MESTRADO EM ONCOLOGIA

ONCOLOGIA LABORATORIAL

In vitro actions of bleomycin on human sperm function and the underlying mechanism of any effects

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M

2023

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

M.ICBAS **2023**

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In vitro actions of bleomycin on human sperm function and the underlying mechanism of any effects

Dissertação de Candidatura ao grau de Mestre em Oncologia (Especialização: Oncologia Laboratorial) submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Financial Support

Ana Beatriz Lobo de Almeida is supported by the 2023 Oncology Research Grant of Liga Portuguesa Contra o Cancro – Núcleo Regional do Norte and the present Master Thesis is included in "*BOAST project: In vitro actions of bleomycin on human sperm function and the underlying mechanism of any effects"*.

Agradecimentos / Acknowledgments

Este trabalho não é só meu, é nosso. É de todos os que, ao longo da vida, se foram cruzando comigo e com os quais eu aprendi. É daqueles que, mesmo sem qualquer envolvimento direto no meu trabalho ajudaram-me a nível pessoal a suportar todos os obstáculos e a festejar todas as conquistas. E é essencialmente de quem acompanhou diariamente esta jornada, este meu enamorar pelo mundo da ciência, o meu fascínio crescente por uma área que eu fui descobrindo por "*portas e travessas*". Desta forma, a eles devo o meu profundo agradecimento. Obrigada!

Em primeiro lugar, e de um modo muito especial, agradeço à minha orientadora Professora Doutora Rosália Sá, com quem tive longas conversas, que me abriu as portas e “me deu a mão” para que eu explorasse este mundo da Oncofertilidade, pelo qual me tenho vindo a apaixonar cada dia mais. Obrigada por todas as oportunidades que me proporcionou, pelos ensinamentos, por todo o apoio e disponibilidade e por me inspirar com a sua ambição e “mente agitada” a querer sempre ver mais além e fazer mais. Muito obrigada, professora!

Agradeço também ao Professor Doutor Mário Sousa, meu coorientador, Diretor do Laboratório de Biologia Celular do Departamento de Microscopia e Group Leader do Biology and Genetics of Reproduction-Molecular and Celular Medicine Group da UMIB, a quem há ano e meio contei os meus objetivos e sonhos e que acabou por me aceitar de braços abertos. Obrigada por ter permitido que eu cumprisse alguns destes sonhos e criasse muitos mais!

Em termos institucionais, agradeço ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto (ICBAS-UP), ao Instituto Português de Oncologia (IPO) do Porto e ao Mestrado em Oncologia, na pessoa da Professora Doutora Carmen Jerónimo, por me terem acolhido e apoiado nestes dois últimos anos. Agradeço também à Unidade de Multidisciplinar de Investigação Biomédica (UMIB) e ao Centro de Genética da Reprodução Prof. Alberto Barros, nas pessoas do Professor Doutor Alberto Barros, diretor, e da Dra. Ana Gonçalves, sem os quais este projeto não seria possível, obrigada por todo o apoio prestado.

Um agradecimento especial é endereçado também ao Liga Portuguesa Contra o Cancro-Núcleo Regional do Norte da (LPCC-NRN), por ter acreditado no nosso projeto e me ter concedido uma das Bolsas de Investigação em Oncologia 2023, a qual me proporcionou tantas e variadas oportunidades que me fizeram crescer como investigadora, pessoa e cidadã deste mundo comum.

Agradeço ao Laboratório de Biologia Celular do Departamento de Microscopia do ICBAS que foi a minha casa durante este período, nas pessoas da Ângela e da Elsa, que

desde o início me mostraram os cantos à casa. Obrigada pela ajuda, palavras amigas e por partilharam comigo muitos ensinamentos. E não posso deixar de agradecer também a todo o Departamento de Microscopia, com um particular agradecimento à Paula Teixeira que desde o primeiro dia me recebeu tão prontamente a mim e aos meus pedidos, e sempre se mostrou disponível a ajudar, e à Doutora Célia Lopes, por toda a simpatia e disponibilidade que sempre teve para me ensinar, ajudar e esclarecer todas as minhas dúvidas.

Agradeço também às minhas companhias diárias que trazem ao laboratório, a alegria e o conforto que qualquer casa precisa, os meus colegas de laboratório, que se tornaram amigos! Ao Joca e ao Gabriel, pela companhia e por se tornarem bons ouvintes dos meus desabafos. À Catarina, a minha compincha preferida, com quem partilhei os meus dias bons e menos bons, as minhas conquistas e stresses, obrigada pela boa-disposição e sinceridade e essencialmente por estares sempre ali, ao meu lado. E à Margarida, minha companheira matutina que garantia sempre que o meu dia começava da melhor forma, obrigada por toda a tua genuinidade e preocupação e por ouvires os meus desabafos sempre com uma palavra de confiança.

Não poderia faltar um agradecimento aos meus amigos, àqueles de uma vida que me conhecem como ninguém e que serão sempre o meu lugar seguro e a todos aqueles que se foram juntando, aqueles que o Porto me deu e que sei que não me largam, nunca.

O agradecimento mais profundo vai para a minha família, o meu núcleo duro, a minha raiz. São ninho e conforto. São as mãos que aplaudem as minhas conquistas, mas também aquelas que me ajudam a levantar, que me empurram para novos desafios e que apertam as minhas com força para me dar confiança para enfrentar os meus medos. Obrigada, nunca será suficiente para agradecer a importância que têm na minha vida e na Mulher que me tornei.

Por fim, agradeço e dedico este trabalho ao meu tio que sei que estará muito orgulhoso do caminho que estou a traçar!

List of Publications and Scientific Communications

The following manuscripts are the outcome of this thesis:

Systematic Review Article

Lobo de Almeida A., Sousa M., Sá R. (2023) Beyond bleomycin-induced pulmonar toxicity its male reproductive toxicity remains unanswered 50 years after this approval. (submitted to an international peer-reviewed journal)

Original Article

Lobo de Almeida A., Gonçalves A., Barros A., Sousa M., Sá R. (2023) Bleomycin *in vitro* exposure decreases markers of human male gamete competence. (submitted to a national prize and to an international peer-reviewed journal)

The research that is part of this thesis has also been presented in the following international and national scientific meetings:

International Scientific Conferences

Lobo de Almeida, A.; Gonçalves, A.; Sousa, M.; Sá, R. The reproductive toxicity of bleomycin. International Federation of Fertility Societies (IFFS) World Congress, 2023. Athens, Greece, September 11, 2023 (Oral Communication).

National Scientific Conferences

Lobo de Almeida, A.; Gonçalves, A.; Barros, A.; Sousa, M.; Sá, R. *In vitro* actions of bleomycin on human sperm function and the underlying mechanism of any effects. 7th Symposium of the MSc in Oncology, Instituto Português de Oncologia do Porto (IPO-Porto), February 13, 2023 (Oral Communication).

Lobo de Almeida, A.; Gonçalves, A.; Barros, A.; Sousa, M.; Sá, R. Bleomycin's effects on human sperm motility and vitality *in vitro*. 16ª edição do IJUP – Encontro de Investigação Jovem da Universidade do Porto, Reitoria da UPorto, May 11, 2023 (Poster Communication).

Lobo de Almeida, A.; Gonçalves, A.; Barros, A.; Sousa, M.; Sá, R. Bleomicina uma "sirga" contra o DNA dos espermatozoides humanos. XXXVIII Jornadas de Estudos da Reprodução, Sociedade Portuguesa de Medicina da Reprodução, Meliã Ria Hotel, Aveiro, May 13, 2023 (Poster Communication).

Lobo de Almeida, A.; Gonçalves, A.; Barros, A.; Sousa, M.; Sá, R. Oncofertilidade: O impacto da bleomicina na capacidade reprodutiva de jovens adultos. 5º Encontro Nacional de Jovens Investigadores em Oncologia, Liga Portuguesa Contra o Cancro, Núcleo Regional do Norte, Porto, September 22, 2023 (Poster Communication).

Resumo

Não obstante da tendência crescente na incidência do cancro a nível mundial, verifica-se igualmente um aumento no número de sobreviventes de cancro devido aos recentes e importantes avanços no rastreio, diagnóstico e tratamento oncológicos. Vindo, portanto, realçar a importância de melhorar a qualidade de vida dos sobreviventes oncológicos.

A paternidade é tida como uma prioridade para uma grande proporção de adolescentes e jovens adultos (AYAs) sobreviventes de cancro. O cancro testicular é o cancro mais comum nos homens nesta faixa etária, no entanto, é também o mais curável, com uma taxa de sobrevivência superior a 90%, principalmente devido à implementação de um regime de quimioterapia que combina bleomicina, etoposido e cisplatina (BEP). Porém, à semelhança de outras terapias, este esquema combinado apresenta gonadotoxicidade, a qual foi também descrita no uso individual de cisplatina e etoposido. Por outro lado, pouco se sabe sobre o efeito da bleomicina, um antibiótico antitumoral amplamente utilizado no tratamento de uma variedade de tumores, no potencial fértil masculino. O seu uso só é restrito devido à toxicidade pulmonar que lhe está intimamente associada.

Neste contexto, a presente tese propõe investigar e compreender os processos subjacentes aos impactos da bleomicina na função espermática humana *in vitro*.

Dada a escassez de informação descrita na literatura, foi efetuada uma revisão sistemática da literatura, aplicando palavras-chave e critérios de inclusão específicos, a fim de compilar toda a informação disponível. Esta pesquisa revelou apenas sete estudos, todos investigações *in vivo* em roedores. Os resultados obtidos sugerem que a bleomicina é gonadotóxica, uma vez que afeta todos os aspetos da função testicular dos roedores, incluindo alterações celulares e hormonais, bem como modificações na histologia e arquitetura dos túbulos seminíferos. A necessidade de mais investigações para determinar se estes efeitos também ocorrem em humanos foi trazida à luz por esta revisão.

À luz disto, foi realizado um estudo *in vitro* onde uma exposição de duas horas à bleomicina foi aplicada a amostras de espermatozoides humanos. Uma análise dos parâmetros seminais, uma avaliação da produção de espécies reativas de oxigênio (ROS), da função mitocondrial do esperma e da integridade do DNA e do acrossoma dos espermatozoides foram realizadas antes e depois do período de incubação. Os resultados obtidos não apenas confirmaram os efeitos deletérios da bleomicina nos parâmetros seminais convencionais previamente documentados em estudos animais *in vivo*, como também acrescentaram novas e importantes informações. Especificamente, a bleomicina mostrou ser inerte no que diz respeito aos níveis de ROS no espermatozoide

humano, mas causa nos mesmos uma disfunção mitocondrial e uma reação acrossómica retardada. Além disso, descobriu-se que a bleomicina provoca danos no DNA, o que é consistente com sua ação antitumoral. A análise ultraestrutural através de microscopia eletrónica de transmissão (TEM) foi usada para confirmar as alterações na cromatina, acrossoma e mitocôndrias dos espermatozoides após a exposição à bleomicina. Esta técnica também revelou, pela primeira vez, um inchaço acentuado das estruturas espermáticas, que já havia sido relatado para os cílios das vias aéreas na fibrose pulmonar induzida pela bleomicina.

Em suma, os resultados do presente estudo revelaram pela primeira vez os efeitos potenciais da bleomicina na fertilidade masculina humana, embora sejam necessárias mais pesquisas para compreender o papel da apoptose e do stress oxidativo (OS) nesta ação deletéria da bleomicina. Além do mais, o presente estudo permite aos profissionais de saúde e aos doentes oncológicos uma discussão e uma tomada de decisão quanto à preservação de fertilidade antes dos tratamentos mais consciente, informada e fundamentada quanto aos potenciais riscos da bleomicina na capacidade fértil masculina.

Palavras-chave: Bleomicina; Quimioterapia; Fertilidade masculina, Espermatozoide humano

Abstract

Despite the growing trend in the incidence of cancer worldwide, there is also an increase in the number of cancer survivors due to recent and important advances in oncological screening, diagnosis and treatment. Therefore, we highlight the importance of improving the quality of life of cancer survivors.

Fatherhood is considered a priority for a large proportion of adolescents and young adults (AYAs) cancer survivors. Testicular cancer is the most common cancer in men in this age group, however, it is also the most curable, with a survival rate of over 90%, mainly due to the implementation of a chemotherapy regimen that combines bleomycin, etoposide, and cisplatin (BEP). However, like other therapies, this combined regimen presents gonadotoxicity, which has also been described in the individual use of cisplatin and etoposide. On the other hand, little is known about the effect of bleomycin, an antitumor antibiotic widely used to treat a variety of tumors, on male fertile potential. Its use is only restricted due to the pulmonary toxicity closely associated with it.

In this context, the present thesis proposes to investigate and understand the processes underlying the impacts of bleomycin on human sperm function *in vitro*.

