic signaling pathways. A retinal model. (submitted)

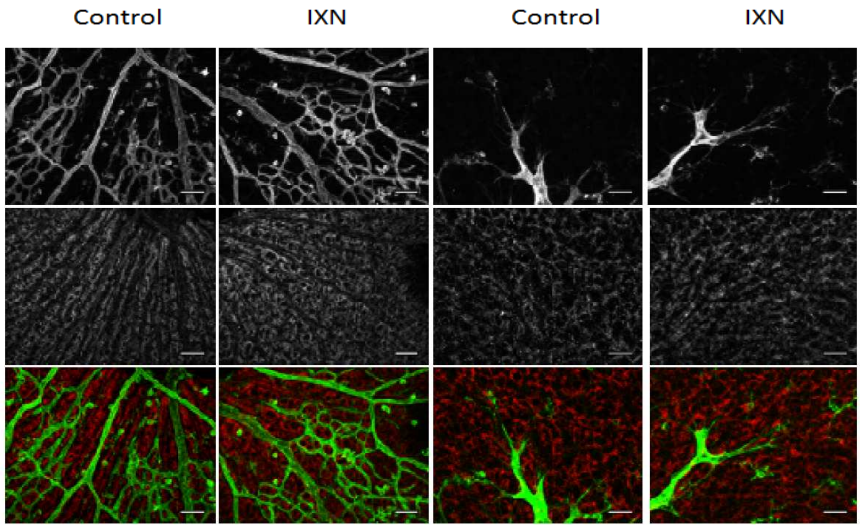

Figure 9- VEGFR-2 expression pattern was modified by IXN. Isolectin B4 (top panels) and anti-VEGFR-2 (middle panels) were used to label the vascular and VEGFR-2 distribution, respectively. Upon IXN treatment, VEGFR-2 maintained its non-vascular distribution but alternatively to the linear arrangement seen in normal conditions, it had a peculiar decoration around immature capillaries, as confirmed in the merged images. In the right panels, images from tip cells show that VEGR-2 expression was much less organized in the vascular-free areas, which may be due to a high dependent nature of the receptor on vessel density and maturity; no differences between groups seemed to exist in these areas. Scale bars, 50 µm (left panels) and 25 µm (right panels).

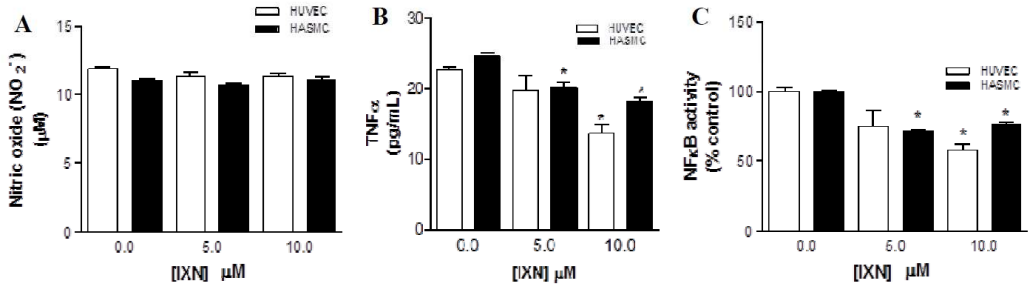

Figure 10- Isoxanthohumol effects on inflammation. HUVEC and HASMC were treated with vehicle (0.0) or 0.1-10 µM isoxanthohumol (IXN) during 24h. Inflammatory markers were then evaluated in extracellular medium. (A) NO level was determined as the concentration of nitrate plus nitrite by Griess reaction and measurements performed at 550 nm. (B) TNFα was measured by ELISA and quantification was performed at 450 nm and 550 nm. (C) NFκB activity was determined using TransAM NFκB p65/p50 transcription factor assay kit and quantification was performed at 450 nm and 650 nm. Results are means ± SEM of independent experiments ($3 \leq n \leq 6$) and are expressed as percentage of control. *$p \leq 0.05$ vs. control.

These findings indicate that IXN exhibits a stronger inhibitory effect on VEGF signaling activation via p-Erk in both EC and VSMC.

Isoxanthohumol downregulates angiopoietins-Tie2 signaling pathways

Ang1 and Ang2 compete for binding to the EC-specific Tie2 receptor, playing a central role in vascular development [Saharinen et al., 2010]. The outcome of Tie2 signaling depends on the ligand interaction. Both Ang1 and Ang2 were found in the extracellular medium of either HUVEC or HASMC, in the presence of vehicle (ethanol 0.1%, 0.0) or IXN treatments (Figure 14 A and B). Ang1 levels (about 600 pg/mL) were higher than Ang2 (about 75 pg/mL) in the extracellular medium in contact with both HUVEC and HASMC. Ang1 was mostly released by HASMC (Figure 14 A) and Ang2 by HUVEC (Figure 14 B). IXN treatment of HUVEC resulted in a progressive reduction of Ang1 and Ang2 in the culture medium reaching the lowest values for 10 µM IXN (39.16 % and 37.5 % reduction, respectively) (Figure 14 A and B). This decrease was coupled with a 35.1% reduction of Ang1 in HASMC for 10 µM IXN treatments, without observed changes in Ang2 released by this cell culture (Figure 14 A and B). Tie2 receptor expression was only observed in HUVEC (Figure 14 D) and accompanied the reduction of angiopoietins, with a statistical significant decrease with 10.0 µM IXN (55.8%) (Figure 14 C).

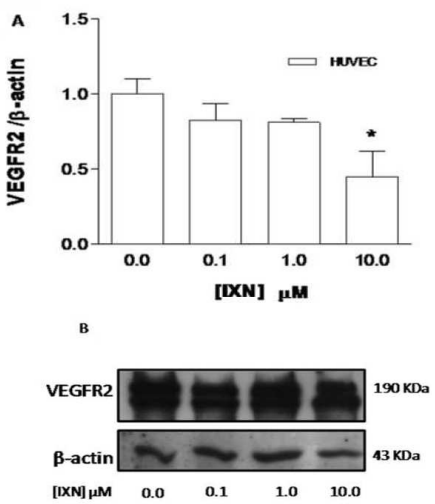

Figure 11- Evaluation of VEGFR2 expression in HUVEC upon treatment with 0.0-10 µM isoxanthohumol (IXN) during 24 h. (A) Graph illustrates the relative band intensity ratio for VEGFR2 after normalization with β-actin. (B) Representative bands obtained after immunostaining. Results are means ± SEM of independent experiments (n=3) and expressed as percentage of control. *p<0.05 vs. control.

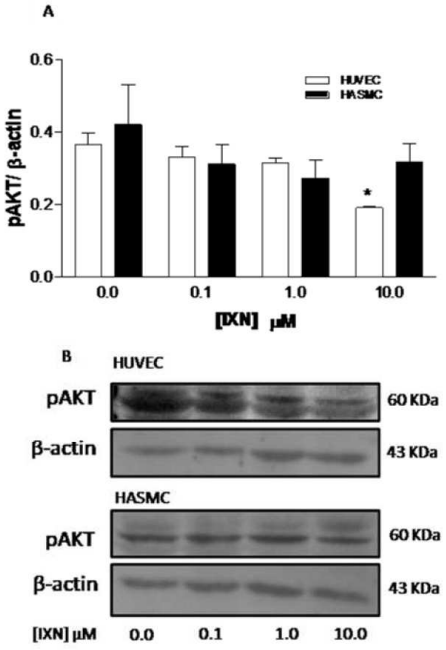

Figure 12- Expression of p-Akt in HUVEC and HASMC lysates after incubation with 0.0-10 µM isoxanthohumol (IXN) during 24 h. (A) Graph illustrates the relative band intensity ratio for p-Akt after normalization with β-actin. (B) Representative bands obtained after immunostaining. Results are means ± SEM of independent experiments (n=3) and expressed as percentage of control. *p<0.05 vs. control.

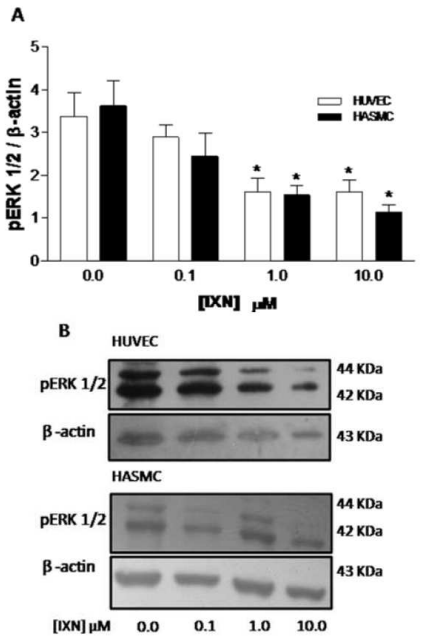

Figure 13- Expression of p-Erk in HUVEC and HASMC lysates after incubation with 0.0-10 µM isoxanthohumol (IXN) during 24 h. (A) Graph illustrates the relative band intensity ratio for p-Erk after normalization with β-actin. (B) Representative bands obtained after immunostaining. Results are means ± SEM of independent experiments (4≤n≤6) and expressed as percentage of control. *p<0.05 vs. control.

Discussion

The vascular protective effects of polyphenols were explained during many years almost exclusively by their antioxidant properties [Rahman et al., 2006; Terao, 1999]. Meanwhile, many other properties of polyphenols have been described such as anti-angiogenic and anti-inflammatory properties of IXN [Negrão et al., 2010], although in ignorance of underlying mechanism. Herein, it is demonstrated that IXN diminished vessel growth, sprouting and stabilization, accompanied by decreased VEGFR2 expression and modulation of Ang-1ie2, Akt and Erk pathways in vascular cells. IXN did also reduce NFkB and TNFα in both HUVEC and HASMC, demonstrating anti-inflammatory properties.

The mouse retina is avascular at birth and the process of vascularization occurs by angiogenesis and develops during the first three weeks. The primary vascular plexus extend from the central retinal artery, radially, in a planar manner, alternating veins and arteries. Vessels at the growing edge of the vascular front are less mature than the central vessels. It is then possible to evaluate several steps of the angiogenic process as well as the maturation of growing vessels along the first week [Dorrell and Friedlander, 2006; Fruttiger, 2007]. Around postnatal day 7, the retinal vessels start to sprout into deeper layers, leading to the formation of a

three-layered system.

The deeper or outer plexus develops by sprouting from the primary plexus, during the second week. The intermediate vascular plexus is the last to be formed from sprouting of superficial and deeper vascular plexus [Uemura et al., 2006].

Treatment with IXN significantly decreased sprouting angiogenesis during the first week after mouse birth. Interestingly, this effect only appears evident at the later time points P5 and P7, probably due to the need of IXN accumulation for a significant outcome in angiogenesis inhibition. These results point out an *in vivo* anti-angiogenic effect of IXN, which is in accordance to previous observations from our group that described a decrease in EC viability, proliferation, invasion and capillary-like structures formation in the presence of IXN and decreased *in vivo* capillary formation [Negrão et al., 2010]. The Akt pathway plays a critical role in the regulation of cell survival. A significant decrease was observed in p-Akt expression (47%) for HUVEC treated with 10 µM IXN. Albini and collaborators verified that other related polyphenol, XN, represses Akt pathway in EC, indicating that it is a target in the molecular mechanism of XN [Albini et al., 2006]. EC proliferation occurs early in angiogenesis and continues as the new capillary elongates. Mitogen-activated protein kinases (MAPK) are a family of Ser/Thr kinases that regulate important cellular processes including cell growth, proliferation and differentiation in response to cellular environment, and mechanical force-induced proliferation [Mojzis et al., 2008]. Growth factors frequently activate one group of this family, the ERK1/2 route. In the presence of IXN, HUVEC decreased p-Erk expression, according to IXN concentration, reaching the highest effect, 52% of inhibition, for the maximal concentration tested (10 µM IXN). The observed inhibition of p-Akt and p-Erk pathways also supports an anti-angiogenic role for IXN. [Oh et al., 1999]. Upon IXN treatment, HUVEC decreased the expression of Tie2 levels and Ang2 production, reinforcing the anti-angiogenic potential of IXN.

Surprisingly, no differences were apparent in the migration of vessels towards the intermediate vascular plexus, suggesting that the initial inhibitory effect of IXN in angiogenesis was overcome during retinal development. The branching process is highly energetically unfavorable and generates reactive oxygen species that seem to decrease vascularization, granting anti-oxidant compounds the possibility of acting as pro-angiogenic agents during this phase [Uno et al., 2010]. Hence, IXN can have a balanced effect on angiogenesis: it is primarily an inhibitor, but due to its anti-oxidant properties [Gerhauser et al., 2002] it can decrease reactive oxygen species and stimulate vessel migration during this phase of development, justifying the fact that IXN did not impair sprouting angiogenesis at P15. Taking together the observations from P5 to P15, IXN seems to have a delay effect on developmental angiogenesis of the retina, rather than a steady anti-angiogenic effect.

During sprouting angiogenesis, a region with dense and uniform capillary plexus is formed behind tip cells. Over time, this vascular plexus starts to be remodeled and mature into a hierarchical vascular tree [Fruttiger, 2007].