Given the scarcity of information described in the literature, a systematic review of the literature was carried out, applying specific keywords and inclusion criteria, in order to compile all available information. This search revealed only seven studies, all *in vivo* investigations in rodents. The results obtained suggest that bleomycin is gonadotoxic, as it affects all aspects of testicular function in rodents, including cellular and hormonal changes, as well as changes in the histology and architecture of the seminiferous tubules. The need for further investigation to determine whether these effects also occur in humans has been brought to light by this review.

In light of this, an *in vitro* study was carried out where a two-hour exposure to bleomycin was applied to human sperm samples. An analysis of seminal parameters, an assessment of the production of reactive oxygen species (ROS), sperm mitochondrial function and the integrity of sperm DNA and acrosome were carried out before and after the incubation period. The results obtained not only confirmed the deleterious effects of bleomycin on conventional seminal parameters previously documented in *in vivo* animal studies, but also added new and important information. Specifically, bleomycin has been shown to be inert with regard to ROS levels in human sperm but causes mitochondrial dysfunction and a delayed acrosome reaction in them. Furthermore, bleomycin has been found to cause DNA damage, which is consistent with its antitumor action. Ultrastructural analysis using transmission electron microscopy (TEM) was used to confirm changes in sperm chromatin, acrosome and mitochondria after exposure to bleomycin. This

technique also revealed, for the first time, a marked swelling of sperm structures, which had previously been reported for airway cilia in bleomycin-induced pulmonary fibrosis.

In summary, the results of the present study revealed for the first time the potential effects of bleomycin on human male fertility, although more research is needed to understand the role of apoptosis and oxidative stress (OS) in this deleterious action of bleomycin. Furthermore, the present study allows healthcare professionals and cancer patients to discuss and make decisions regarding fertility preservation before treatments that are more conscious, informed and well-founded regarding the potential risks of bleomycin on male fertile capacity.

Keywords: Bleomycin; Chemotherapy; Male fertility; Human sperm

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List of Abbreviations

A

AB – Acidic Aniline Blue

ATP – Adenosine Triphosphate

AVR – Acrosome vesicle region

AYAs – Adolescents and young adults

B

Bcl2 – B-cell lymphoma 2 family

BEP – Bleomycin, Etoposide, and Cisplatin

BLH – Bleomycin hydrolase

C

C- – Negative control

C+ – Positive control

CT – Control group

CAT – Catalase

CNPMA – National Council on Medically Assisted Procreation

D

DAPI – 4′,6-diamidino-2-phenylindole

DNA – Deoxyribonucleic acid

E

ER – Equatorial region

EXP group – Experimental group

F

FDA – Food and Drug Administration

Fe^{2+} – Ferrous ion

Fe^{3+} – Ferric ion

FITC – Fluorescein-5-isothiocyanate

FSH – Follicle-stimulating hormone

G

GPx – Glutathione Peroxidase

GSH – Reduced Glutathione

GST – Glutathione S-transferase

I

IC50 – Half maximal inhibitory concentration

H

H_2O_2 – Hydrogen peroxide

HE – Hematoxylin-eosin

L

LH – Luteinizing hormone

M

MDA – Malondialdehyde

MMP – Mitochondrial membrane potential

mRNA – Messenger RNA

N

NRC – National Research Council

O

ODF – Outer dense fibers

OS – Oxidative Stress

OXPHOS – Oxidative phosphorylation

P

PAR – Post-acrosomal region

PAS-H – Periodic Acid-Schiff-Hematoxylin

PNA – Peanut-agglutinin

PBS – Phosphate buffered saline

R

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RPM – Rapid progressive motility

rRNA – Ribosomal RNA

RT – Room Temperature

S

sDNAfrag – Sperm DNA fragmentation

SOD – Superoxide dismutase

SlPM – Slow progressive motility

SPM – Sperm preparation medium

T

TEM – Transmission electron microscopy

TUNEL – Terminal deoxynucleotidyl transferase dUTP nick-end labeling

tRNA – Transfer RNA

W

WHO – World Health Organization

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Chapter I. Introduction and Motivation

1.1 | Oncofertility

Cancer is currently a major public health concern, as one of the leading causes of death worldwide and the top two causes of premature death (before the age of 70) in the majority of nations (112 out of 183) (Mao et al., 2022; Sung et al., 2021).

The most recent projections indicate that there would be 10 million cancer-related deaths worldwide in 2020 and 19.3 million new instances of the disease. Additionally, the predictions are not encouraging. With a projected 28.9 million new cancer cases in 2040, this figure is predicted to nearly treble over the following two decades. However, because of the aging and expanding populations, as well as the rising incidence of risk factors, these projections might not come to pass (Mao et al., 2022; Soerjomataram & Bray, 2021; Sung et al., 2021).

Cancer is not just an adult disease, though. With around 1 million new cases per year worldwide, the incidence of cancer in adolescents and young adults (AYAs; defined as those aged 15 to 39 years) has likewise shown an increased trend in recent decades (Barr, 2011; Janssen et al., 2021; Patterson et al., 2015). Fortunately, more than 80% of people who are diagnosed with cancer at these ages are projected to live for more than 5 years, with cancers like Hodgkin's lymphoma and testicular cancer having a survival rate of more than 90% and accounting for almost 40% of incidence cases at these ages (Barr, 2011; Janssen et al., 2021).

AYAs cancer survivors have been a complicated population group that has received little research despite having a high rate of cancer survivors at these ages, which was primarily made possible by technological and scientific advancements (Chao et al., 2019; Fidler et al., 2019; Janssen et al., 2021; Sung et al., 2021). They are a unique oncology population, unlike children (under 15 years old) and older adults (over 40 years old) (Barr, 2011; Chao et al., 2019), whose cancer populations make it difficult to administer the proper care due to complaints of malpractice from both pediatric and adult oncologists and the requirement for a multiprofessional approach combining knowledge from both specialties (Trama et al., 2016). Compared to children and older people, this cohort exhibits glaring changes in the biology and distribution of disease (Barr, 2011; Docherty et al., 2015). Additionally, adolescence and early adulthood are challenging times of life during which time people experience fast changes in their physical, emotional, cognitive, and social development (Bukowinski et al., 2015; Patterson et al., 2015). In comparison to the general population, all of these growth and modifications increase their risk of morbidities and make them more susceptible to severe long-term side effects of cancer and its therapies as well as a variety of psychosocial difficulties, such as worries about infertility (Barr, 2011; Fidler et al., 2019; Janssen et al., 2021).

One of the major concerns for AYAs cancer survivors is the prospect of having their reproductive capacity compromised or experiencing infertility (Fidler et al., 2019), even though many of them have not yet started their own families or are too young to have even seriously contemplated becoming parents (Patterson et al., 2015). Additionally, this fear and uncertainty is a substantial source of discomfort, negatively affecting the mental health of cancer patients as well as their present and future wellbeing (Cherven et al., 2022; Crawshaw, 2013).

Unfortunately, some cancers, including leukemias, lymphomas, and testicular cancer, can cause infertility situations even before treatments are started. Infertility situations can manifest in men as oligospermia, azoospermia, or decreased sperm parameters (Bîcă et al., 2021; Cherven et al., 2022, 2023; Lautz et al., 2021; Xie et al., 2022), and in women as acute ovarian failure, premature ovarian insufficiency, or premature menopause (Cherven et al., 2022; Janssen et al., 2021). However, the majority of the time, the gonadotoxic effects are brought on by oncologic therapies, such as radiotherapy, surgery, or other forms of chemotherapy (Table I. 1) that target the gonads or are intended to attack cells with high rate of cell division in general, making spermatogenesis a potential target (Cherven et al., 2022; Wallace et al., 2005).

Table I. 1 Male gonadal dysfunction risk estimate when using cytotoxic medicines.

Estimated risk	Drugs	Regimens
High Risk	Busulfan Chlorambucil Chlormethine Cyclophosphamide Dacarbazine Ifosfosfamide Melphalan Procarbazine	MOPP (nitrogen-mustard, vincritine, procarbazine, and prednisone)
Medium risk	Carboplatin Cisplatin Dozorubicin	BEP (bleomycin, etoposide and cisplatin) ABVD (doxorubicin, bleomycin, vinblastine, and dacabarzine)
Low risk	Bleomycin Dactinomycin Vinblastine Vincristine Mercaptopurine Methatrexate	

Adapted by Kawai & Nishiyama, 2019

Fortunately, there is growing awareness of the potential reproductive effects of cancer and its treatments. Despite the rising availability of fertility preservation options and the elevated awareness of healthcare providers, many AYAs do not receive enough information and support (Fidler et al., 2019).

The importance of educating cancer patients about the risks of treatments to their reproductive potential and the possibility of preserving their fertility prior to the start of treatments has recently been highlighted by a number of guidelines and strong recommendations published by medical societies and professional organizations (Del-Pozo-Lérida et al., 2019; Docherty et al., 2015; Rabaça et al., 2020; Trost & Brannigan, 2012; Xie et al., 2022). The American Society of Clinical Oncology, in response, states that "As part of education and informed consent before cancer therapy, oncologists should address the possibility of infertility with patients treated during their reproductive years and be prepared to discuss possible fertility preservation options or refer appropriate and interested patients to reproductive specialists" (Lee et al., 2006). Fortunately, formal fertility preservation programs have been created in many institutions today, increasing the number of patients who can undergo fertility consultations and, as a result, use fertility preservation services (Kawai & Nishiyama, 2019). Evidence, however, indicates that this referral and conversation may not always take place or may not be thorough and correct,

leaving patients ignorant of the true dangers (Fidler et al., 2019; Xie et al., 2022). The urgency of starting treatment, parental overprotection, discomfort on the part of the doctor and patient, and a lack of reliable information from healthcare professionals regarding the risks and preservation of fertility can all contribute to making this conversation more challenging, especially at these ages (Docherty et al., 2015; Fidler et al., 2019; Janssen et al., 2021; Patterson et al., 2015). In reality, people commonly start medications without safeguarding their fertility due to a lack of adequate information (Janssen et al., 2021).

A relatively new and interdisciplinary area called "Oncofertility," which Teresa K. Woodruff coined in 2007, brings all of these questions together. With the ultimate goal of maintaining cancer patients' reproductive function, this specialty bridges the gap between oncology and reproductive medicine (Bîcă et al., 2021; Zaami et al., 2021). This field has consequently grown increasingly significant because to the upward trend in cancer survival rates among patients of reproductive age.

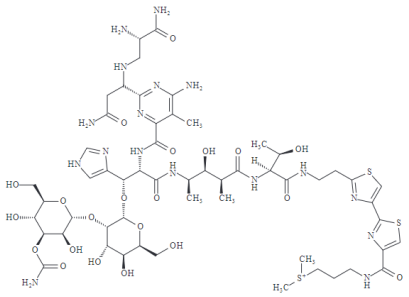
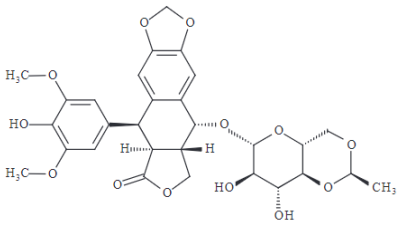
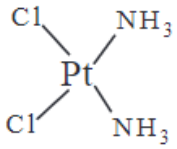
1.2 | Testicular cancer and BEP chemotherapy

Testicular cancer is the most prevalent solid cancer in AYA men (Bagheri-Sereshki et al., 2016; Ghezzi et al., 2016; Khadivi et al., 2020; Marcon et al., 2008), with an estimated 74,500 new cases expected globally in 2020 (Znaor et al., 2022), an incidence that has been rising over time. This tumor is also notable for being the most curable solid tumor (Gilligan et al., 2019), which has led to a steady increase in the number of survivors (Bagheri-Sereshki et al., 2016; Ghezzi et al., 2016; Gilligan et al., 2019; Hamano et al., 2017; Khadivi et al., 2020; Marcon et al., 2008; Maselli et al., 2013).

Testicular cancer 5-year survival rates now reach 90% thanks to early diagnosis, advancements, and improvements in treatment protocols and modalities (Al-Bader & Kilarkaje, 2015; Bagheri-Sereshki et al., 2016; Khadivi et al., 2020; Liu et al., 2015; Marcon et al., 2008; Maselli et al., 2013). Since testicular cancer's origin is still unknown and only strong connections have been found with developmental disorders (cryptorchidism), there are yet no effective preventative methods (Znaor et al., 2022). It is interesting to note that numerous studies have revealed that infertile males also have a higher risk of acquiring testicular cancer, with a 20 times higher incidence (Hamano et al., 2017; Jeruss & Woodruff, 2009). The use of combination chemotherapy, specifically platinum-based chemotherapy, which contains bleomycin, etoposide, and cisplatin (BEP), has been proven effective and is largely to blame for the rise in the survival rates of men with testicular malignancies at all stages (Al-Bader & Kilarkaje, 2015; Bagheri-Sereshki et al., 2016; Hartmann et al., 1999; Khadivi et al., 2020; Marcon et al., 2008; Maselli et al., 2013; Park et al., 2018; Znaor et al., 2022).