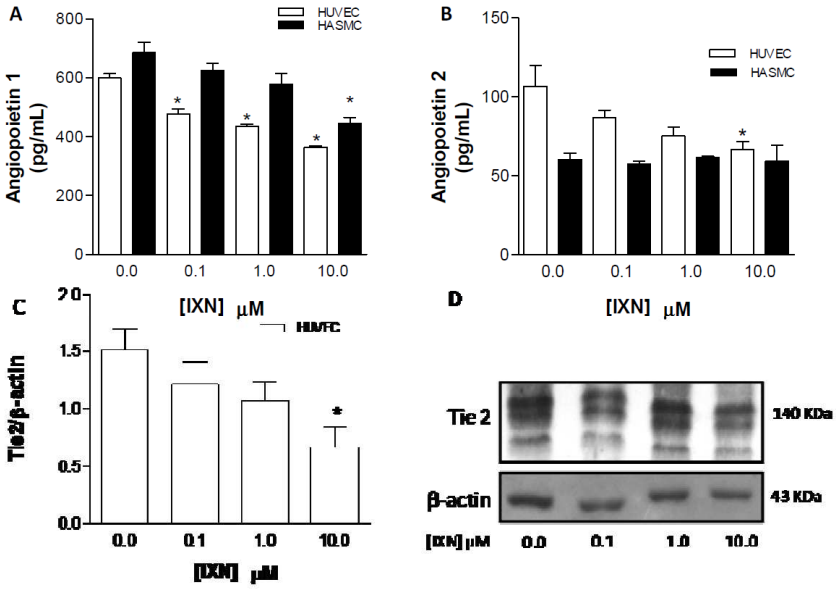

Figure 14- Evaluation of angiopoitein 1, angiopoietin 2 and Tie2 expression after 24 h treatment of HUVEC and HASMC with 0.0-10 µM isoxanthohumol (IXN). (A) Angiopoietin (Ang)1 and (B) Ang2 present in the medium were examined by ELISA and quantification was performed at 450 nm and 550 nm. (C) Tie2 expression in HUVEC was determined by Western blot analysis, graph illustrates the relative band intensity ratio of Tie2 after normalization with β-actin, (D) representative bands obtained after immunostaining. Results are means ± SEM of independent experiments ($4 \leq n \leq 8$) and expressed as percentage of control. *$p<0.05$ vs. control.

Some capillaries are pruned and others are strengthened in order to form functional vessels. The area neighboring arteries is highly sensitive to vascular pruning by leukocytes within a period of partially developed but pericyte-poor vasculature [Ishida et al., 2003]. It seems that dense primary vasculature may result in hyperoxia and reactive oxygen species formation, promoting the expression of inter-cellular adhesion molecule 1 (ICAM-1) at specific sites of the vasculature, activating leukocytes and leading to apoptosis of EC and vascular pruning [Ishida et al., 2003]. Evidence indicates that many inflammatory cells release several factors that act on vascular EC and modulate angiogenesis [Costa et al., 2007]. At this phase, IXN treatment resulted in a less complex and undifferentiated vasculature, with less capillary-free spaces surrounding the main arteries. Once again the anti-inflammatory and anti-oxidant properties of IXN [Gerhauser et al., 2002; Negrão et al., 2010] may suppress hyperoxia-induced ICAM-1 and leukocyte recruitment, diminishing vascular pruning, and giving rise to a more immature vasculature. In these regions, there are particularly important survival and pro-apoptotic players that interfere with vascular pruning and selection, such as leukocytes [Fantin et al., 2010; Ishida et al., 2003], astrocytes [Uemura et al., 2006], microglia [Rymo et al., 2011] and smooth muscle cells [Dorrell and Friedlander, 2006]. The absence of alterations in vessel density and branching under IXN treatment at P5 can probably be also related to its differential action on these stromal and vascular cells.

IXN treatment significantly reduced the average arterioles diameter, probably due to impairment of pericyte coverage. Accordingly, a diminished vessel coverage by αSMA-positive cells along vessel length in IXN treatments at P5 and P7 was observed, especially in the areas of intensive remodeling. This decrease in mural cells coverage does not seem to affect the more central, mature and already covered retinal vessels and is spatially related to the presence of less complex and undifferentiated vasculature. Previous results revealed that another polyphenol, XN, disrupted angiogenic vessels but not stable ones [Negrão et al., 2007], suggesting a protection against the anti-angiogenic action of this compound in vessels where EC-VSMC interaction is established. As observed, after IXN treatment of HASMC, p-Akt expression did not change, confirming that IXN does not seem to affect the viability of VSMC as already described [Negrão et al., 2010]. However, IXN decreased the proliferation and invasion of VSMC [Negrão et al., 2010], which may in part explain the reduced coverage of retina vessels during IXN treatment. In fact, IXN decreased p-Erk expression in HASMC, reaching 68% decrease for the maximal concentration tested (10 μM IXN). Other studies verified that red wine and green tea polyphenol compounds were able to inhibit key events of the angiogenic process like the migration and proliferation of EC and VSMC [Iijima et al., 2002; Oak et al., 2005]. Chen et al., compared the biological actions of five green tea catechins and found apparently different degrees of activation of MAPK, related to polyphenol structure, dose and time of cell exposure [Chen et al., 2000].

Ang1 is produced by VSMC and binds to Tie2 on EC, promoting vascular stabilization [Davis et al., 1996; Zhang et al., 2011]. We observed that Ang1 expression decreased along with increasing IXN concentration, in HASMC, achieving statistical significance only for 10 μM. We also observed *in vitro* concentration-dependent decreasing levels of Tie2 expression in HUVEC in the presence of IXN. The reduction of both Tie2 expression in HUVEC and Ang1 production by HASMC may also explain the reduced retina vessel coverage in the presence of IXN. Moreover, Ang1 can cause vessel enlargement, during a brief postnatal period [Thurston et al., 2005], and the decreased Ang1 expression during IXN treatment can also justify the decreased observed in vessel diameter. Nevertheless, we cannot exclude that the results obtained in HUVEC and HASMC cultures were obtained in the absence of any cell stimulation, a context different from that of retinal angiogenesis. Also, in this *in vitro* model, paracrine effects regarding EC and VSMC, which are not in contact with each other, cannot be observed [Sandhu et al., 2004]. As far as we know, only another study was done regarding polyphenol effects on angiopoitein-Tie2 pathways. The authors evaluated the effects of *in vivo* red-wine consumption on VEGF, angiopoietins and their receptors, in rat erectile tissue [Neves et al., 2010]. Contrary to our results, they found that red-wine treated animals exhibit increased expression of Ang1, Ang2 and Tie2 in corpus cavernosum. The authors suggest that it may be a compensatory mechanism activated by VEGF decrease, to maintain cavernous tissue vascularization. The regulation of the expression of VEGF, angiopoietins and their receptors is very complex and, as above referred, context and tissue dependent. Thus, these results need further *in vivo* confirmation. As seen before with sprouting angiogenesis, at P15 the differences on αSMA-coverage were not so evident, and again, a recovery process seemed to take place.

VEGF is the most potent pro-angiogenic factor, which makes it an attractive target in anti-angiogenic therapies. Most of VEGF angiogenic effects result from VEGFR2 activation [Ferrara et al., 2003]. Accordingly, it was observed that VEGFR2 expression significantly decreased, to 55.16 ± 17.17% of control, after treating HUVEC with 10 μM IXN. This strongly suggests reduced angiogenic activity in the presence of IXN and may also explain the decrease in retinal angiogenesis observed after IXN treatment during the first post-natal 7 days. Corroborating these findings, similar effects on VEGFR2 have been described for cinnamon polyphenols [Lu et al., 2010] and red-wine treated rats [Neves et al., 2010]. The staining of P5 retinas with an anti-VEGFR-2 antibody revealed a receptor distribution mainly restricted to the neural retina, as described before by other groups [Cao et al., 2010; Shih et al., 2003]. The expression of the receptor surrounding the vessels that are being formed evidences the interplay

between endothelial and non-vascular cells that takes place during developmental angiogenesis in retina. IXN modified the pattern of VEGFR-2 expression behind the vascular front, turning it more irregular and suggesting that VEGFR-2 decoration is highly dependent on the vascular distribution and maturity.

The retinal angiogenesis model is valuable for identifying and studying different EC populations, namely phalanx cells (the most quiescent EC, lining consolidated and mature blood vessels), stalk cells (the cells just behind tip cells, that proliferate and elongate the stalk of the sprout and form the lumen) and tip cells (the cells in the tip of the growing vessel, with filopodia that sense the guidance cues in the environment and migrate guiding the vessel sprout) [De Smet et al., 2009]. IXN tends to reduce the number of tip cells and filopodia in the vascular front of retinal angiogenesis, without affecting filopodia length. This can be explained by the already discussed effects of IXN on viability and proliferation of EC and this mild result can possibly be explained, once again, by the dual action of IXN on vessels and stroma. Tip cells are rich in VEGFR2 and migrate through a VEGF gradient. The filopodia from mice treated with IXN were more irregular and not as vertically oriented as expected, which could be owed by the alteration on the pattern of VEGFR2 expression and activation induced by IXN.

Recent studies have helped to clarify the vessel fusion process and identified microglia, or resident tissue macrophages, as 'bridge cells' that promote vascular union [Fantin et al., 2010]. In the present study, we observed a decrease of microglia co-localizing with the fusion sites in IXN-treated mice. The Tie2-expressing macrophages (TEM) are a subset of circulating and tissue-infiltrating monocytes that are innately pro-angiogenic and have lower pro-inflammatory activity [Coffelt et al., 2010; Pucci et al., 2009]. Exposure of TEM to Ang2 drives their polarization to M2-like phenotype increasing TEM's pro-angiogenic activity [Coffelt et al., 2010]. It is speculated that TEM may act as "bridge cells" and if this is the case, the reduction of Ang2 expression by IXN may explain the reduction of macrophages co-localizing with the fusion sites.

Endothelial nitric oxide synthase (eNOS) modulates angiogenesis in response to ischemia, and plays a fundamental role in VEGF-induced angiogenesis. iNOS seems to have a smaller but additive effect on angiogenesis [Nematollahi et al., 2009]. Nevertheless, incubation of HUVEC and HASMC with IXN did not change NO levels in cell culture medium.

TNFα is a cytokine produced in the acute phase reaction of the inflammatory process, mainly by macrophages [Balkwill, 2009]. TNFα exerts regulatory effects on VEGF and on NFκB expression. After treating HUVEC and HASMC with IXN 10μM, secreted TNFα decreased. Other authors had already observed that XN inhibits inflammatory process and TNFα expression, in macrophages [Cho et al., 2008].
NFκB is fundamental in the regulation of several cellular responses like inflammation, stress, proliferation and apoptosis [Naugler and Karin, 2008]. As a redox-sensitive transcription factor, it is in part regulated by the redox status of the cell. Polyphenols, well known anti-oxidants, can be responsible for this regulation. IXN was able to inhibit the NFκB subunits p50/p65 translocation to the nucleus. The inhibition was dependent on IXN concentrations for both HUVEC and HASMC and the effect seemed higher in HUVEC cells. These findings may help to explain IXN potential to prevent inflammation and modulate angiogenesis. Red wine and green tea polyphenols also affect the activity of NFκB, playing an important role in the inflammatory pathways in EC [Canali et al., 2010; Djoko et al., 2007; Manna et al., 2000; Wahyudi and Sargowo, 2007]. Other studies have already described similar effects after treatments with XN but not for IXN [Albini et al., 2006; Negrão et al., 2007].

NFκB regulates the expression of several proteins related to inflammatory and angiogenic processes. Imhof and Aurrand-Lions hypothesized that NFκB was able to promote an inflammatory status in EC, promoting the expression of inflammatory genes, such as ICAM-1, and stimulating Ang2 secretion [Imhof and Aurrand-Lions, 2006]. Therefore, the inhibition of NFκB signaling by IXN may be associated to reduced levels of ICAM-1, explaining the impaired vessel pruning and also the diminished Ang2 expression, contributing to the anti-angiogenic effect of IXN. This effect can also be due to the reduction of TNFα upon IXN treatment, as TNFα seems to induce secretion of Ang2 by endothelium, in a time- and dose-dependent manner, via NFκB [Kim et al., 2000].

Altogether, these results indicate, for the first time, that IXN effects on angiogenesis may, at least in part, be related to modulation of complex interplay between EC, VSMC, inflammatory and stromal cells, regulated by two main pathway axes, VEGF-VEGFR2 and Angiopoietins-Tie2. They also suggest that IXN affects the cell inflammatory status by decreasing two fundamental pro-inflammatory factors, TNFα and NFκB. Finally, the already described IXN effects on EC and VSMC viability, proliferation and migration can be explained by regulation of Akt and primordially Erk pathway.

Acknowledgements

We would like to thank Christiana Katti, Sumathi Sekaran and Russell Foster, from the Nuffield Laboratory of Ophthalmology, University of Oxford, for providing invaluable training in retina dissection.

This study was partially funded by Fundação para a Ciência e Tecnologia, Portugal (FCT) (SFRM/BD/41888/2007; PTDC/SAU-OSM/102083/2008 and PEst-OE/SAU/UI0038/2011) and European Research Advisory Board (ERAB) EA-06 41.

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CAPÍTULO 6

Platelet-mediated flavonoid metabolism.

Interaction with vascular wall cells and implication in vasculopathy

PLoS ONE 5: e9673, 2010

Negrão R, Soares R

Could platelet-accumulating polyphenols prevent tumour metastasis?