The conventional treatment for testicular cancer is BEP chemotherapy, which includes three different drugs with diverse properties and modes of action (Table I. 2). Bleomycin, an antibiotic with anti-cancer properties, causes DNA breaks through a mechanism controlled by free radicals. Etoposide, a member of the topoisomerase II inhibitor class, which blocks topoisomerase II, an enzyme required for activities including DNA replication and transcription. The DNA chain is broken, and topoisomerase synthesis is impeded as a result of this effect. As well as cisplatin an alkylating agent, which targets DNA as its primary target and prevents transcription and replication by attaching an alkyl group to this molecule (Bieber, 2005; Razavi et al., 2019).

Table I. 2 Structures and main mechanisms of action of the three BEP regimen agents.

BEP agent	Structure	Main mechanisms of action
BLEOMYCIN		- DNA breaks - Generation of ROS
ETOPOSIDE		- Inibition of topoisomerase II
CISPLATIN		- Prevention of DNA replication

1.2.1 | Male fertility preservation

Pre-treatment subfertility in the specific situation of testicular cancer is a fact, and the proportion of patients with oligospermia is as high as 52%, compared to 12-30% for other tumors (Kawai & Nishiyama, 2019). Additionally, the testes themselves are thought to be the most susceptible organs in the body to stress from the environment (Khadivi et al.,

2020; Rabaça et al., 2020), and the BEP regimen, the usual treatment for testicular cancer, is not tumor-specific and exhibits gonadotoxicity (Jeruss & Woodruff, 2009).

Only one-fourth of cancer patients maintain their fertility (Hamano et al., 2017). This is likely due to the fact that, according to several studies (Davis et al., 2023), only about 60% of men who are diagnosed with testicular cancer receive fertility counseling prior to starting treatment. The most popular method for preserving fertility in adult males and pubertal men is the cryopreservation of ejaculated semen (Figure I. 1) obtained through masturbation. This process involves collecting semen samples after a time of sexual abstinence, which are then cryopreserved (Del-Pozo-Lérida et al., 2019; Kawai & Nishiyama, 2019).

Figure I. 1 Preserving fertility in postpubertal male patients before beginning oncological therapy.

The aforementioned strategy does not apply to prepubertal-age patients, whose median age at spermarche is 13 years (Brannigan et al., 2021), as they have not yet undergone spermatogenesis and do not thus produce mature sperm. Currently, testicular tissue cryopreservation is the only method available to these patients for preserving their fertility, although it is still purely experimental (Brannigan et al., 2021; Del-Pozo-Lérida et al., 2019; Halpern et al., 2020; Wallace et al., 2005). Even though it is recommended and is being practiced in many nations as the goal of testicular tissue cryopreservation is to protect spermatogonial stem cells with the potential to develop spermatozoa in the future (Moussaoui et al., 2022).

Other experimental strategies are investigated in this field's research. One of them involves inducing the arrest of spermatogenesis or suppressing the secretion of hormones (testosterone and gonadotropins), to protect gonadal tissue against damage caused by treatments. However, because cancer itself may have already caused tissue damage, hormonal gonadoprotection is not advisable for men (Halpern et al., 2020). Additionally, the outcomes offer little value (Brannigan et al., 2021).

More study is still necessary to enable use when a patient is cancer-free and chooses to have children, particularly in the case of prepubescent boys, as there are

currently a limited number of studies available and they also raise ethical and legal concerns (Del-Pozo-Lérida et al., 2019; Moussaoui et al., 2022).

1.2.2 | Impact of BEP chemotherapy on male fertility

The use of BEP is associated with both short- and long-term side effects, including impacts on fertility, as well as potentially fatal toxicities, particularly at the level of cardiovascular disease (Alsdorf et al., 2019; Hartmann et al., 1999).

Numerous animal studies found that the weight of the testis, epididymis, seminal vesicle, and prostate decreased (Bieber et al., 2006; Hashemi et al., 2018; Marcon et al., 2008; Maselli et al., 2012). Despite some non-significant data, it appears that BEP lowers testosterone levels, raises LH levels (Marcon et al., 2008), and either raises (O'Flaherty et al., 2010) or does not affect FSH levels (Al-Bader & Kilarkaje, 2015; Kilarkaje et al., 2013) in terms of reproductive hormone levels. These alterations were linked to the effect of bleomycin on Leydig cells, whose depletion causes a decrease in testosterone production and an increase in LH production (Khadivi et al., 2020; Razavi et al., 2019).

An increase in sperm nuclear and mitochondrial DNA damage, sperm immature chromatin condensation, and changes in seminal parameters (sperm count, vitality, motility, and normal morphology) were additional signs of the gonadotoxic effects of BEP in both human (De Mas et al., 2001; Ghezzi et al., 2016; O'Flaherty et al., 2010; Ståhl et al., 2006) and animal (Bayram et al., 2023; Bieber et al., 2006; Delbes et al., 2007; Hashemi et al., 2018; Khadivi et al., 2020; Kilarkaje et al., 2013; Marcon et al., 2008; Maselli et al., 2012; Moradi et al., 2021; Razavi et al., 2019, 2021) investigations. These changes in sperm characteristics might be either temporary or permanent. According to some research, the sperm DNA damage can continue for up to 24 months after treatments (Bagheri-Sereshki et al., 2016; Ghezzi et al., 2016; O'Flaherty et al., 2010), and some of it may not even be able to be repaired (Delbes et al., 2007). Depending on the dosage and length of the BEP regimen, this can cause azoospermia or oligospermia. Indeed, there is a sizeable portion of cases in which this diagnosis is final and irreversible, even though these scenarios are linked to an infertility diagnosis that is typically temporary, with 50% of patients recovering normal spermatogenesis within 2 years of the end of treatment and 80% after 5 years (Marcon et al., 2008).

It is also challenging to assess how BEP treatment may affect the progeny (Bieber et al., 2006). However, based on the limited research done in rats, BEP treatment appears to reduce progeny survival, which may raise the risk of congenital abnormalities (Bieber et al., 2006; Liu et al., 2015) and a high rate of early postnatal death for male offspring treated with BEP (Maselli et al., 2012).

It is thus advised that conception be avoided throughout the treatment time and for up to two years after treatments in light of the research documenting the adverse effects of chemotherapy and the risk that some damages may be passed on to future generations (Khadivi et al., 2020).

The chemotherapeutic components of the BEP regimen are not solely employed in combination, despite the fact that the majority of chemotherapy procedures are multiagent; consequently, it is crucial to comprehend how each agent particularly impacts fertility (Trost & Brannigan, 2012).

Etoposide is a potential contributor to male infertility since *in vitro* studies on its effects on fertility have shown that it can trigger apoptosis and play a part in the development of oligozoospermia and azoospermia (Baetas et al., 2019; Rabaça et al., 2020). Additionally, because etoposide primarily affects cells during meiosis, structural chromosomal abnormalities in the progeny as well as an increase in sperm aneuploidy have already been described (Bieber, 2005).

Knowledge and documentation of the effects of cisplatin, which affect practically the entire reproductive system, is really good. Because of its ability to trigger apoptosis in these cells, as well as its propensity to raise testosterone levels, cisplatin seems to decrease the number of spermatozoa and spermatids and induce morphological changes in these cells. Furthermore, cisplatin seems to lessen sperm motility and lighten the weight of reproductive organs. Moreover, animal investigations have revealed increased pre- and post-implantation loss, decreased fetal weight, and increased fetus abnormalities and delays at the level of effects on progenesis (Bieber, 2005).

Bleomycin is the substance whose detrimental effects on reproduction are least well understood, and in particular, the effects of bleomycin alone on human sperm have not yet been investigated.

1.3 | Bleomycin

Umezewa and colleagues at the end of the 1960s isolated from cultures of *Streptomyces verticillus* a combination of water-soluble glycopeptides with antitumor and antibacterial properties, which they entitled bleomycin, one of the first anticancer medicines ever discovered (Umezawa & Maeda, 1967).

Bleomycin was initially approved by the Food and Drug Administration (FDA) in 1975 for the treatment of squamous cell carcinomas, malignant lymphomas, and testicular cancer. The FDA has now approved the use of bleomycin for a number of conditions, including the treatment of Hodgkin's disease and non-Hodgkin lymphoma (Brandt & Gerriets, 2023).

Bleomycin has gained a lot of medicinal and clinical interest due to its broad spectrum of cytotoxic activities and lack of myelosuppressive effects (O'Sullivan et al., 2003; Sikic et al., 1985). Due to this, bleomycin is seen to be a desirable addition to a number of combination chemotherapy regimens, such as with doxorubicin, vinblastine, and dacarbazine (ABVD) or BEP (Brandt & Gerriets, 2023; O'Sullivan et al., 2003), being therefore also used to treat cervical, ovarian, melanoma, and sarcoma cancers (Rahaman, 2018; Scholar, 2007).

1.3.1 | Pharmacodynamics

Bleomycin damages DNA through mechanisms reliant on oxygen and metal ions, causing cellular toxicity.

Bleomycin contains at opposite ends of the molecule, the iron-biding region (acid portion) and the DNA-binding region (bithiazole portion). Through the former, bleomycin binds to metal ions, namely iron, in a reduction-oxidation reaction, in which the Fe^{2+} ion is oxidized to Fe^{3+} and oxygen is reduced into reactive oxygen species (ROS). Thus, this medication has the ability to both bind to DNA and create complexes with different metal ions (Della Latta et al., 2015).

Bleomycin can participate in "suicide chemistry" in the absence of DNA, but when DNA is present, these ROS, particularly the highly lethal superoxide and hydroxyl radicals, cleave the C3 to C4 bond of deoxyribose (Brandt & Gerriets, 2023; Della Latta et al., 2015; Lazo, 1999; Reinert et al., 2013). The deoxyribose ring opens as a result of the abstraction of a hydrogen atom from deoxyribose at the C4 position, resulting in the generation of highly cytotoxic free base propenals (Lazo, 1999). These free radicals, specifically the superoxide radical and the hydroxyl radical, both have high toxicity (Lazo, 1999). Following their involvement in cell cycle arrest in the G2 and mitotic phases, these hazardous chemicals also prevent cell replication, which in turn prevents tissue growth and repair (Brandt & Gerriets, 2023; Reinert et al., 2013). This indicates that this medication has a greater overall activity against dividing cells. All three primary classes of RNA - mRNA, tRNA, and rRNA - are also damaged by this substance, which slows their synthesis and reduces protein synthesis (Lazo, 1999; Rahaman, 2018; Reinert et al., 2013).

1.3.2 | Pharmacokinetics

Bleomycin is supplied parenterally, including intramuscularly, subcutaneously, intravenous and intraperitoneally, because its size, positive charge, and structure prevent it from being absorbed through the gastrointestinal tract (Brandt & Gerriets, 2023; Lazo, 1999; Scholar,

2007). Bleomycin is rapidly absorbed and has a high bioavailability when administered through a systemic route. Less than 1% of the drug binds to plasma proteins, and it reaches its peak plasma concentration in about an hour (Brandt & Gerriets, 2023; O'Sullivan et al., 2003).

Bleomycin hydrolase (BLH) performs a series of deamidation and inactivation processes in order to break down the antibiotic (Lazo, 1999). Bleomycin is metabolized by this cysteine protease, a member of the papain superfamily, into an ineffective and harmless compound. By replacing a terminal amine with a hydroxyl group, this non-toxic and DNA cleavage-incompetent form inhibits cytotoxicity and DNA damaging activity (Brandt & Gerriets, 2023; Chen et al., 2012; Della Latta et al., 2015; Lazo, 1999). The skin and lungs, unlike other tissues where bleomycin is present in small amounts, collect this substance because BLH is less active in these tissues. These tissues are therefore particularly vulnerable to the effect of bleomycin (Brandt & Gerriets, 2023; Reinert et al., 2013; Sikic et al., 1985).

Bleomycin is largely eliminated through the kidneys, with 50–70% of the given dose being excreted in the urine, due to a high association between bleomycin and creatinine clearance and a drug clearance that is close to 70 mL/min/m^2. This illustrates how quickly bleomycin leaves the body through urine and plasma (Brandt & Gerriets, 2023; Fyfe & McKay, 2010; Reinert et al., 2013; Scholar, 2007). As a result, how long bleomycin stays in the body is greatly influenced by the patient's renal function. Patients with normal renal function typically have a half-life of about 2 hours, whereas those with impaired glomerular filtration rates may have a half-life of up to 30 hours. Thus, those with lower creatinine clearance may also have much lower bleomycin clearance (Brandt & Gerriets, 2023; Della Latta et al., 2015; Fyfe & McKay, 2010; Lazo, 1999; O'Sullivan et al., 2003; Reinert et al., 2013). Lowering the amount provided to these individuals is important due to the difficulty in removing the drug, which leads to an increase in drug buildup and, ultimately, an increase in toxicity (Reinert et al., 2013). However, the total dose is restricted to between 300 mg and 400 mg, with a significant incidence of pulmonary toxicity observed at higher doses (Aronson, 2016; Blum et al., 1973; Yagoda et al., 1972). The dosage is thus dependent on a variety of factors including the characteristics of the regimen, the tumor, and the patient (e.g. age, weight, and comorbidities).