Nature Rev Cancer 11: 123-124, 2011

Negrão R, Duarte D, Costa R, Soares R

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PLoS one

Platelet-mediated flavonoid metabolism. Interaction with vascular wall cells and implication in vasculopathy

Original Article

Platelet-Mediated Metabolism of the Common Dietary Flavonoid, Quercetin

Platelet-mediated flavonoid metabolism. Interaction with vascular wall cells and implication in vasculopathy

Posted by raqsoa on **21 Mar 2010** at **19:12 GMT**

We read with special interest the paper by Wright et al (1) regarding the involvement of platelets in quercetin metabolism. In this paper, the authors demonstrate that this flavonoid is internalized by platelets, and can be metabolised to its O-methylated derivative, tamarixetin, by platelet enzymatic activity.

The implication of platelets in flavonoid metabolization is of paramount importance, given the role of these cells in vascular pathophysiological processes. In general, natural flavonoids are considered anti-oxidant and anti-inflammatory agents. The vascular system is actually one of the most prone targets for inflammation and oxidative stress development, which ultimately lead to cardiovascular disease and other pathological situations. Recent data from our lab on vascular wall cells showed that beer-derived polyphenols, such as xanthohumol (XN) and its metabolites, isoxanthohumol (IXN) and 8-prenylnaringenin (8PN) interfere with endothelial and smooth muscle cells behaviour. We found that XN and IXN were able to prevent cell growth and migration of both vascular wall cells, and inhibit the assembly of vascular structures in vitro (2). Microarrays also revealed a decrease in pro-inflammatory cytokines and angiogenic growth factors by these two polyphenols, whereas 8PN displayed just the opposite effect (3). More recently, we further showed that these effects were maintained in wound-healing and matrigel plug in vivo models (4). The anti-inflammatory, anti-oxidant and anti-angiogenic effects of XN and IXN render these molecules good therapeutic agents against a huge number of pathological situations in which these processes take place, namely cardiovascular disease, diabetes, and cancer.

Interestingly enough, the fact that polyphenols can be metabolised by platelets opens a new field of research, since these cells interact with endothelial and vascular support cells. The role of platelets in endothelial dysfunction is well established. In addition, platelets are known to induce smooth muscle cell contraction, and enhance migration and proliferation. Besides, platelets play essential roles in inflammatory processes, contributing to leukocyte accumulation and behaviour changes. For instances, platelet adhesion to arterial blood vessel wall contributes to the development of focal arterial atherosclerotic plaques. Therefore, an intimate interaction between platelets and vascular wall cells play crucial roles in pathophysiological conditions.

On the other hand, metabolisation of dietary polyphenols by platelets may contribute to the downregulation of platelet-induced endothelial dysfunction, namely by releasing growth factors and cytokines that interfere with vascular wall cells and other cells like macrophages, modulating the inflammatory process. Tarao Group (5) defends that activated macrophages are a potential target of flavonoids, in the aorta, since metabolites of dietary quercetin accumulate in human atherosclerotic lesions, but not in normal aorta, specially associated with macrophage-derived foam cells, suppressing mRNA expression of the class A scavenging receptor and CD 36. These same authors also observed the methylation of quercetin in macrophage cells.

Although flavonoids plasma concentration seems to be relatively low, these studies also show that flavonoids could accumulate in these target cells at relatively higher concentrations than those that have been generally assumed. In summary, we would like to stress that metabolisation of flavonoids by platelets may interfere with the actions of these compounds in the interplay between these and other blood cells or with vascular wall cells, and somehow influence the progression of vasculopathy.

Finally, as mentioned by the authors, different cell types are also able to methylate, sulphate or glucuronidate flavonoids, including fibroblasts, astrocytes, microglia and enterocytes (1, 3-4, 6). It remains to confirm whether vascular wall cells are also able to metabolise flavonoids. This leads us to hypothesize that different molecules can be present within these cells, presenting eventually distinct effects. As our findings point out, XN and its metabolite 8PN display opposite actions in endothelial and smooth muscle cell cultures. This may drive to conflicting effects if these molecules are used for therapeutic or preventive purposes.

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CORRESPONDENCE

LINK TO ORIGINAL ARTICLE

Could platelet-accumulating polyphenols prevent tumour metastasis?

Rita Negrão, Delfim Duarte, Raquel Costa and Raquel Soares

We read with great interest the Review by Gay and Felding-Habermann (Contribution of platelets to tumour metastasis. *Nature Rev. Cancer* **11**, 123–124 (2011))[1], which discussed the observation that cancer patients usually present signs of thrombosis, which is much more severe during metastatic progression. These authors suggest that platelets may protect tumours from immune elimination and enhance adhesion to blood vessels and tumour cell proliferation, which renders them useful for distant metastasis. According to these authors, platelets could be regarded as putative targets for cancer therapy. Platelets have a major role in haemostasis, by repairing minor vascular injuries and thrombosis[2]. Platelets are also recognized by their important roles in the angiogenic process, given their release of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF2; also known as bFGF), epidermal growth factor (EGF) and interleukin-1β (IL-1β) upon activation.

We have been studying the effects on angiogenesis and inflammation of a group of naturally derived compounds, polyphenols, which have established anti-oxidant, anti-inflammatory, anti-angiogenic and antitumour properties. The topical administration of two of these molecules, xanthohumol or isoxanthohumol (IXN), resulted in a reduction in systemic inflammatory factors and angiogenesis in granulation tissue in rat wound-healing assays[3]. Furthermore, we also demonstrated that xanthohumol reduces cancer progression and inflammation in a mouse xenograft breast cancer model[4]. Emerging evidence has revealed that these compounds can be internalized by platelets, in which they undergo further metabolism by platelet enzymatic activity[2]. Interestingly, polyphenols may also interfere with platelet adhesion to the blood vessel wall, which is associated with the development of atherosclerotic plaques.

It is therefore conceivable that polyphenol accumulation within platelets in blood circulation may be considered as an anticancer mechanism of polyphenols. In agreement, the release of metabolized polyphenols by active platelets[2] decreases local tumour cell viability, proliferation and invasion and increases apoptosis[4]. This effect may further be potentiated through the anti-inflammatory and anticancer properties that are characteristic of these compounds.

In conclusion, similar to anti-microbial pharmacological agents that are in clinical use, such as neutrophil-accumulating macrolides (which are then released in an active form at the infection site[5]), the accumulation of polyphenols in platelets could prevent tumour metastasis.

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doi:10.1038/nrc3004-c1

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Competing interests statement
The authors declare no competing financial interests. **[Au: Please return forms]**

DISCUSSÃO

DISCUSSÃO

A angiogénese é fundamental durante o desenvolvimento do organismo e, apesar de, em geral, estar quiescente no adulto, ocorre em situações fisiológicas como o ciclo reprodutivo na mulher ou a cicatrização. É um processo complexo que envolve a interacção entre diferentes tipos de células como por exemplo as células que constituem os vasos sanguíneos, EC e células murais, e as células inflamatórias, sendo regulado pelo balanço entre factores pró-angiogénicos e inibidores da angiogénese, podendo estar desregulado em determinadas situações patológicas [Carmeliet, 2004; Raza e colaboradores, 2010].

São várias as patologias associadas a alterações na angiogénese. O cancro, a aterosclerose, a psoríase e a retinopatia diabética estão associados a uma angiogénese excessiva [Costa e colaboradores, 2007; Folkman, 2007a; Soares e Costa, 2009]. Pelo contrário, na isquemia do miocárdio, na doença arterial periférica, e nalgumas patologias neurológicas, a angiogénese é deficiente [Carmeliet, 2005]. A inflamação crónica e a angiogénese são dois processos que ocorrem muitas vezes associados em várias destas patologias.

A ingestão de uma dieta rica em polifenóis diminui a incidência de patologias como as doenças cardiovasculares, a diabetes tipo 2, as doenças neurodegenerativas e o cancro [Bravo, 1998; Dell'Agli e colaboradores, 2004; Gerhauser, 2005; Oak e colaboradores, 2003; Soleas e colaboradores, 1997; Stevens e Page, 2004]. Os polifenóis manifestam uma actividade biológica vasta, incluindo propriedades anti-oxidantes, anti-inflamatórias e anti-carcinogénicas [Araújo e colaboradores, 2011; Monteiro e colaboradores, 2009; Oak e colaboradores, 2005; Rahman e colaboradores, 2006; Stevens e Page, 2004]. O efeito anti-angiogénico de polifenóis naturais tem sido também alvo de estudo nos últimos anos, dada a relação entre a angiogénese e um cada vez maior número de patologias. Apesar da intensa investigação sobre a modulação da angiogénese pelos polifenóis, muito ainda se encontra por esclarecer, nomeadamente no que diz respeito à influência na comunicação EC-células murais, fundamental no processo angiogénico.

O objectivo deste trabalho foi, por isso, compreender como os polifenóis actuam nas EC e nas VSMC, de modo a regular a angiogénese e a inflamação, assim como confirmar o seu efeito na angiogénese em modelos animais e identificar vias moleculares que possam estar envolvidas nesta regulação. Este

conhecimento pode ajudar a fundamentar eventuais recomendações alimentares e possibilitar avanços para uma futura utilização de produtos ricos em polifenóis na profilaxia e/ou terapêutica.

Dado que a parede dos vasos sanguíneos é constituída por dois tipos de células, as EC e as células murais, intervenientes no processo angiogénico, o estudo envolveu inicialmente estes dois tipos de células: EC de cordão umbilical humano, HUVEC, e VSMC de aorta fetal humana, FLTR. As HUVEC são frequentemente utilizadas para estudar a angiogénese *in vitro*. Estas células proliferam activamente e mantêm a morfologia e as propriedades funcionais da generalidade das EC [Gifford e colaboradores, 2004]. Apesar de EC de diferentes tecidos terem funções especializadas de acordo com o tecido e o ambiente envolvente [Chi e colaboradores, 2003] e de existir alguma heterogeneidade ao longo da árvore vascular [Staton e colaboradores, 2009], as EC em cultura perdem, na sua maioria, o fenótipo específico do órgão de origem à medida que proliferam [Gifford e colaboradores, 2004]. As FLTR são uma linha celular imortalizada que mantém a morfologia e as características funcionais das VSMC de aorta de indivíduos adultos [Martin e colaboradores, 2001]. Mesmo as VSMC maduras mantêm a capacidade de se desdiferenciarem e perderem o seu fenótipo contráctil, regressando a um estado de proliferação e migração, quando em cultura, de acordo com o ambiente que as rodeia [Chaterji e colaboradores, 2010]. A utilização destes dois tipos de células permitiu investigar separadamente o efeito dos compostos de interesse em cada tipo celular.

Num estudo muito interessante, Albini e colaboradores demonstraram que o XN inibe a capacidade de proliferação, migração e invasão de EC e a angiogénese *in vivo* [Albini e colaboradores, 2006]. No entanto, nada foi descrito para o efeito deste polifenol nas células murais. Desta forma foi considerado pertinente testar o efeito do XN nas VSMC e verificar simultaneamente se nas condições utilizadas os efeitos descritos para as EC se mantinham (**Capítulo 1**). Os resultados obtidos confirmaram que o XN diminui, nas condições testadas, a viabilidade e a capacidade invasiva das HUVEC, aumentando simultaneamente a sua apoptose, tal como tinha já sido descrito por Albini para as EC [Albini e colaboradores, 2006] e nalguns estudos para diversas células tumorais [Colgate e colaboradores, 2007; Dorn e colaboradores, 2010; Gerhauser, 2005]. O Matrigel® é um extracto de membrana basal de sarcoma de Ratinho rico em laminina, colagénio tipo IV, entactina, factores de crescimento e outras proteínas, utilizado como matriz para a cultura de células e tecidos, sobretudo em estudos de proliferação, invasão e diferenciação. A cultura de HUVEC em Matrigel® e o seu tratamento com XN

inibiu também a capacidade das HUVEC se diferenciarem e se organizarem em estruturas tubulares, um processo crítico para a formação de novos vasos sanguíneos, confirmando o seu potencial como agente anti-angiogénico. Verificou-se ainda que o tratamento com XN induziu resultados semelhantes nas FLTR. O XN diminuiu a viabilidade e a capacidade invasiva das FLTR, aumentando também a sua apoptose. Este efeito do XN nas FLTR é por si só interessante, na medida em que abre a possibilidade deste polifenol poder ser utilizado em patologias associadas a proliferação e invasão anómalas das VSMC, como acontece por exemplo na aterosclerose e na reestenose [Costa e colaboradores, 2007; Griffioen e Molema, 2000; Moulton, 2001]. Diversos grupos de investigação têm sugerido que a utilização de fármacos anti-angiogénicos que actuem simultaneamente nas EC e células murais pode trazer benefícios superiores no tratamento de tumores, dado que a terapia anti-VEGF resulta muitas vezes numa resistência dos vasos sanguíneos tumorais, estabilizados pelas células murais e pelo aumento da sobrevida celular via Ang1/Tie2 [Bergers e colaboradores, 2003; Erber e colaboradores, 2004]. Além disso, ocorre frequentemente uma revascularização muito rápida dos tumores após a suspensão do tratamento com anti-VEGF. O espaço vazio deixado pela morte do endotélio e que continua protegido pelas células murais parece ser rapidamente preenchido por novas EC após a suspensão da terapia anti-VEGF [Mancuso e colaboradores, 2006]. Por esta razão, as propriedades demonstradas pelo XN nos dois tipos de células que constituem o vaso sanguíneo merecem ser realçadas e são a favor da sua eficácia anti-angiogénica.