1.3.3 | Adverse effects

Bleomycin generates extremely low myelosuppression in the bone marrow, in contrast to the majority of chemotherapy drugs (Lazo, 1999; Sikic et al., 1985). Bleomycin does have a number of common adverse effects, including nausea (11% of patients reported it), fever (24%), and chest pain (28%). Additionally, side effects such as diarrhea, hair loss,

vomiting, hyperthermia, headaches, exhaustion, poor appetite, and others are mentioned as being typical of various chemotherapies (Rahaman, 2018; Scholar, 2007). Myocardial infarction, stroke, and Raynaud's phenomenon are a few of the sporadic vascular events that have also been recorded (Reinert et al., 2013).

The two tissue-specific toxicities, on the other hand, are caused by the low concentrations of BLH in the skin and lungs that were previously mentioned. The most frequent side effects, which often manifest when cumulative doses surpass 200 mg, are flagellate erythema, hyperpigmentation, and hyperkeratosis (Chen et al., 2012; Fyfe & McKay, 2010; Reinert et al., 2013). Despite this, these skin toxicities - while potentially unpleasant - are not linked to an increased risk of morbidity and mortality and typically do not signal the end of treatment (Fyfe & McKay, 2010).

Bleomycin directly acts on alveolar endothelium and epithelial cells, causing bleomycin pulmonary toxicity, which can result in epithelial apoptosis, intra-alveolar inflammation (including macrophage activation and subsequent cytokine release), as well as collagen deposition (Lazo, 1999). The death rate for pulmonary fibrosis caused by bleomycin is between 3 and 5% in those affected, despite the incidence being less than 10% (Bestanchuk et al., 2021; Brandt & Gerriets, 2023; Reinert et al., 2013). Aging, greater bleomycin doses, and patient renal function all raise the chance of experiencing this side effect (Fyfe & McKay, 2010). The most serious side effect of bleomycin is hence pulmonary damage via pulmonary fibrosis due to its poor prognosis. Bleomycin has already established itself as a substance used in animal model research to generate lung fibrosis in a quick and consistent manner due to this causal relationship between bleomycin and pulmonary fibrosis (Lazo, 1999).

Fertility issues and respiratory diseases has been associated (Furlow, 2014), nevertheless, in contrast with the bleomycin-induced lung fibrosis well described and studied, its effects *in vivo* or *in vitro* on human male fertility remain unknown.

1.4 | Sperm, a marker of male fertility

Human sperm has been the subject of numerous studies to better understand its structure and functions ever since it was identified in the late 1960s as the male gamete in charge of transporting paternal genetic material (Creasy & Chapin, 2013; Kumar & Singh, 2021; Sharma & Agarwal, 2011). A list of data is now available from the examination of the number, quality, and functionality of human spermatozoa, which enables the prediction of male fertility ability. This makes semen analysis the first and most important test to determine male fertility. It comprises an evaluation of variables such as sperm count, vitality, motility, and morphology. Sperm is a great marker to track the effects of

substances and medications on the male reproductive system since changes to these parameters directly relate to changed fertility capacity (Nallella et al., 2006; Razavi et al., 2019).

The majority of human andrology laboratories around the world adhere to the WHO recommendations (WHO, 2021), which outline the methodologies and benchmark values that should be used. Due to the WHO criteria's limitations in terms of accurately predicting male fertility, additional sperm features have essentially been assessed in the research community (Tello-Mora et al., 2018).

The process of spermatogenesis, which is intricate and hormone-dependent, results in spermatozoa (Figure I. 2). A diploid cell undergoes mitotic and meiotic divisions on testis seminiferous tubules to become a haploid, highly specialized cell (Creasy & Chapin, 2013; Kumar & Singh, 2021; Sharma & Agarwal, 2011). In addition to these cell divisions during spermatogenesis, additional processes such as acrosomal cap development, chromatin condensation, and tail formation are also required (Sharma & Agarwal, 2011; Sunanda et al., 2018). Since stem spermatogonia, which can perpetually grow or divide, is present in the testis, the process of spermatogenesis in humans begins at puberty and lasts the rest of a man's life (Creasy & Chapin, 2013; Shalet, 2009; Sharma & Agarwal, 2011).

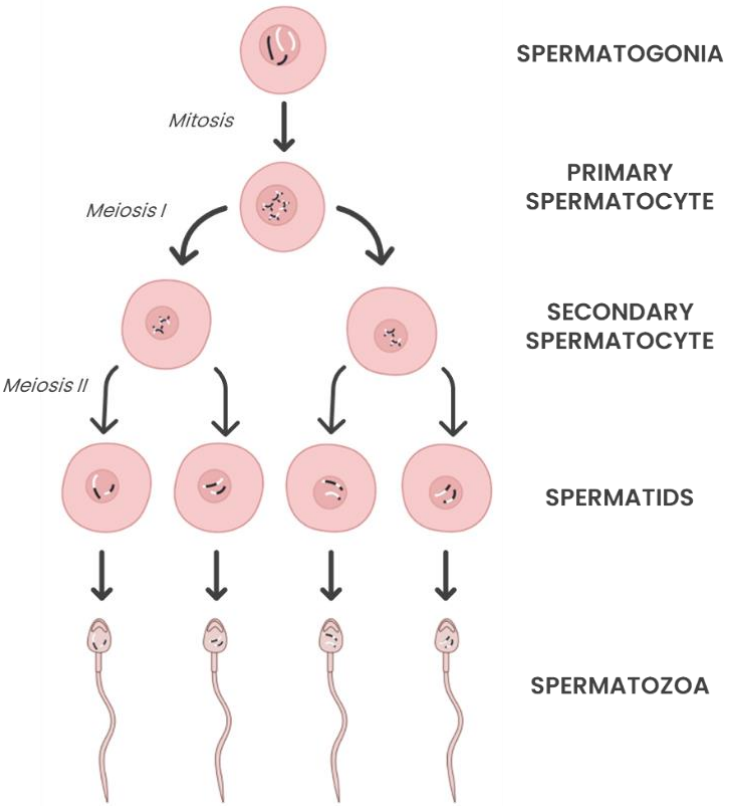


Figure I. 2 The process of cell division during spermatogenesis is shown schematically.

Spermatozoa have a distinctive structural organization and a compacted form with a small quantity of cytoplasm and a large nucleus, with a typical oval-shaped head (3-5 µm length and 2-3 µm width), a midpiece (7-8 µm), and a tail (45 µm) (Sharma & Agarwal, 2011; Sunanda et al., 2018).

The sperm head, where the nucleus is capped by the acrosome and plasma membrane, is occupied by densely packed DNA as a result of chromatin changes in which DNA-binding proteins called histones are replaced by transition proteins and then by protamines, encouraging extreme compaction of DNA and protecting it from potential damage (Figure I. 3) (Arafa et al., 2020; Sunanda et al., 2018). As a result, modifications to chromatin condensation may increase sperm DNA damage susceptibility (Muratori & De Geyter, 2018).

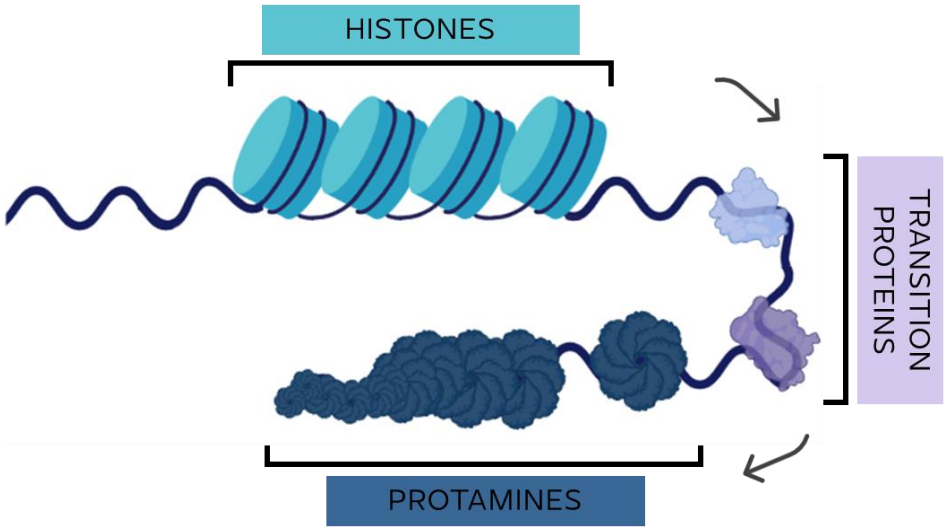


Figure I. 3 Schematic representation of chromatin modifications that occur throughout spermatogenesis.

The sperm flagellum is made up of three parts: the midpiece, the major piece, and the endpiece, and it develops in a regular manner from the base of the nucleus (Figure I. 4). The central component of the flagellum is the axoneme. This is, in the midpiece, surrounded by outer thick fibers and 50 to 75 mitochondria, which are critical for sperm movement and capacitation, whereas the remaining flagellum is encircled by a fibrous sheath (Barbagallo et al., 2020; Inaba, 2003; Kumar & Singh, 2021; Sharma & Agarwal, 2011; Sunanda et al., 2018). The axoneme is the most critical component for sperm motility, made up of proteins like radial spokes and dynein arms as well as a cytoskeletal structure made of axial microtubule filaments in a 9+2 organization with 9 outer duplets of a complete and an incomplete tubule and a core microtubule duplet (Figure I. 4) (Inaba, 2003; Kumar & Singh, 2021).

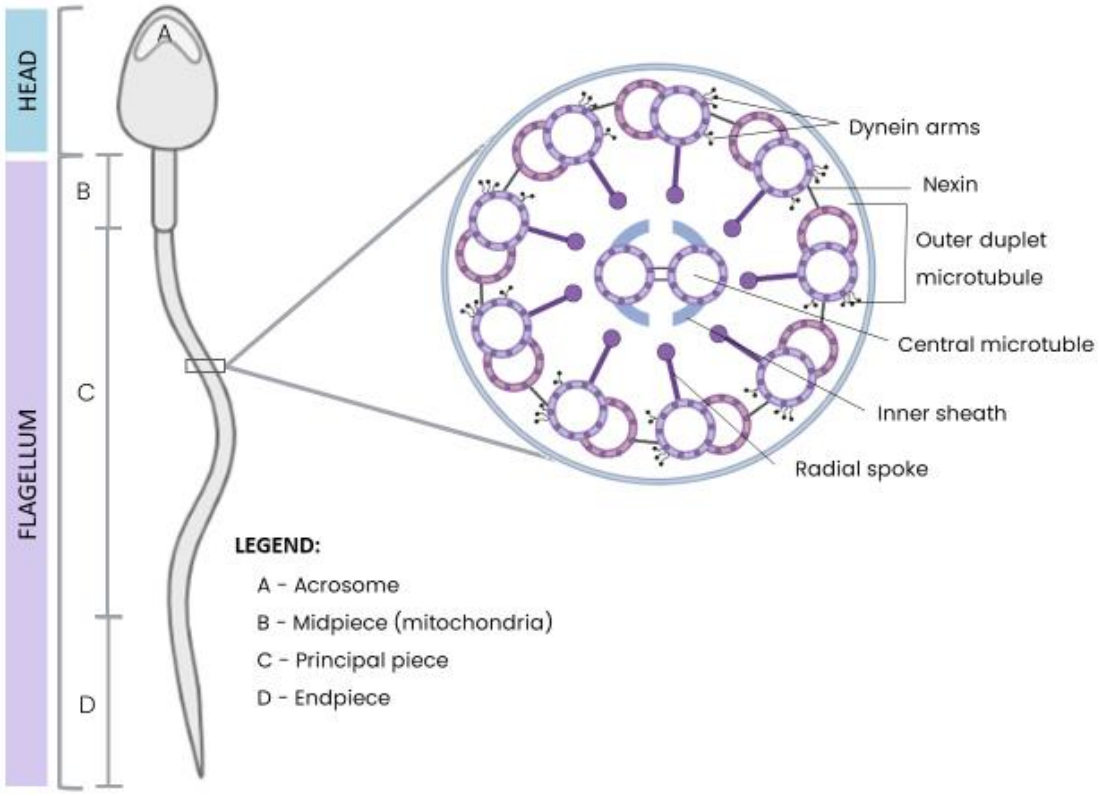


Figure I. 4 Schematic representation of sperm (left side) and axoneme (right side).

Once spermatogenesis is complete in the testis, spermatozoa are immotile and do not develop the ability to migrate until they reach the epididymis (Inaba, 2003). Sperm motility is essential for maintaining male fertility and thus a key factor in determining seminal quality (Agnihotri et al., 2016; Barbagallo et al., 2020). Nonetheless, sperm motility as well as other metabolic processes including hyperactivation, capacitation, and acrosome response depend on the synthesis of adenosine triphosphate (ATP) (Kumar & Singh, 2021).

Spermatozoa also have mitochondria, which are known as the "power house" of an eukaryotic cell (Kumar & Singh, 2021). For every molecule of glucose, mitochondria, which are restricted to the sperm midpiece, make 30 ATP molecules through oxidative phosphorylation (OXPHOS) (Barbagallo et al., 2020; Kumar & Singh, 2021; Paoli et al., 2011). The energy produced by this process is thought to be used for spermiogenesis, sperm maturation, and activation of sperm motility (Kumar & Singh, 2021). However, the fibrous sheath provides a rich source of glycolytic enzymes, enabling glycolysis, a different metabolic pathway for sperm ATP synthesis. Only two ATP molecules are produced as a result of this glycolic route for every oxidized glucose molecule. Studies indicate that, in contrast to OXPHOS, glycolysis produces energy that is mostly required for hyperactivated sperm motility (found in spermatozoa during fertilization), capacitation, and acrosome response (Kumar & Singh, 2021; Piomboni et al., 2012).

According to Bagallo et al. (2020), the characterization of the primary energy source for sperm motility is still up for debate. Despite acknowledging the significance of glycolysis as an energy source, several studies contend that the majority of the energy required for sperm movement is generated by mitochondrial respiration (Paoli et al., 2011). Others emphasize the glycolytic pathway as the primary energy generation mechanism for sperm motility in addition to the critical significance of mitochondria in energy production for the same function (Kumar & Singh, 2021). Regardless of the energy source, one of the causes of subfertility/infertility and unsuccessful fertilization is insufficient or defective sperm motility (Chakraborty & Saha, 2022).

1.5 | Thesis Aims

Bearing in mind the increasing need to safeguard patient quality of life, adjusting treatments and creating tools that allow for the sustainability of cancer care in the future, faced with the high applicability of bleomycin in oncologic therapies, essentially in AYAs, it is imperative to address one primary question: How does isolated bleomycin affect the human male reproductive function?

This works thus aimed to address this subject in two phases, which are escribed in the following two chapter:

Chapter II – Systematic review article: Compile all of the published research that discusses the impact of bleomycin alone on the male reproductive system, then carefully consider and debate the information that each and every study's findings can offer. This will serve as the foundation for comprehending what needs to be done and acknowledged in relation to the potential toxicity of bleomycin to reproduction.

Chapter III – Original article: Since there is a clear correlation between changes in sperm parameters and reproductive outcomes, sperm are considered the best marker to track the effects of bleomycin on human male reproductive function, despite the challenge of evaluating the medication's effects on human male fertility *in vivo*. Thus, bleomycin's effects on human sperm function will be investigated *in vitro* for the first time. Specifically, the effects of bleomycin will be assessed on sperm vitality, motility, DNA integrity, chromatin condensation, production of reactive oxygen species (ROS), mitochondrial membrane potential (MMP), and acrosome status in sperm. An ultrastructural analysis will also be conducted to further elucidate and illustrate some of these parameters. By better understanding the mechanisms underlying potential reproductive effects and predicting patients' reproductive function, this study will enable better management of fertility preservation by educating patients and healthcare professionals about the risks associated with this medication.

Chapter II. Beyond bleomycin-induced pulmonary toxicity its male reproductive toxicity remains unanswered 50 years after this approval

[Systematic Review Article]

This chapter is based on the following submitted manuscript:
Lobo de Almeida, A., Sousa, M., Sá, R. (2023) Beyond bleomycin-induced pulmonary toxicity, its male reproductive toxicity remains unanswered 50 years after its approval

Abstract

Aim: Cancer treatment long-term complications for men include testicular dysfunction and possible infertility. Nonetheless, the evidence for this risk for various chemotherapies is insufficient. This is the case with bleomycin, which, aside from its well-studied lung toxicity, has received little research attention for its effect on male reproduction. This systematic review sought evidence for the effects of bleomycin-cancer treatment on testicular function and fertility.

Methods: PubMed and Web of Science were searched using specific search terms, and seven animal studies met all inclusion criteria and provided an analysis of bleomycin gonadotoxicity.

Results: Overall, the results suggest that bleomycin is gonadotoxic. It has the same effect on the nuclear DNA integrity of rodent sperm as it does on cancer cells. Bleomycin-induced changes in rodent testis histopathology are most noticeable at seminiferous tubule structure level. Bleomycin also significantly decreases rodents´ seminal conventional parameters and testosterone levels, the latter of which is consistent with increased induced degeneration of Leydig cells. Seminiferous tubule necrosis and alterations in tunica albuginea thickness in rodent testis are signs of a bleomycin-induced inflammatory process similar to the pulmonary one.

Conclusion: More research, particularly human research, is required to provide strong evidence on how bleomycin affects human testicular function, including hormone function; and whether is gonadotoxic, whether it has a transient or permanent effect; how it may affect offspring; and how it can be reduced. Despite this, the current findings have important implications for clinicians in counseling patients about fertility risk and guiding requirements for fertility preservation strategies.

Keywords: Bleomycin; Chemotherapy; Testicular function; Sperm analysis; Male fertility

2.1 | Introduction

Cancer is currently a major health challenge, with 19.3 million new cases worldwide in 2020 according to the most recent estimates (Sung et al., 2021). Fortunately, thanks to advances in oncologic treatments, the overall cancer survival rate has glimpsed a significant increase in recent years (Siegel et al., 2021; Vakalopoulos et al., 2015). With this increase in survival, oncologic care focuses more on the morbidity observed in some patients, as well as on the long-term side effects of the treatments (Brannigan et al., 2021; Howell & Shalet, 2001). With increased focus on patient quality of life, new approaches and more personalized medicine emerge to try to circumvent the limitations and side effects of conventional treatments. Chemotherapy, on the other hand, remains the primary medical strategy for cancer treatment (Brannigan et al., 2021).

Chemotherapeutic agents target tumor cells in a variety of ways, but the majority of them are designed to target cells that divide quickly, making spermatogenesis a potential target (Brannigan et al., 2021; Vakalopoulos et al., 2015; Wallace & Hamish, 2011).

When considering life after cancer treatment, one of the main concerns for men of reproductive age or younger is whether they will be able to have children in the future (Vakalopoulos et al., 2015), given that more than half of male oncologic survivors want to have children (Yumura et al., 2022).

The agent used and the dose received are linked to testicular damage (Howell & Shalet, 2001, 2005). Furthermore, the patient's age and stage of testicular maturity may have an effect on the strength of the deterrent effect (Howell & Shalet, 2005). Testicular dysfunction is a common and troubling long-term side effect of cytotoxic chemotherapy. There have been reports of azoospermia and oligospermia as a result of certain chemotherapies (Howell & Shalet, 2001, 2005; Wallace & Hamish, 2011), and the resumption of spermatogenesis has proven unpredictable, as evidenced by cases of severe and permanent infertility (Vakalopoulos et al., 2015).

Unfortunately, the majority of chemotherapy protocols are multiagent (Trost & Brannigan, 2012), making it difficult to determine how one agent, in particular, affects fertility.

Bleomycin, which has a high-performance range partly because it has no myelosuppressive effects, is mostly used in combination regimens to treat diseases such as Hodgkin's and non-Hodgkin's lymphoma, as well as testicular cancer (Brandt & Gerriets, 2023; O'Sullivan et al., 2003).

The most serious toxicity associated with this glycopeptide with antibacterial and cytotoxic properties is pulmonary toxicity (Brandt & Gerriets, 2023; Reinert et al, 2013). Although the reproductive toxicity of the major combination chemotherapies that included

bleomycin has been extensively studied and described, individualized bleomycin has a limited amount of literature on it.

In light of this, this review predisposes to review the literature regarding the individual contribution of bleomycin exposure on testicular function and, as a result, fertility, creating a compilation of the state of the art on this topic and an important tool for future considerations and research.

2.2 | Bleomycin's Origins

In the late 1960s, Umezewa and colleagues isolated from cultures of *Streptomyces verticillus* a complex of water-soluble glycopeptides with antitumor and antibacterial properties that gave rise to bleomycin, one of the first anticancer agents (Umezawa & Maeda, 1967). Bleomycin was approved by the Food and Drug Administration (FDA) for the first time in 1975 to treat testicular cancer, squamous cell carcinomas, and malignant lymphomas. The FDA now approves bleomycin for a number of uses, including the treatment of both Hodgkin's disease and non-Hodgkin lymphoma (Brandt & Gerriets, 2023). Bleomycin has piqued the interest of researchers and clinicians due to its broad spectrum of cytotoxic effects and lack of myelosuppressive effects (O'Sullivan et al., 2003; Sikic et al., 1985). Bleomycin is also considered an appealing addition to several combination chemotherapy regimens, such doxorubicin, vinblastine, and dacarbazine (ABVD) or BEP (Brandt & Gerriets, 2023; O'Sullivan et al., 2003).

Bleomycin-induced cellular toxicity is caused by DNA damage, which occurs as a result of oxygen- and metal-ion-dependent mechanism. Bleomycin's iron-binding region (acid portion) and DNA-binding region (bithiazole portion) are located at the molecule's opposite ends (Figure II. 1).

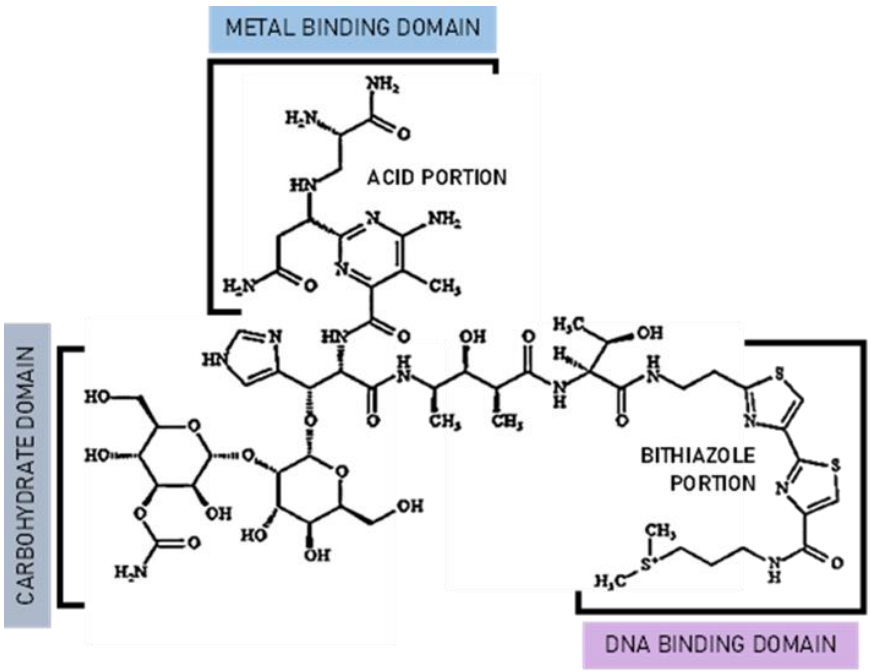


Figure II. 1 Structure of bleomycin identifying main DNA and metal binding domains.

Bleomycin binds to metal ions, specifically iron, in this last region, resulting in a reduction-oxidation reaction that generates reactive oxygen species (ROS). Bleomycin can engage in "suicide chemistry" in the absence of DNA, but in the presence of DNA, these reactive species, including highly toxic superoxide and hydroxyl radicals, cleave the C3 to C4 bond of deoxyribose (Brandt & Gerriets, 2023; Della Latta et al., 2015; Lazo, 1999; Reinert et al, 2013). These free radicals then cause the deoxyribose ring to open as a result of the abstraction of a hydrogen atom, which resulted in the generation of highly cytotoxic free base propenals (Lazo, 1999). These toxic products then participate in cell cycle arrest during the G2 and mitotic phases, halting cell replication and, as a result, tissue growth and repair (Brandt & Gerriets, 2023; Reinert et al, 2013). This compound also inhibits the synthesis and mediates damage to all three major classes of RNA – mRNA, tRNA, and rRNA – and thus protein production (Lazo, 1999; Rahaman, 2018; Reinert et al, 2013).

Bleomycin is not absorbed through the gastrointestinal tract due to its large size, positive charge, and structure, so it is administered parenterally, including intramuscularly, subcutaneously, intravenous, and intraperitoneally (Brandt & Gerriets, 2023; Lazo, 1999; Scholar, 2007). Bleomycin is rapidly absorbed and has high bioavailability, with less than 1% of the compound binding to plasma proteins and reaches its peak plasma concentration in approximately 1 hour (Brandt & Gerriets, 2023; O'Sullivan et al., 2003).