O processo angiogénico tem início na dissociação da adesão EC-células murais por um estímulo angiogénico, permitindo a migração das EC, e termina com o restabelecimento da ligação EC-células murais, que contribui para a maturação e estabilização do vaso sanguíneo. As células murais produzem VEGF e à medida que rodeiam as EC também as vão protegendo, dado que o VEGF é necessário à sobrevivência das EC. A sinalização Ang1/Tie2 e PDGFB/PDGFRβ contribui também para a estabilização do vaso, fundamental para o seu bom funcionamento [Armulik e colaboradores, 2005; Bergers e Song, 2005]. O revestimento inadequado do endotélio por células murais resulta frequentemente em derrame ou ruptura vascular.

A observação de que o XN pode actuar sobre os dois tipos de células que formam os vasos sanguíneos levantou uma questão importante, o XN poderá ser útil como anti-angiogénico se actuar preferencialmente sobre os vasos em formação e não sobre os vasos já estruturados e que são

estabilizados por uma maior cobertura de VSMC. Neste contexto, foi testado o efeito do XN em estruturas tubulares formadas por co-culturas de HUVEC e FLTR em Matrigel®. As estruturas tubulares formadas resultaram em cordões mais densos e, surpreendentemente, o tratamento destas estruturas com XN resultou, não na redução do número de estruturas tubulares, como tinha acontecido com as culturas de HUVEC, mas no aumento do número e estabilidade destas estruturas. Este resultado foi mais tarde confirmado em ensaios *ex vivo* com anéis de aorta de Rato cultivados em Matrigel®. A cultura de anéis de aorta em Matrigel® reproduz o *sprouting* angiogénico em culturas tridimensionais e permitiu avaliar o efeito do XN na presença de estruturas mais complexas: um vaso sanguíneo maduro em que o endotélio está ligado às VSMC pela membrana basal e é revestido por várias camadas de VSMC, estando ainda presentes outras células como fibroblastos e macrófagos [Auerbach e colaboradores, 2000; Nicosia, 2009]. O tratamento com XN, à semelhança do que foi descrito para as estruturas tubulares formadas em co-culturas de HUVEC e FLTR, conduziu a um ligeiro aumento do *sprouting* vascular a partir do anel de aorta [Costa , 2009]. Este resultado reforça a ideia de que o XN parece afectar essencialmente os vasos em formação e não os vasos maduros, já estabilizados. A comunicação entre as EC e VSMC parece assim fortalecer a estrutura vascular protegendo as células do efeito do XN. De facto, as células murais produzem VEGF necessário à sobrevida das EC e é possível que a Ang1, que é constitutivamente produzida pelas células murais, active por ligação ao Tie-2 expresso nas EC vias de sinalização envolvidas na sobrevida celular, como a via do Akt e simultaneamente bloqueie a actividade do NFκB [Imhof e Aurrand-Lions, 2006]. De acordo com isto, foi recentemente sugerido que o contacto directo entre EC e VSMC quiescentes inibe a resposta inflamatória desencadeada pelo TNFα [Wallace e Truskey, 2010]. A activação do processo inflamatório leva à produção de Ang2 pelo endotélio, que, por ligação ao Tie2 promove a angiogénese [Fiedler e colaboradores, 2006]. A diminuição do NFκB levaria à diminuição do estado inflamatório das EC e da libertação de Ang2 e a uma inibição do processo angiogénico. Assim, a diminuição do NFκB observada após o tratamento dos dois tipos de células com XN parece ser um importante mecanismo envolvido no efeito anti-angiogénico observado no tratamento das células com XN e na estabilização dos vasos sanguíneos.

Os resultados obtidos permitiram verificar não só que o XN afecta igualmente as EC e as VSMC, diminuindo a sua viabilidade, capacidade invasiva e diferenciação em estruturas capilares, mas sobretudo que o tratamento com XN parece afectar os vasos angiogénicos mais imaturos, protegendo a

estrutura vascular já estabelecida. Parece também razoável admitir que o NFκB possa ser um regulador deste efeito, assim como o sistema angiopoietinas-Tie2.

A cerveja é praticamente a única fonte alimentar fornecedora de XN e durante o seu fabrico a maior parte do XN presente no extracto de lúpulo sofre isomerização térmica, dando origem ao IXN [Magalhães e colaboradores, 2009; Stevens e colaboradores, 1999], que aparece assim em maiores concentrações. Esta é uma das razões pelas quais a maior parte das cervejas comerciais tem níveis muito baixos de XN (0,002-1,2 mg/L) e níveis mais elevados de IXN (0,04-3,44 mg/L) [Gerhauser, 2005]. A 8PN também aparece no lúpulo e na cerveja (0,001-0,24 mg/L) [Gerhauser, 2005] e é uma flavanona prenilada obtida por isomerização do desmetilxanto-humol ou por desmetilação do IXN. A 8PN é o fitoestrogénio mais potente que foi isolado até hoje sendo agonista dos receptores de estrogénios, o que indica que possa ser usada em situações patológicas hormono-dependentes como na osteoporose e em complicações associadas à menopausa [Milligan e colaboradores, 2002]. No organismo, o XN pode ser convertido em IXN por ciclização ácida no estômago e o IXN pode ser O-desmetilado pelo citocromo P450 hepático humano, originando 8PN [Guo e colaboradores, 2006; Nikolic e colaboradores, 2005], que também se pode formar na presença da microflora intestinal humana [Hanske e colaboradores, 2010; Possemiers e colaboradores, 2005]. No entanto, dada a presença quase exclusiva destes polifenóis no lúpulo, a sua presença na dieta humana ocorre apenas por consumo de cerveja e, mais recentemente, de alguns alimentos fortificados ou suplementados produzidos pela indústria alimentar. Embora a biodisponibilidade destes compostos seja controversa, Bolca e colaboradores verificaram que após a suplementação da dieta humana com 6,12 de mg de XN, 3,6 mg de IXN e 0,3 mg de 8PN por dia durante 5 dias, foram encontrados níveis plasmáticos de 4,99 de nM XN, 14,86 de nM IXN e 2,20 de nM de 8PN e concentrações no tecido mamário da ordem dos pmol/g de tecido [Bolca e colaboradores, 2010]. O IXN é portanto o flavonóide prenilado que provavelmente atinge maiores concentrações no organismo humano [Bolca e colaboradores, 2010]. Pareceu-nos por isso importante fazer um estudo comparativo destes três flavonóides na modulação da angiogénese e da inflamação, *in vitro,* utilizando EC e VSMC (**Capítulo 2**). Para os ensaios foram utilizadas culturas de HUVEC e de VSMC de aorta humana, HASMC.
Estes ensaios permitiram verificar (e confirmar no caso do XN) que os polifenóis testados modulam de modo diferente várias etapas do processo angiogénico. O XN e o IXN exerceram efeitos semelhantes,

diminuindo, de um modo geral, a viabilidade, a proliferação e a capacidade invasiva das EC e das VSMC, e aumentando a apoptose dos dois tipos de células. Estes polifenóis diminuíram ainda a formação de estruturas capilares na presença de Matrigel®. O IXN parece ser uma molécula com potencial interesse em situações patológicas associadas a uma excessiva proliferação das VSMC, demonstrando também actividade anti-angiogénica, como tinha já sido observado para o XN. Curiosamente, o efeito do IXN tendeu a ser mais pronunciado nas HUVEC, enquanto o XN parece actuar mais eficazmente nas HASMC. Surpreendentemente, a 8PN, cuja estrutura é idêntica à do IXN, apresentou efeitos praticamente opostos a este, aumentando a viabilidade e a proliferação das HUVEC e diminuindo a sua apoptose, ao mesmo tempo que estimulou a formação de estruturas tubulares de HUVEC em Matrigel®.

Embora os estudos *in vitro* forneçam informação valiosa, a respectiva interpretação e extrapolação para a situação *in vivo* deve ser cuidadosa. Por essa razão, foram realizados ensaios com o intuito de verificar se os efeitos observados para o XN, o IXN e a 8PN se mantinham em dois modelos animais de angiogénese. Um modelo menos fisiológico, simulador de uma situação patológica com um estímulo angiogénico forte, que resulta do implante, no tecido subcutâneo de Ratinho, de Matrigel® adicionado de VEGF. Pode ainda adicionar-se ao Matrigel® substâncias cujo efeito na angiogénese se pretenda estudar. O Matrigel® cria uma estrutura envolvente, próxima do ambiente natural, criando o suporte para o desenvolvimento da resposta angiogénica [Auerbach e colaboradores, 2003]. O outro modelo utilizado foi o modelo de cicatrização cutânea [Eming e colaboradores, 2007a]. O processo de cicatrização é um processo fisiológico necessário à reparação e regeneração de tecido danificado. Este processo depende da interacção entre a inflamação, o stresse oxidativo e a angiogénese [Soares, 2009]. A cicatrização desenvolve-se normalmente sem complicações, mas a resolução da inflamação é crítica e fundamental para o processo [Eming e colaboradores, 2007b]. Nos mamíferos, a cicatrização resulta da ocorrência de três fases, a hemostase e inflamação, a formação do tecido granuloso e, por fim, a reepitelização e remodelação do tecido [Tan e colaboradores, 2007]. O processo angiogénico é estimulado imediatamente aquando da formação do trombo plaquetário, através da libertação de factores angiogénicos pelas plaquetas, e é amplificado pela hipóxia e inflamação, os dois principais estímulos angiogénicos, levando à produção de VEGF, um potente factor angiogénico. Por outro lado, o VEGF promove a permeabilidade vascular permitindo a libertação de factores inflamatórios (que atingem a concentração máxima por volta do terceiro dia), assim como de nutrientes no local da incisão,

facilitando a proliferação e sobrevida das EC e a formação de novos vasos sanguíneos que aparecem no local da cicatrização por volta do dia 3 a 5 após a incisão [Kagawa e colaboradores, 2009]. A angiogénese é depois sustentada por EC, VSMC, macrófagos, fibroblastos e pela ECM, que produzem factores de crescimento, proteases e factores angiogénicos. Na fase final da cicatrização, a produção de todos estes factores vai sendo gradualmente reduzida até regressar aos níveis basais, promovendo a estabilização dos vasos formados [Chung et al., 2010]. O processo de cicatrização proporciona assim uma oportunidade para estudar a modulação da angiogénese e da inflamação pelos polifenóis, assim como a inter-relação entre estes dois processos. A utilização destes dois modelos permitiu confirmar as propriedades anti-angiogénicas do XN e do IXN. A aplicação tópica destes dois polifenóis na zona de cicatrização resultou na redução do número de vasos sanguíneos, sobretudo a nível dos microvasos, e numa redução da zona de cicatrização. Estes resultados sugerem que o efeito do XN e do IXN ocorre sobretudo sobre os vasos angiogénicos ainda em formação e não sobre os vasos estabilizados. Foi curioso observar que a inibição do crescimento vascular foi bastante mais pronunciada na presença de um forte estímulo angiogénico, o VEGF, como ocorreu nos implantes de Matrigel®, tendo estes polifenóis inibido significativamente o estímulo angiogénico do VEGF. Os resultados observados nos dois modelos animais confirmaram que o XN e o IXN possuem características anti-inflamatórias. A resposta inflamatória é uma consequência inevitável da danificação tecidular. Embora durante muito tempo o processo inflamatório tenha sido considerado indispensável a uma adequada cicatrização e à homeostasia cutânea na danificação celular, a constatação de que a inflamação pode retardar a cicatrização e o estudo de diversas patologias em que ocorrem lesões de difícil cicatrização associadas a um estado de inflamação crónica, como por exemplo na diabetes e insuficiência venosa, sugerem que o controlo da fase inflamatória parece ser crucial na modulação da cicatrização [Eming e colaboradores, 2007b]. A actuação simultânea do XN e do IXN nos processos inflamatório e angiogénico pode ser a chave para uma terapia mais eficaz nas lesões crónicas e num grande número de patologias actuais em que a angiogénese e a inflamação estão desreguladas. As lesões crónicas são também microambientes pró-oxidativos e a excessiva produção de ROS pode, para além de danificar as estruturas celulares, potenciar vias de sinalização pró-inflamatórias [Eming e colaboradores, 2007b]. Embora o stresse oxidativo não tenha sido avaliado neste ensaio, é possível que as características anti-oxidantes do XN e do IXN também tenham permitido uma melhor resolução da cicatrização. Por outro lado, o IXN pareceu

ter um efeito mais pronunciado do que o XN. Dado que as características anti-angiogénicas do IXN não tinham ainda sido devidamente estudadas, estes resultados permitem sugerir que, embora as atenções da comunidade científica e da indústria alimentar estejam centradas no XN, o IXN possa vir a ser considerado, pelas razões expostas, uma molécula com potencial anti-angiogénico valorizável, até porque existe em maior quantidade no plasma e nos tecidos humanos após consumo de alimentos que o contenham, ou que contenham XN [Bolca e colaboradores, 2010].