Bleomycin metabolism consists of a series of deamidation, and inactivation reactions catalyzed by bleomycin hydrolase (BLH) (Lazo, 1999). This cysteine protease from the papain superfamily metabolizes bleomycin into an inactive and nontoxic

molecule. This inactive amino causes a terminal amine to be replaced with a hydroxyl group, inhibiting DNA damage activity and cytotoxicity (Brandt & Gerriets, 2023; Chen et al., 2012; Della Latta et al., 2015; Lazo, 1999). Unlike most tissues, where bleomycin is present in trace amounts, the skin and lungs accumulate it. This is due to the slower rate of bleomycin degradation in these tissues, which is caused by decreased activity of bleomycin hydrolase (Brandt & Gerriets, 2023; Reinert et al, 2013). The tissue-specific toxicity is explained by the low levels of BLH in the skin and lungs. The most common side effects that occur when cumulative doses exceed 200 mg are flagellate erythema, hyperpigmentation, and hyperkeratosis (Chen et al., 2012; Fyfe & McKay, 2010; Reinert et al, 2013). However, while these skin side effects are not associated with an increased risk of morbidity and death and typically resolve when treatment is discontinued, the same cannot be said for bleomycin-induced pulmonary toxicity, namely pulmonary fibrosis (Della Latta et al., 2015; Fyfe & McKay, 2010). Although the incidence is less than 10%, the mortality rate for bleomycin-induced pulmonary fibrosis is between 3 and 5% in those affected (Fyfe & McKay, 2010). As a result, the most common limitation of this treatment is pulmonary toxicity.

As a result of a strong correlation between bleomycin and creatinine clearance, with a drug clearance approaching 70 mL/min/m^2, bleomycin is primarily eliminated via the kidneys, with the majority of the drug excreted in the urine (Brandt & Gerriets, 2023; Reinert et al, 2013; Scholar, 2007; Fyfe & McKay, 2010). Thus, the length of time bleomycin remains in the body is largely determined by the patient's renal function. Bleomycin has a half-life of about 2.5 hours in patients with normal renal function, but it may be longer in patients with reduced glomerular filtration rates, implying that patients with low creatinine clearance may have significantly lower bleomycin clearance (Brandt & Gerriets, 2023; Della Latta et al., 2015; Lazo, 1999; O'Sullivan et al., 2003; Reinert et al, 2013; Fyfe & McKay, 2010) and, as a result, an increase in drug's toxicity (Reinert et al, 2013).

The dosage is determined by a variety of factors, including the regimen's, tumor's, and patient's characteristics (e.g., age, weight, and comorbidities), but the total dose is limited to 300 - 400 mg, with a significant incidence of pulmonary toxicity at higher doses (Aronson, 2016; Blum et al., 1973; Yagoda et al., 1972).

2.3 | Methods

2.3.1 | Search strategy

This review was designed according to the guidelines of the 2020 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. To find original clinical and experimental studies describing the testicular damage caused by bleomycin, PubMed and Web of Science were searched up to May 23, 2023. The following search terms were quested in the titles and abstracts: “bleomycin” AND “sperm” OR “semen” OR “hormone” OR “luteinizing hormone (LH)” OR “follicle-stimulating hormone (FSH)” OR “testosterone” OR “testis” AND “fertility” OR “reproduction” and related terms.

Due to the scarcity of research describing the isolated effects of bleomycin on the male reproductive system, Google Scholar was also added with the goal of maximizing comprehensiveness and mitigating publication bias (Gusenbauer & Haddaway, 2020). Because Google Scholar provides over 389 million reports, the search was more targeted and concise, yielding clearer results by restricting the following search terms to just the title.

It should be noted that because there are not many articles written on the subject, no time constraints were imposed on the date of publication of the papers. A screening of relevant article bibliographies was also performed to find additional studies.

2.3.2 | Selection process and the eligibility criteria

First, duplicates were removed, and the titles and abstracts of the publications found were independently screened against eligibility criteria to determine whether they appeared to be researching bleomycin-induced testicular damage. Following that, full texts were examined to ensure they met the below eligibility criteria.

The inclusion criteria were:

- Articles that investigate bleomycin-induced testicular damage, including sperm analysis and/or reproductive hormone evaluation;
- Original articles containing *in vivo* and *in vitro* research.

The exclusion criteria for the review were:

- Review articles;
- Articles written in languages other than English;
- Articles focusing solely on female fertility.
- Articles that include research on combination regimens rather than isolated bleomycin.

Mention that one study (Ojo, 2017) was discovered during the bibliographic screening of the reference lists of previously included articles, but despite meeting all of the inclusion criteria, it was excluded from this review. This decision was made because it was unable to correctly interpret the results obtained due to informational gaps and inconsistencies in this article's "Material and Methods" and "Results" sections, which preclude any reliable analysis.

In total, 7 manuscripts were included in the final analysis as presented in the PRISMA diagram (Figure II. 2).

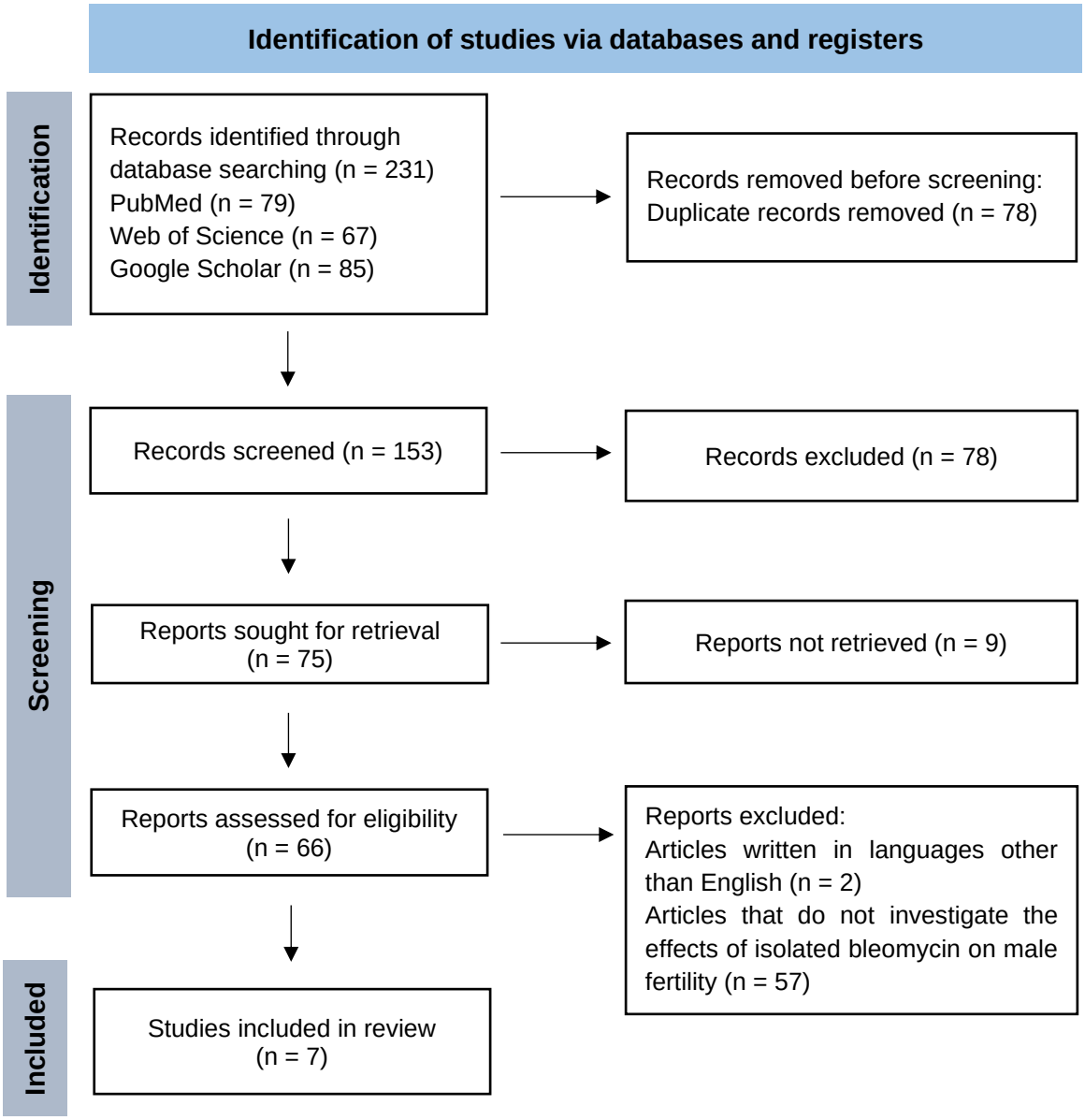


Figure II. 2 PRISMA 2020 flow diagram of article search and selection strategy to assess the effects of isolated bleomycin and reproductive function.

2.3.3 | Data extraction

The study's design (such as sample size, whether it was conducted on humans or animals, the dosage and route of bleomycin administration), as well as the specific reproductive outcomes available (such as semen parameters and hormone levels), were gathered and recorded.

2.4 | Results

2.4.1 | Studies assessing testicular function after treatment with bleomycin

This systematic review identified seven studies reporting the effect of bleomycin exposure on male fertility. Table II. 1 summarizes the studies' design.

Table II. 1 Summary of the study design and fertility outcomes evaluated in each of the studies included in this review.

Study	Design				Fertility Outcomes
	Animals	Control group	Experimental group	Schedule	
Lu & Meistrich, 1979	Male C3HHeB/FeJ mice	Saline IP	Bleomycin (> 0.01 mL/g) IP	Single dose	Sperm count Histopathology
Carreau et al., 1988	Male Sprague-Dawley rats	Saline IP	Bleomycin (20 mg/kg) IP	Single dose	Hormone analysis Histopathology
Amirshahi et al., 2014	10 male Wistar rats	Saline IP	Bleomycin (10 mg/kg) IP	2x/week/48 days	Semen analysis Hormonal analysis Antioxidant/oxidant status
Thwaini, 2015	10 male Wistar rats	Saline IP	Bleomycin (15 mg/kg) IP	3x/week/8 weeks	Sperm count Hormonal analysis Histopathology
Belhan et al., 2019	8 male rats	Saline IT	Bleomycin (7.5 mg/kg) IT	Single dose	Semen analysis
Yaghutian Nezhad et al., 2021	8 male Balb/c mice	Saline IP	Bleomycin (10 mg/kg) IP	Daily/35 days	Semen analysis Histopathology Antioxidant/oxidant status Genetic expression
Ibrahim et al., 2023	10 Albino male rats	Distilled water IP	Bleomycin (1.5 U/kg) IP	2x/week/3 weeks	Semen analysis Hormonal analysis Antioxidant/oxidant status

Abbreviations: IP, intraperitoneal administration; IT, intratracheal administration.

The rodents were used in all seven studies. Of these, 5/7 (71%) studies were conducted on rats (Carreau et al., 1988; Amirshahi et al., 2014; Thwaini, 2015; Belhan et al., 2019; Ibrahim et al., 2023), while 2/7 (28%) were conducted on mice (Lu & Meistrich, 1979; Yaghutian Nezhad et al., 2021). Different strains of rats and mice were used. Aside from an experimental group in which the animals received bleomycin injections, all studies

included a control group in which the animals received injections of saline solution (Lu & Meistrich, 1979; Carreau et al., 1988; Amirshahi et al., 2014; Thwaini, 2015; Belhan et al., 2019; Yaghutian Nezhad et al., 2021) or distilled water (Ibrahim et al., 2023) instead of bleomycin. Administration by injection included the intraperitoneal route in 6 studies (Lu & Meistrich, 1979; Carreau et al., 1988; Amirshahi et al., 2014; Thwaini, 2015; Yaghutian Nezhad et al., 2021; Ibrahim et al., 2023) and the intratracheal route in one study (Belhan et al., 2019). Within each study, the same route of administration was used for both experimental and control groups.

The outcomes under investigation covered a wide range of factors important for male fertility. The most studied outcome was sperm count, which was evaluated in 6/7 studies (except Carreau et al. (1988)), and in four of them (Amirshahi et al., 2014; Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021), a more thorough sperm analysis that included parameters such as vitality, motility, and morphology, among others, was also performed. In 5 studies (Amirshahi et al., 2014; Carreau et al., 1988; Ibrahim et al., 2023; Thwaini, 2015; Yaghutian Nezhad et al., 2021), the reproductive hormones (testosterone, and/or LH, and/or FSH) were also examined in the animals' serum, making hormonal analysis another thoroughly investigated aspect. Furthermore, in 4 of the studies (Carreau et al., 1988; Lu & Meistrich, 1979; Thwaini, 2015; Yaghutian Nezhad et al., 2021), histopathological analysis was performed to look for any potential morphological, anatomical, and cellular changes caused by bleomycin treatment in testicular tissue and other related organs. In 3 of the studies (Amirshahi et al., 2014; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021), an additional analysis was performed to assess the antioxidant/oxidant status of the testicular tissue by measuring enzymes and specific markers. A single study also looked at the gene expression of pro- and anti-apoptotic genes (Yaghutian Nezhad et al., 2021).