A 8PN, contrariamente ao que estava descrito [Pepper e colaboradores, 2004], demonstrou características pró-angiogénicas sustentadas, nos ensaios *in vitro* e *in vivo*. Estimulou de um modo bastante marcado a formação de novos capilares durante o processo de cicatrização, ao mesmo tempo que aumentou o estímulo inflamatório, não tendo sido observadas diferenças na formação de vasos sanguíneos nos implantes de Matrigel® quando aplicada em conjunto com o VEGF, comparativamente com os implantes de Matrigel® só com VEGF. Apesar de terem sido já descritos efeitos bifásicos para a 8PN na proliferação e viabilidade de células tumorais [Brunelli e colaboradores, 2007], e embora seja necessário estudar mais profundamente os mecanismos envolvidos, uma possível explicação para os efeitos pró-angiogénicos observados prende-se com o efeito estrogénico atribuído à 8PN [Milligan e colaboradores, 2002; Schaefer e colaboradores, 2003], que é um potente agonista do receptor alfa dos estrogénios [Brunelli e colaboradores, 2007]. De facto, os estrogénios induzem a proliferação das EC, contribuindo para a formação dos vasos sanguíneos [Soares e colaboradores, 2003], o que sugere que a 8PN possa ter efeito pró-angiogénico, por um mecanismo semelhante ao dos estrogénios. Muita investigação tem sido realizada com o intuito de descobrir potenciais agentes anti-angiogénicos na tentativa de combater o cancro, tendo já resultado a aprovação para a utilização terapêutica de alguns fármacos. A terapia pró-angiogénica tem sido, também explorada. A descrição das propriedades pró-angiogénicas *in vitro* e *in vivo* da 8PN reveste-se assim também de um interesse especial dada a possibilidade de este composto poder vir a ser utilizado em patologias com angiogénese deficiente, como na isquemia do miocárdio, na doença arterial periférica e nalgumas patologias neurológicas. De facto, foi já demonstrado que os estrogénios são neuroprotectores e que a via VEGF/Akt/eNOS desempenha um papel importante na angiogénese cerebral, mantendo o fluxo sanguíneo adequado para que os estrogénios possam chegar às diversas regiões cerebrais. Simultaneamente, os receptores alfa e beta dos estrogénios estão também envolvidos na regulação do VEGF [Jesmin e colaboradores,

2010a]. Os estrogénios são ainda cardioprotectores, e actuam via receptor alfa dos estrogénios regulando a transcrição do VEGF e, assim, o desenvolvimento da microvasculatura cardíaca [Jesmin e colaboradores, 2010b]. Deste modo, a possibilidade de a 8PN poder ser utilizada como estimulador da angiogénese em determinadas patologias merece ser explorada. Interessante é também o facto da 8PN ser quimicamente muito semelhante ao IXN, apenas diferindo no C5. A 8PN tem um hidroxilo em C5 e o IXN um substituinte O-metilado. É curioso que a desmetilação transforme um composto com reduzida capacidade estrogénica e características anti-angiogénicas (IXN), num potente fitoestrogénio com características pró-angiogénicas (8PN) [Nikolic e colaboradores, 2005]. De facto polifenóis com estruturas químicas muito semelhantes podem ter efeitos biológicos muito distintos ou mesmo opostos, o que recomenda prudência na utilização indiscriminada de suplementos alimentares ricos em polifenóis.

A indústria alimentar tem demonstrado muito interesse pelo estudo das propriedades bioactivas dos polifenóis dadas as características protectoras e promotoras da saúde que têm sido atribuídas a estes compostos. A possibilidade de produzirem suplementos alimentares ou alimentos fortificados com polifenóis descritos como benéficos surge como uma oportunidade de atrair consumidores informados. A indústria cervejeira tem estado particularmente atenta aos estudos com o XN, tendo testado diversas alterações metodológicas na produção de cerveja, com o intuito de a enriquecer neste polifenol, o que permitiu a criação e comercialização na Alemanha de duas cervejas fortificadas com XN [Xan, 2011]. O crescente interesse pela fortificação alimentar com polifenóis motivou a realização de um estudo em ratos envolvendo o consumo de uma cerveja fortificada com XN, com o intuito de avaliar se o consumo deste tipo de alimento pode ser benéfico em situações associadas a alterações na angiogénese e na inflamação (**Capítulo 3**). Para responder a esta questão foi utilizado o modelo de cicatrização, que permite a avaliação da modulação da angiogénese e da inflamação. Os animais ingeriram cerveja *stout* fortificada com XN ao longo das cinco semanas de estudo, sendo a quantidade diária de cerveja ingerida considerada equivalente a cerca de três cervejas por dia, para um homem adulto. Com o objectivo de verificar eventual toxicidade, dada a elevada concentração de XN na cerveja fortificada (10 mg/L), foram realizadas, no final do ensaio, análises serológicas, que não demonstraram evidência de alterações no funcionamento hepático e do metabolismo glicídico e lipídico, assim como uma melhoria no estado

redox global nos animais que consumiram cerveja *stout* fortificada com XN. Embora a cicatrização seja um processo fisiológico em que ocorre uma regulação recíproca entre a inflamação e a angiogénese, o que resulta normalmente na regeneração do tecido danificado, patologias muito diferentes podem estar na origem do aparecimento de lesões crónicas, difíceis de cicatrizar, como resultado de uma progressão descontrolada das várias fases da cicatrização, desde a inflamação e angiogénese até à formação do tecido de granulação e à reepitelização. De facto, a maior parte das lesões crónicas não progride e permanece na fase inflamatória [Loots e colaboradores, 1998], observando-se elevada infiltração de macrófagos e um perfil inflamatório excessivo como no caso das lesões cutâneas dos doentes diabéticos [Loots e colaboradores, 1998; Rodero e Khosrotehrani, 2010], simultaneamente caracterizadas por baixos níveis de VEGF e uma insuficiente angiogénese. Embora o número de EC nas lesões diabéticas não esteja diminuído, parece que estas não se conseguem organizar em vasos sanguíneos funcionais [Qiao e colaboradores, 2011]. Por outro lado, as úlceras crónicas associadas à insuficiência venosa são caracterizadas não só por um estado pró-inflamatório mas também por uma produção de VEGF excessiva e sustentada, o que origina uma elevada permeabilidade vascular e excessiva transudação na zona da úlcera, resultando na degradação e ruptura vascular [Shoab e colaboradores, 1999]. Uma melhor regulação da fase inflamatória pode assim favorecer a cicatrização de lesões com um perfil inflamatório excessivo, promovendo a transição para a fase de granulação. Nalgumas patologias o aumento da angiogénese pode favorecer a cicatrização, enquanto noutras esta pode ser melhorada por uma diminuição do estímulo angiogénico. Parece óbvio, no entanto, que nem sempre um aumento da angiogénese está relacionado com uma melhor perfusão do tecido danificado e correspondente cicatrização, dado que apenas um processo angiogénico controlado conduz à formação de vasos funcionais. A diminuição dos factores inflamatórios e dos níveis de VEGF no soro dos animais, observada após ingestão de cerveja fortificada com XN, parece ter criado condições que permitiram uma revascularização adequada do tecido danificado. A hipótese de que o XN possa exercer uma fina modulação da fase inflamatória, adequada ao microambiente da área lesada que vai sendo modificado, permitindo uma regulação mais eficaz da angiogénese e promovendo uma melhor cicatrização, é bastante atractiva. De acordo com esta hipótese, o consumo de cerveja *stout*, e sobretudo de cerveja *stout* fortificada com XN, melhorou a cicatrização do tecido danificado. A zona da cicatrização encontrava-se com melhor resolução, apesar do número de vasos formados tender a diminuir, de

acordo com a redução da concentração do VEGF verificada. O estado redox sistémico também melhorou, o que de acordo com o que tinha sido sugerido no capítulo 2, parece estar relacionado com as propriedades anti-oxidantes do XN e pode também ter contribuído para a melhor resolução da cicatrização, dado que as lesões são também microambientes pró-oxidativos, como já referido [Eming e colaboradores, 2007b]. Outra observação interessante realizada no decurso deste estudo foi as diferenças na morfologia do tecido adiposo na zona de cicatrização cutânea, após o consumo de cerveja *stout* e de cerveja *stout* fortificada com XN. O consumo destes dois tipos de cerveja revelou uma tendência para aumentar o número de adipócitos e diminuir a sua área. Não se conhece ainda a função que estes adipócitos desempenham no processo de cicatrização, no entanto a hipertrofia dos adipócitos tem sido associada a estados pró-inflamatórios [Monteiro, 2009; Monteiro e colaboradores, 2006]. Alguns polifenóis têm sido descritos como inibidores competitivos das sulfotransférases dos estrogénios, aumentando a disponibilidade dos estrogénios por inibição da sua metabolização [Harris e colaboradores, 2004; Kirk e colaboradores, 2001; Otake e colaboradores, 2000]. Este mecanismo poderia explicar a alteração observada na morfologia do tecido adiposo, dado que a maior disponibilidade de estrogénios levaria a um aumento da proliferação de pré-adipócitos e da adipogénese, favorecendo uma redistribuição do tecido adiposo [Monteiro, 2007]. A ser confirmado o aumento do número e a redução do tamanho dos adipócitos após o consumo de cerveja fortificada com XN pode traduzir-se em mais um efeito benéfico deste polifenol, que se encontra aliás em concordância com a diminuição dos factores inflamatórios séricos observada nestes animais. Os adipócitos comunicam com EC e células inflamatórias através da produção de várias citocinas que modulam a angiogénese [Cao, 2010], podendo por isso estar envolvidos no processo de cicatrização, por modulação da inflamação e da angiogénese. A possibilidade de o XN induzir um aumento hiperplásico dos adipócitos em vez do seu aumento de tamanho, e as consequências desta modificação, necessita no entanto, de ser mais aprofundada.

Os resultados sugerem que a ingestão de cerveja fortificada com XN exerceu efeitos quer a nível local quer a nível sistémico, diminuindo a resposta inflamatória, favorecendo a resolução da fase inflamatória e conduzindo consequentemente a um processo angiogénico mais controlado, o que melhorou a cicatrização. De acordo com estas observações, o consumo de alimentos fortificados, embora requeira ainda prudência e necessite de ser testado em modelos de lesão patológica, pode vir a ter interesse em

doentes com dificuldades de cicatrização ou mesmo em patologias em que a exacerbação da resposta inflamatória resulta em cicatrizes hipertróficas ou cicatrizes queloides, até porque tem sido descrita para alguns polifenóis a acumulação em locais de excessiva inflamação [Kawai e colaboradores, 2008b]. Apesar da suplementação nutricional com diversos polifenóis começar a atrair a atenção do público, a possibilidade de surgirem efeitos tóxicos não deve ser negligenciada, sendo necessário ponderar bem as doses utilizadas e as diferenças no metabolismo individual, antes da implementação dos polifenóis como aditivos alimentares. A eventual utilização de flavonóides prenilados como agentes terapêuticos farmacológicos moduladoras da angiogénese e da inflamação pode vir a ser interessante.

Embora os flavonóides prenilados apenas existam num grupo restrito de alimentos naturais, outros flavonóides, como os flavanóis, são mais abundantes nos alimentos. No grupo dos flavanóis, a atenção da comunidade científica têm-se concentrado sobretudo na EGCG, que existe em elevadas concentrações no chá verde e para a qual têm sido demonstradas propriedades anti-carcinogénicas, anti-oxidantes e anti-angiogénicas. A catequina tem sido muito menos estudada, dado que parece exercer efeitos biológicos menos pronunciados. No entanto, a catequina encontra-se presente em variadíssimos tipos de alimentos muito comuns como sejam os frutos e bebidas como o chá verde, o vinho tinto e a cerveja. A catequina pode por isso ser ingerida com maior frequência e em maior quantidade, podendo eventualmente acumular-se, e atingir concentrações capazes de desencadear efeitos biológicos. De facto, descrições recentes têm sugerido que é possível ocorrer acumulação de polifenóis em membranas, células e tecidos, após exposição continuada a estes compostos [Bolca e colaboradores, 2010; Kawai e colaboradores, 2008b] e que as catequinas conseguem ligar-se às membranas fosfolipídicas por ligações relativamente fortes [Kajiya e colaboradores, 2002]. Tendo em conta estas considerações, propusemo-nos estudar o efeito da catequina nas células vasculares, avaliar a eficácia da catequina na regulação da angiogénese e da inflamação, e verificar se a modulação destes processos pela catequina variava com diferentes estímulos angiogénicos (**Capítulo 4**).

Para isso utilizou-se inicialmente, como nos casos anteriores, culturas de HUVEC e HASMC e realizaram-se estudos em anéis de aorta. Observou-se que a catequina aumentou a viabilidade das HUVEC e das HASMC, provavelmente por diminuição da apoptose destas células, tendo também diminuído a proliferação dos dois tipos de células. Embora de um modo não significativo, a catequina parece interferir nos processos de migração e invasão celular de um modo diferente, estimulando-os nas

HUVEC e diminuindo-os nas HASMC. De acordo com estes resultados, a catequina, tal como o XN e o IXN, parece ser um polifenol com potencial utilização em patologias associadas a uma excessiva migração e invasão das VSMC, como a reestenose e a aterosclerose. A catequina existe em concentrações muito relevantes no vinho tinto [Manach e colaboradores, 2004; Scalbert e Williamson, 2000], cuja ingestão moderada tem efeito protector das doenças cardiovasculares [Soleas e colaboradores, 1997; Stoclet e colaboradores, 2004], e é provavelmente um dos polifenóis que contribui para este efeito protector [Bell e colaboradores, 2000]. De um modo geral, a catequina não alterou a formação de estruturas capilares em culturas de EC em Matrigel® nem o *sprouting* angiogénico a partir de anéis de aorta. Ao mesmo tempo, a catequina diminuiu dois importantes moduladores do processo inflamatório, o TNFα em ambas as células e o NFκB nas HASMC. O TNFα é uma citocina inflamatória com produção estimulada na fase aguda da inflamação e que está envolvida na inflamação sistémica. O TNFα estimulada o NFκB que regula diversos genes envolvidos na sobrevida e na proliferação celulares, no processo inflamatório e na angiogénese, como o VEGF [Naugler e Karin, 2008].

Estes resultados parecem sugerir que embora a catequina possa desencadear uma resposta anti-inflamatória, não parece alterar nas condições testadas, o processo angiogénico e a relação EC-VSMC, promovendo eventualmente a estabilização das células vasculares.

Na tentativa de esclarecer melhor esta questão, dado que recentemente foi proposto que a catequina tem propriedades anti-angiogénicas [Guruvayoorappan e Kuttan, 2008], e de perceber se a resposta era ou não dependente do estímulo angiogénico, foram realizados ensaios em dois modelos animais, o modelo de cicatrização e o modelo de implantes de Matrigel®. Os resultados observados permitiram concluir que a catequina, de facto, não alterou o processo inflamatório e angiogénico, numa situação fisiológica, o que está de acordo com os resultados obtidos para as culturas de HUVEC em Matrigel® e de anéis de aorta. Surpreendentemente, na presença de um estímulo fortemente angiogénico, como o VEGF, adicionado ao Matrigel® implantado no tecido subcutâneo de ratinho, a catequina reduziu em cerca de 50% o número de vasos formados e ainda mais a actividade da N-acetilglucosaminídase, uma enzima presente em macrófagos activados.

Estes resultados levam-nos a propor que a catequina exerce efeitos distintos na angiogénese, inibindo-a apenas na presença de um forte estímulo angiogénico, como o VEGF. A ausência de efeitos da catequina

na vasculatura madura realça o seu potencial interesse no tratamento de patologias em que ocorre um forte estímulo angiogénico.

Os polifenóis testados parecem exercer os seus efeitos nalguns pontos-chave da modulação da angiogénese: alterando a viabilidade e proliferação das EC e VSMC, alterando a capacidade de diferenciação das EC, desestabilizando ou consolidando a relação EC-VSMC e modulando o processo inflamatório. Foram, por isso, estudadas algumas vias moleculares intervenientes nestes processos e realizados estudos que permitissem esclarecer o efeito destes compostos na própria organização vascular durante o processo angiogénico (**Capítulo 5**).

No Ratinho, a retina é avascular aquando do nascimento e os vasos sanguíneos desenvolvem-se durante as três semanas seguintes, num processo reprodutível temporal e espacialmente [Dorrell e colaboradores, 2002]. Durante a primeira semana é formada uma camada superficial de vasos, que irradiam a partir da artéria central da retina em direcção à periferia da retina, sendo os vasos centrais mais maduros do que os da periferia. Por volta do oitavo dia após o nascimento (P8), os vasos sanguíneos começam a crescer em direcção à camada mais profunda. É durante a terceira semana de desenvolvimento que é formada uma nova camada de vasos por migração de vasos sanguíneos da camada superficial e da camada profunda, em direcção a uma zona intermédia [Dorrell e colaboradores, 2002]. O desenvolvimento da vasculatura da retina é um processo extraordinariamente ordenado e regulado e a formação das várias camadas de vasos sanguíneos que caracterizam a irrigação da retina envolve a interacção de diversos tipos de células como as EC, células da glia, células murais e células neuronais [Fruttiger, 2007; Uemura e colaboradores, 2006]. O *sprouting* vascular é mediado por um gradiente de VEGF libertado pela rede de astrócitos pré-formada, sendo o crescimento do vaso sustentado pelo estabelecimento de ligação entre várias EC e entre as EC e células murais ou células do estroma [Dorrell e Friedlander, 2006; Gariano e Gardner, 2005]. Este modelo de *sprouting* angiogénico, embora tenha particularidades do tecido onde ocorre, é considerado um bom modelo para estudar a angiogénese [Ozduman e colaboradores, 2010; Uemura e colaboradores, 2006]. Como os vasos sanguíneos se desenvolvem após o nascimento, este modelo permite o acesso à manipulação de todo o processo angiogénico, em diversos tempos de acordo com a fase a investigar, e a observação simultânea dos vasos em diversas fases de maturação. Como o desenvolvimento das diversas camadas vasculares obedece a padrões regulares e reprodutíveis, são facilmente identificadas eventuais alterações. É

também um modelo frequentemente utilizado para o estudo de substâncias exógenas, resultante da procura de uma terapia sistémica ou local. Foi por isso utilizado o modelo da angiogénese da retina para avaliar o efeito do polifenol testado na organização vascular e na própria progressão angiogénica. O IXN foi o polifenol escolhido porque demonstrou propriedades anti-inflamatórias e anti-angiogénicas consistentes ao longo dos trabalhos já discutidos e simultaneamente é, dos flavonóides prenilados estudados, o mais abundante após a metabolização humana [Bolca e colaboradores, 2010].

Os estudos de sinalização celular, realizados em culturas de HUVEC e HASMC previamente tratadas com IXN, permitiram verificar que o IXN diminuiu significativamente nas HUVEC a sinalização via Akt, importante na regulação da sobrevivência celular [DeBusk e colaboradores, 2004]. A proliferação da EC está associada ao início da angiogénese e decorre depois à medida que o vaso vai crescendo. A sinalização via Erk1/2, que regula o crescimento, a proliferação e a diferenciação celulares [Chen e colaboradores, 2005; Mojzis e colaboradores, 2008] diminuiu nos dois tipos de células após o tratamento com o IXN. O VEGF regula a viabilidade das EC, a diferenciação das EC nos seus vários fenótipos durante o *sprouting* angiogénico, a proliferação das células *stalk* que reagem à concentração de VEGF na sua proximidade, através da ligação ao VEGFR2 e a migração dos vasos sanguíneos por ligação aos receptores VEGFR2 das células *tip*, sensíveis ao gradiente de concentração de VEGF [De Smet e colaboradores, 2009; Ferrara e colaboradores, 2003; Gerhardt e colaboradores, 2003]. O tratamento com IXN diminuiu a expressão do VEGFR2 nas EC, assim como a expressão de Ang2 e do seu receptor Tie2. A Ang2 é libertada pelas EC na presença de níveis elevados de VEGF ou perante um estímulo inflamatório e promove, por ligação ao Tie2, a desestabilização do vaso sanguíneo e o *sprouting* angiogénico [Fiedler e colaboradores, 2006; Kim e colaboradores, 2000; Kim e colaboradores, 2006]. Estes resultados estão de acordo com os resultados descritos no capítulo 2, que sugeriam que o IXN diminuía a viabilidade, a proliferação e a diferenciação das EC em estruturas semelhantes a capilares, atribuindo a este polifenol características anti-angiogénicas. Observando a retina de ratinhos sujeitos a injecções intra-peritoneais diárias com IXN, verificou-se que durante a primeira semana após o nascimento ocorreu uma diminuição do *sprouting* angiogénico, sendo visível o aumento do raio avascular da camada superficial da retina, diminuindo o comprimento quer das artérias quer das veias formadas ao longo desta camada de vasos. Observou-se ainda uma tendência para diminuir o número de células *tip* e de *filopodia*. As alterações observadas no *sprouting* angiogénico da retina após o

tratamento com IXN confirmaram as propriedades anti-angiogénicas do IXN verificadas por alteração da viabilidade, proliferação e diferenciação das EC e estão de acordo com a diminuição da expressão de p-Akt, p-Erk1/2, VEGFR2, Ang2 e Tie2. Outro ponto sensível à acção deste polifenol parecia ser a ligação EC-VSMC, que permite a estabilização do vaso sanguíneo [Gerhardt e Betsholtz, 2003; Hughes e colaboradores, 2006]. Para além dos efeitos já descritos na expressão da p-Akt e p-Erk1/2 nas EC, verificou-se que o IXN, embora não tenha alterado a expressão de p-Akt nas VSMC, diminuiu a expressão de p-Erk1/2 nestas células e diminuiu também a expressão de Ang1. A Ang1, por ligação ao Tie2 expresso nas EC, promove a ligação VSMC-EC e a estabilização e maturação do vaso sanguíneo [Fukuhara e colaboradores, 2009; Zhang e colaboradores, 2011], podendo ainda durante o primeiro mês de desenvolvimento intervir no aumento do diâmetro vascular [Thurston e colaboradores, 2005]. Em sintonia com estes resultados, observou-se nas retinas de ratinhos tratados com IXN uma diminuição da cobertura dos vasos com células murais e uma diminuição do diâmetro das artérias já formadas, que podem ser explicadas pela diminuição da proliferação das células murais e da sua capacidade de expressar a Ang1. No entanto, curiosamente, quer no caso da diminuição do *sprouting* angiogénico, quer no caso da diminuição da cobertura dos vasos com células murais o processo parece apenas ser retardado, uma vez que há visivelmente reversão dos efeitos observados durante a primeira semana, no final da segunda semana de desenvolvimento. Embora não exploradas durante este trabalho, são conhecidas as características anti-oxidantes dos polifenóis em geral e do IXN em particular [Gerhauser e colaboradores, 2002]. A retina é um tecido metabolicamente muito activo, com abundância em ácidos gordos polinsaturados nas membranas celulares e grande exposição à luz, sendo por isso vulnerável às ROS [Kalt e colaboradores, 2010; Rhone e Basu, 2008]. Por outro lado, o *sprouting* angiogénico e a formação das várias camadas vasculares na retina são processos energeticamente desfavoráveis e geradores de ROS, que parecem diminuir a vascularização, sendo possível que o IXN como anti-oxidante favoreça o processo e actue nesta fase como pró-angiogénico, revertendo os efeitos observados durante a primeira semana [Uno e colaboradores, 2010]. Foi também interessante verificar que os vasos já estabelecidos inicialmente, na parte central da retina, não foram afectados pelo efeito do IXN, o que reforça a ideia de que o IXN poderá afectar os vasos ainda imaturos, angiogénicos, sem afectar a vasculatura já estabelecida. Esta observação pode ser de uma enorme relevância, tendo em conta a eventual utilização deste polifenol no tratamento de patologias em que a angiogénese se encontre

excessivamente estimulada. O último ponto que se pretendeu avaliar, como alvo de acção deste polifenol, foi a modulação do processo inflamatório. O IXN diminuiu a expressão de TNFα e de NFκB, nas EC e nas VSMC, o que explica não só as características anti-inflamatórias observadas anteriormente para o IXN, mas também alguns dos efeitos observados nas retinas dos animais tratados com IXN. De facto, foi já referido que a angiogénese e a inflamação são processos intrinsecamente relacionados e que surgem associados em diversas patologias. A cooperação entre as células inflamatórias e a angiogénese na retina é extraordinariamente evidente, o que realça a importância da utilização deste modelo quando se pretende estudar os dois processos. Nas retinas dos animais tratados com IXN observou-se uma redução do *sprouting* angiogénico, como previamente referido. Tem sido descrito que os macrófagos e a microglia produzem factores solúveis estimuladores do s*prouting* angiogénico [Fantin e colaboradores, 2010; Rymo e colaboradores, 2011]. A diminuição do estímulo inflamatório pode por isso comprometer este processo. O NFκB promove a expressão de genes pró-inflamatórios como o da molécula de adesão intercelular 1 (ICAM-1) e estimula a secreção de Ang2 [Imhof e Aurrand-Lions, 2006]. Durante a fase do *pruning* angiogénico, ocorre a apoptose controlada das EC mediada pela adesão de leucócitos activados ao endotélio através da ICAM-1 presente nas EC [Ishida e colaboradores, 2003]. O tratamento com IXN diminuiu também o *pruning* vascular durante a angiogénese da retina, conduzindo à formação de uma rede vascular mais desorganizada e menos diferenciada, de acordo com a diminuição do TNFα e de NFκB. Um estudo recente [Fantin e colaboradores, 2010] veio acrescentar mais um ponto de ligação entre o processo inflamatório e angiogénico. Segundo os autores, numa situação de hipóxia os macrófagos residentes nos tecidos são conduzidos até à proximidade das células *tip*, libertando moléculas que alteram a diferenciação das EC, preparando-as para a fusão e servindo assim de ponte para a anastomose de dois vasos em formação. De acordo com esta hipótese, observou-se na retina a redução de células da microglia na zona de fusão entre duas células *tip*, após tratamento com o IXN. A angiogénese na retina tem especificidades próprias do tecido, e a angiogénese fisiológica ou patológica são contextualmente diferentes, por isso os resultados devem ser interpretados com prudência, até porque as vias moleculares foram estudadas em culturas isoladas de EC e de VSMC, onde os efeitos parácrinos não foram avaliados [Sandhu e colaboradores, 2004]. No entanto, dada a consistência dos resultados obtidos ao longo de todo o trabalho, mesmo em modelos diferentes, parece possível especular que o IXN diminuiu o crescimento vascular, o *sprouting* angiogénico e a estabilização

dos vasos sanguíneos, provavelmente por uma diminuição da expressão de VEGFR2, da modulação do sistema-Ang-Tie2 e por redução da sinalização via Akt e Erk nas células vasculares. O IXN também diminuiu o NFκB e o TNFα em culturas, demonstrando propriedades anti-inflamatórias que parecem consolidar as propriedades anti-angiogénicas do IXN. A actual terapia com factores anti-angiogénicos, como os anticorpos anti-VEGF, levanta problemas devido aos efeitos secundários que desenvolve como a hipertensão e a interferência com a revascularização do miocárdio em doentes com risco de isquemia cardíaca. A perda dos efeitos neurotróficos e vasculotrópicos do VEGF pode também exacerbar a perda neuronal e a isquemia no olho de doentes diabéticos, por exemplo. Alguns dos fármacos inibidores do VEGF, já validados terapeuticamente, parecem poder induzir uma adaptação do tumor conduzindo à sua progressão e a uma maior malignidade, aumentando a sua capacidade invasiva e de metastização. Assim, a utilização de compostos que combatam a natureza multifactorial de diversas patologias, poderia ter mais capacidade de sucesso, do que a utilização de fármacos que envolvem apenas um alvo. Nesse sentido, o IXN parece ser uma molécula com potencial interesse.