Furthermore, 5/7 studies had as their primary goal the evaluation of the action of potential protective agents in combination with bleomycin, with positive results for all compounds tested (Table II. 2).

Table II. 2 Protective agents evaluated against bleomycin-induced reproductive toxicity in male rodents.

Study	Protective agent evaluated	Outcome
Amirshahi et al., 2014	Royal jelly	Co-administration could effectively mitigate bleomycin's negative effects by inhibiting oxidative processes and restoring the antioxidant defense system
Thwaini, 2015	Valsartan	
Belhan et al., 2019	Theophylline	
Yaghutian Nezhad et al., 2021	Thymoquinone	
Ibrahim et al., 2023	Vitamin E and Selenium	

2.4.2 | Histopathological evidence for testicular function

Testes are crucial to male fertility, but because of their location, this organ is regarded as the most vulnerable organ against external stress (Khadivi et al., 2020). Additionally, the testis' germinal epithelium is particularly sensitive to the detrimental effects of chemotherapy (Wallace & Hamish, 2011). Table II. 3 exposes the main results of the histopathological evaluation performed in 4/7 studies included in this review, comparing in each study the exposed group with the respective control group.

Table II. 3 Summary of the histopathology outcomes in male rodents following bleomycin exposure.

Study	Histopathology	
Lu & Meistrich, 1979	Interstitial tissue or structure of tubules	NC#
Carreau et al., 1988	Testis weight/body weight ratio	NC*
Thwaini, 2015	Vacuolation of seminiferous tubules	↗#
	Detachment of basal membrane	↗#
	Number of spermatogonia and spermatocytes	↘#
	Degeneration of Leydig cells	↗#
Yaghutian Nezhad et al., 2021	Diameters of seminiferous tubules (µm)	↘*
	Thickness of tunica albuginea (µm)	↗*
	Thickness of germinal epithelium (µm)	NS
	Testis weight/body weight ratio (mg)	↘*
	Number of spermatogonia, spermatocytes, spermatids, and Sertoli cells per tubule	↘*
	Necrosis in seminiferous tubules	↗*

Abbreviations: NC, no change.
The arrows ↗ and ↘ indicate an increase and a decrease, respectively.
Statistical analysis: * significant; NS, non-significant; # significance omitted from the original manuscript.

Lu and Meistrich published the first study on the histopathology of the testes after bleomycin exposure in 1979. Testes were fixed in Bouin's solution and stained with periodic acid-Schiff-hematoxylin (PAS-H) for their study. The sections revealed no obvious changes in interstitial tissue or tubule structure (Lu & Meistrich, 1979).

More recently, Yaghutian Nezhad et al. (2021) conducted the most detailed histopathological evaluation. After testis biopsy, fixation in Bouin's solution, and hematoxylin-eosin (HE) stain of 5 µm sections, they discovered a significant increase in the thickness of the tunica albuginea, the thick fibrous capsule composed primarily of structural collagens that surrounds the testis, despite non-significant changes in germinal epithelium thickness (Yaghutian Nezhad et al., 2021). This analysis also revealed a significant reduction in the diameters of the seminiferous tubules, as well as a change in their normal structure, and clear signs of severe necrosis (Yaghutian Nezhad et al., 2021). Aside from these changes, these authors also mentioned a significant decrease in the number of spermatids and Sertoli cells, as well as a significant decrease in the number of spermatogonia and spermatocytes, as Thwaini (2015) previously reported. Thwaini's histological studies also included testes removal and fixation in Bouin's solution, as well as HE staining of 5 µm paraffin sections. Seminiferous tubule vacuolation, basal

membrane detachment, and Leydig cell degeneration were also described by these authors (Thwaini, 2015).

Yaghutian Nezhad et al. (2021) also reported a significantly lower testis weight/body weight ratio after bleomycin exposure contradicting Carreau's (1988) conclusion of no changes regarding this parameter.

2.4.3 | Semen analysis

The most fundamental method of evaluating male (in)fertility is sperm analysis. As a result, epididymal sperm was assessed in six of the studies included in this review (Table II. 4).

Table II. 4 Summary of the evaluation of seminal parameters in male rodents following bleomycin exposure and comparison of results of seminal analysis to World Health Organization (WHO) reference limits for the manuscript's year of publication.

Study	Semen Parameters		Study's values		WHO reference cut-off values
			CT Mean ± SD	EXP Mean ± SD	
Lu & Meistrich, 1979	Sperm count (fraction of untreated control)	↘#			
Amirshahi et al., 2014	Sperm count (x10^6/mL)	↘*	37.36 ± 1.15	25.77 ± 0.82	15 [a]
	Vitality (%)	↘*	86.96 ± 0.90	67.46 ± 1.24	58 [a]
	Motility (total) (%)		92.27 ± 0.37	87.54 ± 0.73	40 [a]
	Rapid progressive	↘*			
	Slow progressive	↘*			
	Non-progressive	NS			
	Immotile	NS			
	DNA fragmentation (%)	↗*			
	Mature chromatin condensation (%)	↘*			
Thwaini, 2015	Sperm count (sperm/mg of epididymis head)	↘*			
Belhan et al., 2019	Sperm count (x10^6/mL)	↘*	81.75 ± 1.75	46.50 ± 16.81	15 [a]
	Vitality (%)	↘*	85.38 ± 0.74	64.25 ± 0.88	58 [a]
	Motility (total) (%)	↘*	67.50 ± 2.67	43.37 ± 2.58	40 [a]
	Normal morphology (%)	↘*	87.13 ± 0.83	71.13 ± 0.83	4 [a]
Yaghutian Nezhad et al., 2021	Sperm count (x10^6/mL)	↘*			
	Vitality (%)	↘*			
	Motility (%)				
	Rapid progressive	↘*			
	Slow progressive	↘*			
	Non-progressive	↗*			
	Immotile	↗*			
	Normal morphology (%)	↘*			
	Mature chromatin condensation (%)	↘*			
Ibrahim et al., 2023	Sperm Count (x10^6/mL)	↘*	61.67 ± 4.41	35.00 ± 2.52	16 [b]
	Motility (total) (%)	↘*	46.00 ± 8.89	24.10 ± 3.16	42 [b]
	Normal morphology (%)	↘*	90.47 ± 1.12	87.10 ± 0.59	4 [b]

Abbreviations: CT, control group; EXP, experimental group; SD, standard deviation.
The arrows ↗ and ↘ indicate an increase and decrease, respectively.
Statistical analysis: * significant; NS, non-significant; # significance omitted from the original manuscript.
[a] (WHO, 2010).
[b] (WHO, 2021).

All of the studies that looked at sperm by spermiogram shared only one key metric, sperm count, and the results were consistent: bleomycin reduces sperm count (Amirshahi et al., 2014; Belhan et al., 2019; Lu & Meistrich, 1979; Ibrahim et al., 2023; Thwaini, 2015; Yaghutian Nezhad et al., 2021). In all of the studies, the standard method of measuring sperm concentration with a hemocytometer counting chamber by microscope observation was used, with only the diluent solution changing.

Amirshahi et al. (2014), Belhan et al. (2019), and Yaghutian Nezhad et. al. (2021) used eosin-Y and nigrosine stain to assess sperm vitality, which is a determinant of seminal quality. All of these studies found a decrease in sperm vitality (Amirshahi et al., 2014; Belhan et al., 2019; Yaghutian Nezhad et al., 2021).

Morphology was assessed in three studies using different stain techniques, including eosin-nigrosin (Belhan et al., 2019), diff-quick (Yaghutian Nezhad et al., 2021) and eosin-Y (Ibrahim et al., 2023). The decrease in normal morphology was consistent across all studies (Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021).

For four of the articles reviewed (Amirshahi et al., 2014; Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021), sperm motility was also assessed using random fields microscopy. While Belhan's (2019) and Ibrahim's (2023) studies only report to a significant decrease in total motility rate, Amirshahi et al. (2014) and Yaghutian Nezhad et al. (2021) took into account the WHO's four-category system, namely, rapidly progressive (≥ 25 m/s), slowly progressive (< 25 m/s), non-progressive, and immotile (WHO, 2010). As a result, they reported a significant decrease in rapidly and slowly progressive motility (Amirshahi et al., 2014; Yaghutian Nezhad et al., 2021), and an increase (Yaghutian Nezhad et al., 2021) in non-progressive and immotile spermatozoa.

A traditional semen analysis includes the previously mentioned parameters. The World Health Organization (WHO) defines the standards and reference limits for the analysis of sperm parameters, specifically on human samples, and the first laboratory manual for sperm analysis was published in 1980, with the 6th Edition released in 2021 (WHO, 2021). As a result, to ensure a thorough comparison of the results obtained by the various authors, we combined their results after bleomycin exposure to the WHO reference values in relation to the manuscripts' year of publication for each seminal parameter. Regardless, this was only applied to three studies because the other three (Lu & Meistrich, 1979; Thwaini, 2015; Yaghutian Nezhad et al., 2021) that performed sperm analysis did not reveal quantitative results, only qualitative results.

Aside from the decrease in some experimental group parameters in comparison to the control group, studies reported quantitative results that were superior when compared

to the WHO cut-off reference value. The exception was Ibrahim's (2023) finding, in which animals' sperm showed reduced total motility after bleomycin exposure that was lower than the WHO cut-off reference value (WHO, 2021). Furthermore, Ibrahim's study evaluated the recovery of seminal parameters after 60 days of treatment, concluding that none of the seminal parameters evaluated showed a significant recovery after this period (Ibrahim et al., 2023).

2.4.4 | Sperm DNA damage

As shown in Table II. 4, two studies also evaluated sperm DNA integrity, including the determination of sperm DNA fragmentation (Amirshahi et al., 2014) and the status of chromatin condensation (Amirshahi et al., 2014; Yaghutian Nezhad et al., 2021).

Amirshahi et al. (2014), using the acridine orange test and the aniline blue assay (AB), found a significant increase in the percentage of spermatozoa with DNA fragmentation and a decrease in normal sperm chromatin condensation, respectively. The findings of Amirshahi's (2014) on chromatin condensation were consistent with those of Yaghutian Nezhad and colleagues (2021) using the same technique.

2.4.5 | Reproductive hormone levels

Four of the seven studies included in this systematic review investigated hormonal status (Table II. 5).

Table II. 5 Summary of reproductive hormone function in male rodents following bleomycin exposure and comparison of results of reproductive hormone function to male human and mice reference limits.

Study	Reproductive Hormones		Study's values		Human reference range [a]	Mice reference range [b]
			CT Mean ± SD	EXP Mean ± SD		
Carreau et al., 1988	Testosterone (%)	↘*	100	27	NA	NA
Amirshahi et al., 2014	Testosterone (ng/mL)	↘*	4.28 ± 0.23	2.47 ± 0.17	1.73 – 7.78	0.66 – 5.4
Thwaini, 2015	LH (mIU/mL)	NS	5.3 ± 1.1	5.0 ± 0.9	1.2 – 8.6	-
	FSH (mIU/mL)	NS	5.6 ± 1.0	5.4 ± 1.0	1.3 – 19.3	-
	Testosterone (nmol/L)	↘*	2.8 ± 0.08 0.81 ± 0.02	1.37 ± 0.02 0.40 ± 0.006	- 1.73 – 7.78	- 0.66 – 5.4
Yaghutian Nezhad et al., 2021	LH (IU/mL)	↗*				
	FSH (IU/mL)	↗*				
	Testosterone (ng/mL)	↘*				
Ibrahim et al., 2023	LH (mIUu/mL)	↘*	11.27 ± 0.37	6.53 ± 0.85	1.2 – 8.6	-
	FSH (mIU/mL)	↘*	2.49 ± 0.29	0.76 ± 0.03	1.3 – 19.3	-
	Testosterone (ng/mL)	↘*	4.65 ± 0.29	0.53 ± 0.02	1.73 – 7.78	0.66 – 5.4

Abbreviations: CT, Control group; EXP, Experimental group; SD, standard deviation; FSH, Follicle Stimulating Hormone; LH, Luteinizing Hormone; NA, not applicable; (-) not available.
The arrows ↗ and ↘ indicate an increase and decrease, respectively.
Statistical analysis: * significant; NS, non-significant.
[a] (North Bristol NHS Trust, 2022).
[b] (Rocky Mountain Diagnostics, Inc, 2023)

In both clinical diagnostic and research settings, hormonal status has been primarily assessed using immunoassays (Carmina et al., 2019). These assays involve an antigen-antibody reaction and may include immunoradiometric assay, which was used by Amirshahi et al. (2014), Yaghutian Nezhad et al. (2021) and Carreau et al. (1988), and enzyme-linked immunosorbent assay (ELISA), which was used by Ibrahim et al. (2023) and Thwaini (2015).