Apesar de muitos estudos sugerirem que os polifenóis contribuem para a manutenção da saúde e prevenção de patologia no indivíduo adulto e durante o envelhecimento [Araújo e colaboradores, 2011; Dell'Agli e colaboradores, 2004; Faria e colaboradores, 2011; Gerhauser, 2005; Holst e Williamson, 2008; Keating e colaboradores, 2009; Monteiro, 2007; Rossi e colaboradores, 2008], a baixa biodisponibilidade atribuída de um modo geral aos polifenóis e a dificuldade de associar no Homem um efeito protector directamente a um composto têm levantado dúvidas sobre a sua eficácia [Halliwell, 2007; Kay, 2010]. No entanto, descrições recentes parecem sugerir que os polifenóis podem atingir concentrações *in vivo* capazes de desencadear respostas biológicas (**capítulo 6**).

Apesar de todos os benefícios atribuídos a estes compostos, os polifenóis são xenobióticos, não se devendo desprezar a sua eventual toxicidade. De acordo com este pensamento, têm recentemente sido atribuídas aos polifenóis propriedades horméticas, uma vez que parecem exercer efeitos benéficos a baixas concentrações, estimulando os sistemas protectores celulares e reforçando os mecanismos de defesa, enquanto que em concentrações mais elevadas são citotóxicos [Fraga e colaboradores, 2010; Gems e Partridge, 2008; Holst e Williamson, 2008; Lambert e colaboradores, 2007; Murado e Vazquez, 2007; Tosetti e colaboradores, 2009]. As propriedades horméticas dos polifenóis podem ter especial relevância na homeostasia vascular [Siow e Mann, 2010].

Os polifenóis são absorvidos a nível intestinal, tendo sido identificados no plasma e em diversos órgãos [Manach e colaboradores, 2004]. A acumulação nos órgãos e tecidos e os tempos de semi-vida plasmática parecem variar com o tipo de polifenol e não apenas com a quantidade ingerida, variando também com a espécie animal e entre indivíduos da mesma espécie [Khan e Mukhtar, 2007; Possemiers e colaboradores, 2007]. Tem ainda sido referida por diversos investigadores a importância do contexto nutricional em que os polifenóis são ingeridos para o seu efeito biológico. Alguns efeitos observados após a administração isolada de um polifenol podem ser significativamente diferentes do efeito associado à ingestão desse mesmo polifenol, no seu contexto alimentar habitual, em que são ingeridos simultaneamente outros polifenóis ou nutrientes [Scholz e Williamson, 2007].

Apesar da concentração atingida pelos polifenóis no sangue e nos tecidos ser bastante baixa, tem sido proposto que a concentração de polifenóis em determinadas estruturas celulares ou tecidos do organismo atinja valores relativamente elevados, o que pode explicar a sua bioactividade [Han e colaboradores, 2006; Kawai e colaboradores, 2008b; Terao e Piskula, 1999; Wright e colaboradores, 2010]. A produção recente de anticorpos capazes de reagirem com alguns polifenóis ou seus metabolitos [Kawai e colaboradores, 2008a; Wyns e colaboradores, 2011] tem sido particularmente importante na identificação das estruturas celulares às quais os polifenóis se conseguem ligar e consequentemente em estudos sobre a distribuição destes compostos. O polifenol resveratrol liga-se a locais específicos na epiderme, provavelmente um receptor celular, bloqueando mecanismos intracelulares reguladores da apoptose e prevenindo o envelhecimento da pele, num processo que é independente da sua actividade sequestradora de ROS [Bastianetto e colaboradores, 2010]. Foi também descrito que este polifenol se liga a locais específicos da membrana plasmática de células de cérebro de Rato. Estes locais de ligação são de natureza proteica, saturáveis, e diferentes polifenóis têm afinidades diferentes para este local de ligação, de acordo com o efeito neuroprotector do composto [Han e colaboradores, 2006]. As procianidinas, outro tipo de polifenóis, também interagem com proteínas da membrana celular e com zonas membranares ricas em colesterol, alterando as características da membrana celular e regulando a sinalização celular [Fraga e Oteiza, 2011]. Os polifenóis e seus metabolitos permanecem no sistema circulatório durante tempo suficiente para que possam interagir com as células da corrente sanguínea. Foi sugerido pelo grupo de Junji Terao que os macrófagos activados são um potencial alvo dos polifenóis na aorta dado que, após ingestão oral, metabolitos do

polifenol quercetina acumulam-se nas placas ateroscleróticas humanas, mas não em aortas saudáveis, associando-se especialmente a macrófagos que constituem as células esponjosas [Kawai e colaboradores, 2008a; Kawai e colaboradores, 2008b]. Os polifenóis podem assim acumular-se neste tipo de células e locais em concentrações relativamente superiores às que são geralmente assumidas para estes compostos, temdo por base as concentrações plasmáticas. Um estudo recente demonstrou que a quercetina é internalizada e metabolizada, enzimaticamente, nas plaquetas, num derivado O-metilado [Wright e colaboradores, 2010]. As plaquetas parecem proteger os tumores da eliminação imunológica no sistema circulatório, promovendo a adesão do tumor aos vasos, a proliferação das células tumorais e a metastização tumoral através da corrente sanguínea [Gay e Felding-Habermann, 2011], constituindo interessantes alvos na terapia tumoral e anti-metastização. De acordo com estas evidências, é tentador propor que a acumulação dos polifenóis nas plaquetas em circulação pode ser um dos mecanismos pelos quais os polifenóis actuam como anti-angiogénicos e anti-carcinogénicos, podendo ser libertados aquando da activação das plaquetas e diminuir a viabilidade, a proliferação e a invasão das células tumorais no local, aumentando a sua apoptose. Este efeito poderia ainda ser potenciado pelas propriedades anti-inflamatórias e anti-angiogénicas que os polifenóis manifestam. Se assim fosse, a acumulação dos polifenóis nas plaquetas protegeria da metastização tumoral, à semelhança de agentes anti-microbianos actualmente utilizados na terapêutica, como os macrólidos, que se concentram nos neutrófilos e que são depois libertados na sua forma activa no local da infecção [Mandell e Coleman, 2001]. As plaquetas interagem com EC e VSMC e o seu papel na disfunção endotelial também é conhecido. As plaquetas induzem a contracção das VSMC e aumentam a sua migração e proliferação, estimulam a acumulação de leucócitos e deste modo o desenvolvimento de placas de ateroma. Deste modo a acumulação e metabolização de polifenóis nas plaquetas poderia também proteger da disfunção endotelial e da aterosclerose. Não é ainda conhecido se as células que constituem os vasos sanguíneos são capazes de metabolizar ou acumular polifenóis, mas a possibilidade de os polifenóis poderem de facto acumular-se nas células que circulam na corrente sanguínea é por si só aliciante. O esclarecimento desta hipótese não só ajudaria a compreender melhor algumas propriedades atribuídas aos polifenóis, como as propriedades anti-angiogénicas e anti-inflamatórias, como poderia permitir utilizar esta via como estratégia terapêutica.

CONCLUSÕES E PERSPECTIVAS FUTURAS

Da análise global dos resultados obtidos neste trabalho, acima discutidos, podem ser retiradas as seguintes conclusões:

1- O XN possui características anti-angiogénicas e anti-inflamatórias *in vitro*, diminuindo a viabilidade, a proliferação e a capacidade invasiva das EC e das VSMC.
   O IXN apresentou efeitos *in vitro* semelhantes ao XN, nos dois tipos de células.
   O efeito do IXN parece ser mais pronunciado nas EC e o do XN nas VSMC.
2- As propriedades anti-angiogénicas e anti-inflamatórias do XN e do IXN foram confirmadas *in vivo* com o modelo de implante de Matrigel® e no modelo de cicatrização. O efeito destes polifenóis parece ser sobretudo sobre os vasos angiogénicos e não sobre os vasos estabilizados.
3- A 8PN manifestou características pró-angiogénicas nos ensaios *in vitro*, aumentando a viabilidade e a proliferação das células endoteliais, diminuindo a sua apoptose, e promovendo a formação de estruturas semelhantes a capilares em culturas de EC em Matrigel®. *In vivo* a 8PN parece estimular a formação de novos capilares assim como o estado inflamatório durante o processo de cicatrização.
4- A ingestão de cerveja fortificada com XN num modelo animal exerceu efeitos quer a nível local quer a nível sistémico, favorecendo a resolução da fase inflamatória durante o processo de cicatrização, e conduzindo consequentemente a um processo angiogénico mais controlado, o que melhorou a cicatrização.
5- A catequina exerceu efeitos distintos na angiogénese, inibindo-a apenas na presença de um forte estímulo angiogénico, como o VEGF, sem afectar a vasculatura pré-existente.
6- O IXN diminuiu o crescimento vascular, o *sprouting* angiogénico e a estabilização dos vasos sanguíneos, provavelmente por uma diminuição da expressão de VEGFR2, da modulação do sistema-Ang-Tie2 e por redução da sinalização via Akt e Erk nas células vasculares. O IXN também diminuiu o NFκB e o TNFα em culturas celulares de EC e de VSMC, demonstrando propriedades anti-inflamatórias que parecem consolidar as propriedades anti-angiogénicas do IXN.

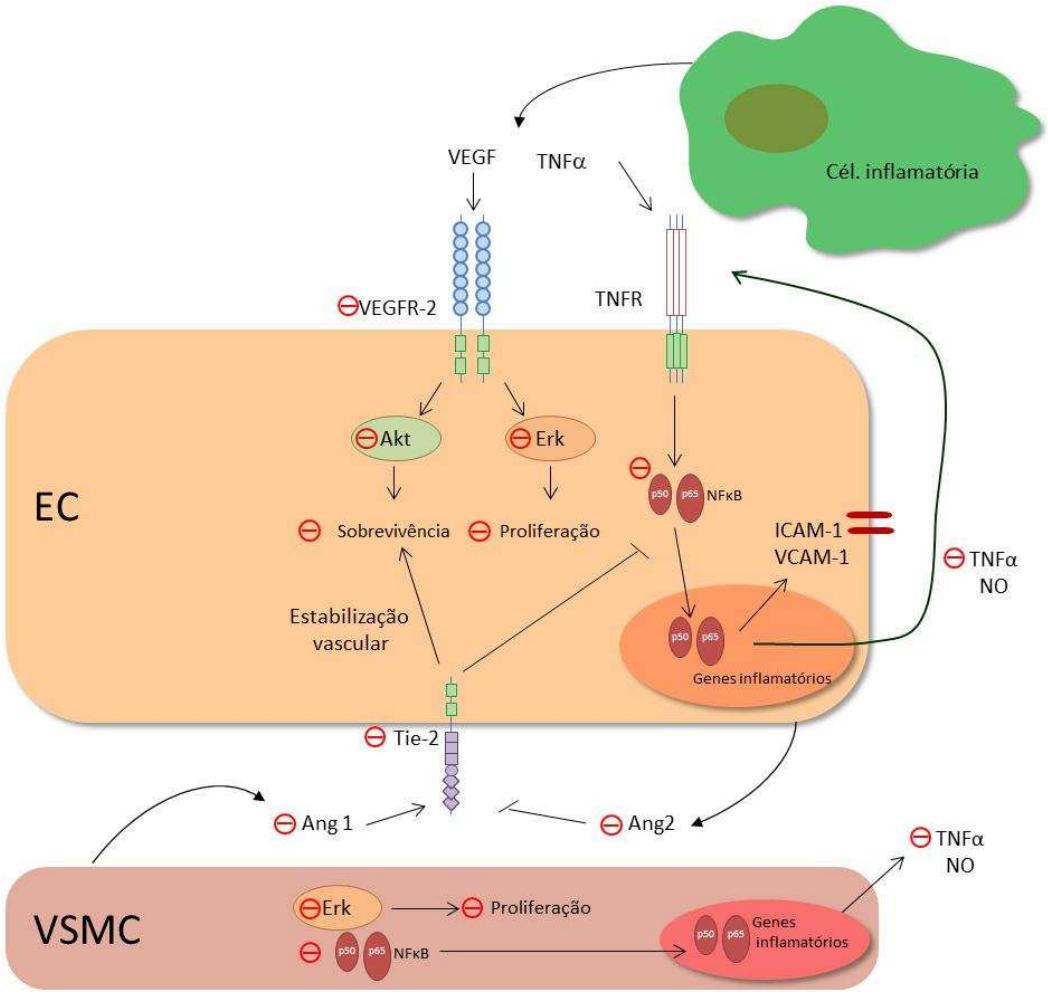

Figura 6 – Representação esquemática dos mecanismos propostos de intervenção do isoxanto-humol (IXN) na modulação dos processos angiogénico e inflamatório. ⊖ Inibição pelo IXN.

Apesar da baixa biodisponibilidade atribuída de um modo geral aos polifenóis, descrições recentes parecerem sugerir que os polifenóis podem atingir concentrações *in vivo* capazes de desencadear respostas biológicas, sendo acumulados e metabolizados em determinadas células como os macrófagos e as plaquetas.

Esta possibilidade vem fortalecer a nossa hipótese de que os polifenóis com propriedades anti-inflamatórias, nomeadamente o IXN, possam ir modulando os microambientes celulares na sua proximidade, restringindo a inflamação e proporcionando uma melhor regulação do processo angiogénico, na medida em que esta regulação pode ir sendo afinada, à medida que o microambiente celular se vá alterando.

A actuação simultânea destes polifenóis nos processos inflamatório e angiogénico pode ser a chave para uma terapia mais eficaz nas lesões crónicas e num grande número de patologias actuais em que a angiogénese e a inflamação estão desreguladas.

Foi ainda possível constatar que polifenóis com estruturas químicas muito semelhantes podem ter efeitos biológicos muito distintos ou mesmo opostos, o que recomenda prudência na utilização indiscriminada de suplementos alimentares ricos em polifenóis.

Como sempre acontece em qualquer trabalho científico, muito ficou ainda por fazer.

De modo a continuar o objectivo a que nos propusemos de estudar a modulação da angiogénese e inflamação por polifenóis, seria agora interessante confirmar *in vivo* a modulação pelo IXN das vias moleculares para as quais se observaram alterações da sinalização em culturas de células vasculares, assim como confirmar os locais específicos de regulação resultantes da acção do IXN. Pretendemos ainda aprofundar o efeito pró-angiogenico da 8PN e verificar se esse efeito é ou não dependente dos estrogénios. Os resultados obtidos com a 8PN podem revestir-se de particular interesse em patologias caracterizadas por um défice angiogénico. A alteração da relação estabelecida entre as EC e as VSMC é um dos primeiros efeitos observados nos vasos de diversas patologias como na retinopatia diabética e nalgumas patologias associadas à degradação neurovascular. No cancro tem também sido sugerido que uma melhoria da ligação EC-VSMC poderia diminuir a metastização tumoral. Por isso, o estudo da modulação pelos polifenóis dos factores e vias moleculares envolvidas nesta ligação merece ser mais aprofundado.

Finalmente, a confirmação do efeito dos polifenóis na inflamação e angiogénese em modelos animais de patologia, como por exemplo em animais diabéticos é imperativo para dar consistência aos resultados agora obtidos.

O conhecimento mais aprofundado da interacção dos diversos polifenóis com os componentes celulares e suas consequências biológicas contribuirá seguramente para o desenvolvimento de estratégias nutricionais e farmacológicas orientadas no sentido da promoção da saúde e da prevenção da doença.

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RESUMO

A angiogénese é um processo fundamental durante o desenvolvimento do organismo e, apesar de, em geral, quiescente no adulto, ocorre em situações fisiológicas como o ciclo reprodutivo na mulher ou a cicatrização. É um processo complexo, que envolve a interacção entre diferentes tipos de células como as células endoteliais, as células murais, as células inflamatórias e as células do estroma. É regulada pelo balanço entre factores activadores e inibidores deste processo podendo, em determinadas situações patológicas, estar desregulado, como na aterosclerose, no cancro, na diabetes, na isquemia do miocárdio. A inflamação crónica e a angiogénese são dois processos que ocorrem muitas vezes associados em várias destas patologias e evidências cada vez maiores sugerem que a inflamação é um estimulador de diversas fases do processo angiogénico. A ingestão de uma dieta rica em polifenóis diminui a incidência de patologias como as doenças cardiovasculares, o cancro, a diabetes tipo 2 e as doenças neurodegenerativas. Os polifenóis manifestam uma actividade biológica vasta, incluindo propriedades anti-oxidantes, anti-inflamatórias, anti-tumorais e anti-angiogénicas.

Neste trabalho estudou-se a acção de polifenóis sobre as células endoteliais e de músculo liso vascular, de modo a compreender como os polifenóis actuam nestas células e regulam a angiogénese e a inflamação. Foram também utilizados modelos animais com o intuito de compreender as repercussões da possível modulação destes processos em condições fisiopatológicas.

O xanto-humol diminuiu, de um modo geral, a viabilidade, a proliferação e a capacidade invasiva das células endoteliais e de músculo liso vascular, e aumentou a apoptose dos dois tipos de células. Este polifenol diminuiu ainda a formação de estruturas capilares na presença de Matrigel®. O tratamento com xanto-humol pareceu afectar os vasos angiogénicos mais imaturos, sugerindo um efeito protector da estrutura vascular já estabelecida. Resultados muito semelhantes foram obtidos com o isoxanto-humol. Curiosamente, o efeito do isoxanto-humol tendeu a ser mais pronunciado nas células endoteliais, enquanto o xanto-humol pareceu actuar mais eficazmente nas células de músculo liso vascular. As propriedades anti-angiogénicas e anti-inflamatórias destes polifenóis foram confirmadas *in vivo* no modelo de implante de Matrigel® e no modelo de cicatrização. A aplicação tópica destes dois polifenóis na zona de cicatrização resultou na redução do número de vasos sanguíneos, sobretudo a nível dos microvasos, e numa redução da zona de cicatrização, sugerindo que o efeito destes compostos é sobretudo sobre os vasos angiogénicos ainda em formação e não sobre os vasos estabilizados.

Os resultados obtidos com a 8-prenilnaringenina pelo contrário demonstraram que este polifenol possui características pró-angiogénicas sustentadas, nos ensaios *in vitro* e *in vivo*. A 8-prenilnaringenina

aumentou a viabilidade e a proliferação das células endoteliais e diminuiu a sua apoptose, ao mesmo tempo que estimulou a formação de estruturas tubulares de células endoteliais em Matrigel® e aumentou a formação de novos capilares e o estímulo inflamatório durante o processo de cicatrização. Numa outra abordagem, foi testado o efeito de um alimento fortificado com xanto-humol no modelo de cicatrização. Os resultados sugerem que a ingestão de cerveja fortificada com xanto-humol exerceu efeitos quer a nível local quer a nível sistémico, diminuindo a resposta inflamatória, favorecendo a resolução da fase inflamatória durante o processo de cicatrização, e conduzindo consequentemente a um processo angiogénico mais controlado, o que melhorou a cicatrização.

A catequina, outro polifenol mais abundante a nível alimentar exerceu efeitos distintos na angiogénese, inibindo-a apenas na presença de um forte estímulo angiogénico, como o VEGF. A ausência de efeitos observada nos tratamentos com catequina na vasculatura madura realça o seu potencial interesse no tratamento de patologias em que ocorre um forte estímulo angiogénico.

A utilização do modelo de *sprouting* angiogénico da retina e de culturas de células endoteliais e de músculo liso vascular permitiram ainda constatar que o isoxanto-humol diminuiu o crescimento vascular, o *sprouting* angiogénico e a estabilização dos vasos sanguíneos, provavelmente por uma diminuição da expressão de VEGFR2, da modulação do sistema-Ang-Tie2 e por redução da sinalização via Akt e Erk nas células vasculares. O isoxanto-humol também diminuiu o NFκB e o TNFα em culturas celulares de células endoteliais e de músculo liso vascular, possuindo propriedades anti-inflamatórias que parecem consolidar as suas propriedades anti-angiogénicas.

Apesar da baixa biodisponibilidade atribuída de um modo geral aos polifenóis, descrições recentes parecerem sugerir que os polifenóis podem atingir concentrações *in vivo* capazes de desencadear respostas biológicas, sendo acumulados e metabolizados em determinadas células como os macrófagos e as plaquetas. As propriedades anti-angiogénicas e anti-inflamatórias demonstradas ao longo deste trabalho para o xanto-humol e isoxanto-humol e para a catequina em situações de forte estimulação pelo VEGF, assim como as características pró-angiogénicas da 8-prenilnaringenina, podem sustentar um aconselhamento nutricional ou constituir um alvo terapêutico/preventivo muito interessante em patologias em que a angiogénese e a inflamação se encontram desreguladas.

ABSTRACT

Angiogenesis is a crucial process in development and in adult physiological events such as female reproductive cycle or wound healing. It is a complex multi-step process which involves multiple cell-to-cell interactions among endothelial, mural, inflammatory and stromal cells. The dynamic balance between pro- and anti-angiogenic factors underlies the strict regulation of all angiogenic processes. The loss of this balance is believed to be responsible for the development of several pathologies, such as atherosclerosis, cancer, diabetes and myocardial ischemia, or their associated complications. Angiogenesis and chronic inflammation are interdependent in several of these pathologies and a great amount of evidence suggests that inflammation acts as a stimulator of several stages of the angiogenic process.

It is known that polyphenol-rich diet consumption reduces the incidence of cardiovascular diseases, cancer, type 2 diabetes and neurodegenerative diseases. Polyphenols are indeed bioactive compounds which have shown to possess anti-oxidant, anti-inflammatory, anti-cancer and anti-angiogenic properties.

In this work we studied the effect of polyphenols on endothelial and vascular smooth muscular cells, in order to unravel the molecular mechanisms for polyphenol action, particularly in what concerns the regulation of angiogenesis and inflammation. We also studied the impact of the modulation of these processes in physiopathological conditions, using experimental animal models.

Xanthohumol was able to reduce endothelial and vascular smooth muscle cell viability, proliferation, invasive capacity and to stimulate apoptosis in both cell types. This polyphenol also reduced capillary-like structure formation in the presence of Matrigel®. Xanthohumol exposure seemed to affect the more immature angiogenic vessels, suggesting a protective effect over previously established vascular structures. Similar results were obtained with isoxanthohumol. Curiously, this polyphenol seemed to be more effective on endothelial cells, whereas xanthohumol seemed to be more effective on vascular smooth muscle cells.

The observed anti-angiogenic and anti-inflammatory effects of xanthohumol and isoxanthohumol were further confirmed *in vivo* using the Matrigel® plug and the wound healing models. Topical administration of either polyphenols on the healing area produced a reduction in the number of blood vessels, particularly at the microvessel level, and a reduction in the total area of healing, suggesting that xanthohumol and isoxanthohumol act mainly upon newly forming angiogenic vessels and not upon stabilized ones.

Contrary to xanthohumol and isoxanthohumol, 8-prenylnaringenin exerted a sustained pro-angiogenic activity both *in vitro* and *in vivo*. It increased the viability and proliferation and decreased apoptosis of endothelial cells. Simultaneously it stimulated the organization of endothelial cells in tubular structures, over Matrigel®, and enhanced both new capillary formation and the pro-inflammatory stimulus during the wound healing process.

In another context, we also tested the effects of a xanthohumol-enriched diet using the wound healing experimental model. Our results suggest that the ingestion of xanthohumol-fortified beer exerted both local and systemic effects, namely decreasing inflammatory response, facilitating inflammatory phase resolution during wound healing thus leading to a strictly controlled angiogenic process that improved healing.

Catechin, a common polyphenol in human diet, had distinct effects on angiogenesis, inhibiting it only when a strong angiogenic stimulus like VEFG was applied. The lack of effect observed when mature vasculature was treated with catechin highlights its potential interest in the treatment of pathologies where a strong angiogenic stimulus exists.

The retina *sprouting* angiogenic model, endothelial cell culture and vascular smooth muscle cell culture allowed to observe that isoxanthohumol decreased vascular growth, the angiogenic sprouting and the boll vessel stabilization. These effects may be due to a decrease of VEGFR2 expression, to a modulation of the Ang-Tie2 system and/or by a signaling reduction of Akt and Erk pathways in vascular cells. Isoxanto-humol also decreased NFκB and TNFα in endothelial and vascular smooth cells, showing anti-inflammatory properties which seems to reinforce the anti-angiogenic properties of this polyphenol.

Despite the low bioavailability attributed to polyphenols in general, recent works suggest that these compounds can achieve *in vivo* concentrations able to trigger biological responses, and that they could be accumulated and metabolized in some cells like macrophages and platelets. The anti-angiogenic and anti-inflammatory properties of xanthohumol, isoxanthohumol and catechin in a situation of strong stimulus by VEGF, as well as the pro-angiogenic characteristics of 8-prenylnaringenin may support nutritional recommendations or may constitute an interesting therapeutic/preventive target in pathologies where angiogenesis and inflammation are deregulated.

“A pedra não tem esperança de ser outra coisa que não pedra.
Mas ao colaborar, ela congrega-se e torna-se templo.”
Antoine de Saint-exupéry, in Cidadela

“Ainda que de abóbadas imperfeitas, porque livres.”
Ruy Belo