Both testosterone and sperm are produced by the testes, and both processes are regulated by the hormones LH and FSH. As a result, testosterone levels in the blood were measured in all five studies that looked at reproductive hormone function after bleomycin exposure (Amirshahi et al., 2014; Carreau et al., 1988; Ibrahim et al., 2023; Thwaini, 2015; Yaghutian Nezhad et al., 2021). All of these studies point to a significant decrease in testosterone levels following bleomycin treatment.

However, while testosterone serum levels were found to be concordant, LH and FSH serum levels were not. Only the three most recent manuscripts measured LH and FSH serum levels after bleomycin exposure (Ibrahim et al., 2023; Thwaini, 2015; Yaghutian Nezhad et al., 2021). Bleomycin did not cause significant changes in LH and FSH levels in Thwaini's study (2015), implying that endocrine cells are resistant to this

cytotoxicity. Yaghutian Nezhad et al. (2021) discovered an increase in both hormone serum levels, whereas Ibrahim et al. (2023) discovered a significant decrease.

Ibrahim also assessed the serum hormone levels after 60 days of treatment, finding no significant changes in testosterone levels but a significant decrease in FSH levels and an increase in LH levels (Ibrahim et al., 2023).

Carreau and colleagues (1988) also performed an *in vivo* assay to evaluate the interference of LH in testosterone levels and the recovery of testosterone levels after 5, 10, and 30 days of bleomycin injection. Unlike Ibrahim's (2023) findings, the results of this experiment revealed that serum testosterone levels drop sharply after 5 days but gradually, though not completely, recover after 30 days. Furthermore, as measured by crude testicular cell suspensions, the treated and untreated groups' basal Leydig cell testosterone synthesis decreased after 5 days and recovered completely after 30 days. Under *in vitro* LH stimulation, testosterone production in the untreated group's cellular suspension was also investigated. The respective medium was supplemented with various concentrations of bleomycin, and the results revealed that, under LH stimulation, testosterone levels decrease with an increase in bleomycin concentration, which does not occur in the absence of this hormonal stimulation (Ibrahim et al., 2023).

Similarly to the sperm analysis, we compared the hormone levels obtained after bleomycin exposure to human reference values (Table II. 5). Apart from the experimental group's FSH levels and the control group's LH levels in Ibrahim's (2023) study, which are below the minimum and above the maximum of the range, respectively, all studies' LH and FSH levels were included in the human reference range.

Testosterone is the only hormone for which reference ranges exist for both humans and mice. Though Amirshahi's testosterone levels in both the control and experimental groups are normal in comparison to both ranges (Amirshahi et al., 2014), only the experimental group's animals became deficient in this hormone when compared to the range for mice in Thwaini's (2015) study. In this study, both the control and experimental groups' animals had lower testosterone levels than the human reference range (Thwaini, 2015). Finally, according to Ibrahim's findings, the experimental group's (Ibrahim et al., 2023) animals had lower testosterone levels than the control group's mice, putting them below the recommended levels for this hormone. Finally, only in Ibrahim's research was a significant decrease in testosterone levels in the experimental group compared to the control group observed, which was sufficient to lower the mice's testosterone reference levels (Ibrahim et al., 2023).

2.4.6 | Antioxidant/oxidant status in testicular function

Three studies (Amirshahi et al., 2014; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021) investigated the effect of bleomycin on the antioxidant/oxidant status of the testes by measuring levels of malondialdehyde (MDA; lipid peroxidation) and three antioxidant enzymes: catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) (Table II. 6).

Table II. 6 Summary of testicular tissue antioxidant/oxidant status in male rodents following bleomycin exposure.

Study	Antioxidant/oxidant status	
Amirshahi et al., 2014	MDA (µmol/g tissue)	↗*
Yaghutian Nezhad et al., 2021	MDA (nmol/mg protein)	↗*
Ibrahim et al., 2023	MDA (nmol/g)	↗*
	CAT (u/g)	↘*
	GPx (mu/mL)	↘*
	SOD (u/mg)	↘*

Abbreviations: MDA, malondialdehyde; CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase.
The arrows ↗ and ↘ indicate an increase and decrease, respectively.
Statistical analysis: * significant; NS, non-significant.

Different methodologies were used to assess the levels of lipid peroxidation. The standard determination of MDA based on the production of a pink pigment as a result of the reaction between one molecule of MDA with two molecules of thiobarbituric acid (Dutta et al., 2019) was performed by Amirshahi et al. (2014) and Yaghutian Nezhad et al. (2021). Ibrahim et al. (2023), on the other hand, used specific biodiagnostic kits based on the Kjeldahl method (Sáez-Plaza et al., 2013) a reference method for the determination of these proteins according to the assessment of organic nitrogen, involving a three-step process (digestion, distillation, and titration).

Bleomycin-induced oxidative stress (OS) in the testes was observed in all studies by increasing lipid peroxidation (Amirshahi et al., 2014; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021), with only one study observing a decrease in the physiological levels of the antioxidant enzymes CAT, GPx, and SOD (Ibrahim et al., 2023). This most recent study looked at the recovery of these proteins 60 days after treatment and found that there were no significant changes in MDA levels after this time, but all of the antioxidant enzymes tested showed a significant decline, which may indicate a possible recovery of these levels (Ibrahim et al., 2023).

2.4.7 | Expression of apoptosis-related genes

Only one study (Yaghutian Nezhad et al., 2021) looked at the expression of two distinct genes, Bcl2L1 and Bax, which act as apoptosis inhibitor and activator of the intrinsic apoptotic signaling pathway, respectively (Table II. 7).

Table II. 7 Summary of gene expression in male rodents following bleomycin exposure.

Study	Gene expression	
Yaghutian Nezhad et al., 2021	Bcl2L1 (apoptotic inhibitor)	↘
	Bax (apoptotic activator)	NS

The arrows ↗ and ↘ indicate an increase and decrease, respectively.
Statistical analysis: * significant; NS, non-significant.

To assess the expression of apoptotic genes, RNA extraction from testes was performed, followed by cDNA synthesis to allow for the realization of a real-time RT-PCR. The levels of Bax gene expression did not differ significantly between the experimental and control groups, whereas Bcl2L1 expression decreased significantly in the presence of bleomycin.

2.5 | Discussion

Bleomycin causes testicular injury, according to all seven studies included in this systematic review, resulting in changes in sperm parameters, sperm DNA integrity, hormone secretion, testis histology, and antioxidant/oxidant status of the studied rodent models' testicular tissue.

Despite the small number of studies examining the specific impact of bleomycin isolated on male fertility, the findings suggest that this drug may have a negative effect on sperm count (Amirshahi et al., 2014; Belhan et al., 2019; Lu & Meistrich, 1979; Ibrahim et al., 2023; Thwaini, 2015; Yaghutian Nezhad et al., 2021), vitality (Amirshahi et al., 2014; Belhan et al., 2019; Yaghutian Nezhad et al., 2021), motility (Amirshahi et al., 2014; Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021), morphology (Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021) and mature chromatin condensation (Amirshahi et al., 2014; Yaghutian Nezhad et al., 2021), as well as reduce the testosterone production and secretion (Carreau et al., 1988; Ibrahim et al., 2023; Thwaini, 2015; Yaghutian Nezhad et al., 2021), or increase MDA levels, a sign of high lipid peroxidation (Amirshahi et al., 2014; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021). Certain parameters, however, were only studied in a single publication, making it impossible to draw valid conclusions about how this drug will affect the integrity of sperm

DNA, antioxidant enzyme levels, or gene expression. Furthermore, there were some differences in study design (dosages and injection schedule) between studies, which, along with the variety of strains involved, help to explain the differences in results between studies but make any valid conclusion more difficult to infer.

Dosage selection is an important consideration in the design of the reproductive toxicity study. This decision should be based on preliminary studies and literature on the drug's pharmacodynamic and pharmacokinetic studies (ICH, 2005). The selection of the dosage and the schedule of bleomycin's administration were not explained in the articles reviewed, which could explain the observed heterogeneity. As a result, future studies should always be advised to provide this information, and preliminary studies should be performed and included in manuscripts in the case of previous missing data.

The route of administration chosen should be similar to that used in humans (ICH, 2005), as it is critical in the pharmacokinetic, pharmacodynamic, and toxicity of pharmacological agents (Al Shovaib et al., 2020). Unfortunately, intraperitoneal (IP) administration was used in the majority of the studies reviewed, and bleomycin was administered intratracheally (IT) in one study, in contrast to the intravenous administration used in humans (Al Shovaib et al., 2020). The IP route is one of the most commonly used in rodent studies because it is simple and quick, but it is only used in clinic in a few cases. Though this is a valid option when the goal of the study is to evaluate the drug's effects rather than its pharmacokinetics (Al Shovaib et al., 2020), its use in experimental studies is dubious. The IT route is commonly used for bleomycin when the goal of the study is to create an *in vivo* model of pulmonary fibrosis (Barbayianni et al., 2018). As a result, future studies that purport to mimic human exposure should be advised to prefer drug administration via intravenous or intraperitoneal injections when the former is not possible.

Another important point to mention is the number of animals used in the study. Though the number of animals was sufficient in five of the seven manuscripts included in this review, two of them (Carreau et al., 1988; Lu & Meistrich, 1979) failed to mention this aspect of the study design, making it difficult to assess the validity of the results obtained in these studies.

Histopathology of the testis is considered to be the most sensitive method of detecting the effects of pharmacological compounds on spermatogenesis and providing an assessment of their male reproductive toxicity (Hess & Moore, 1993; ICH, 2005; Lanning et al., 2002; Vidal & Whitney, 2014). The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use defined it as a good pathological and histopathological examination of the male reproductive organs, the use of Bouin's fixation and paraffin embedding, and the avoidance of formalin for testes (Hess

& Moore, 1993; ICH, 2005; Lanning et al., 2002; McLachlan et al., 2007). The authors of the articles reviewed (Lu & Meistrich, 1979; Thwaini, 2015; Yaghutian Nezhad et al., 2021) chose Bouin's fixation solution and paraffin embedding media that allowed for detailed and adequate detection of differences between treated and control tissue (Hess & Moore, 1993; Lanning et al., 2002). The HE stain, which has a wide range of applications and is simple to use, is an excellent method for analyzing testicular morphology on paraffin sections and is thus sufficient for cell identification and location (ICH, 2005; Lanning et al., 2002; McLachlan et al., 2007). Furthermore, the PAS-H stain on the testis provides useful information about the stage of spermatogenesis (Lanning et al., 2002). As a result, both stain techniques should be used in a histopathological analysis to determine the toxicity of a compound. However, in none of the studies examined, these two techniques were used concurrently, with HE staining being the most used. Unfortunately, the biggest issue was the disparity in interpretations (Hess & Moore, 1993), which resulted in heterogeneity of the aspects reported in each study, making valid inferences impossible. As a result, it is critical to identify the key aspects that must be observed across studies in order to determine whether there are common alterations caused by the treatment/compound across studies. Several articles (McLachlan et al., 2007; Sasaki et al., 2011) have been published that provide some enumeration of specific types of histopathological lesions or morphologic changes in order to provide a uniformization of the testis histopathological states. In the future, a distinct list should be included in a universal guideline or recommendation for the evaluation of testicular toxicity, assisting in the clarification and comparison of results.

Carreau et al. (1988) and Yaghutian Nezhad et al. (2021) calculated the testis weight/body weight ratio and compared it between untreated and treated groups, allowing them to predict testicular function, which is an important indicator in the study of drug's adverse effects. Testicular weight is less affected by body weight than most other tissues, and its decrease reflects germ cell loss as well as seminiferous tubule lumen contraction, in contrast to increased weight, which indicates an increase in fluid content (Lanning et al., 2002). Although Carreau et al. (1988) reported no changes in the testis weight/body weight ratio in 1988, Yaghutian Nezhad et al. (2021) reported a significant decrease in this ratio after bleomycin exposure more than 30 years later. This discordance in observations is most likely due to differences in bleomycin administration schedules and dosages used. Carreau et al. (1988) administered bleomycin to the experimental group's animals in a single dose, whereas Yaghuttian Nezhad et al. (2021) administered it to them daily for 35 days, despite the lower dose.

Despite the fact that Lu and Meistrich (1979) described interstitial tissue and the structure of seminiferous tubules without any changes in 1979, all subsequent reports (Thwaini, 2015; Yaghutian Nezhad et al., 2021) mentioned bleomycin-induced changes in these structures. It should be noted that Lu and his colleague (Lu & Meistrich, 1979) used a single dose of lower doses of bleomycin in their assays, which could explain the absence of structural alterations.

The testicular parenchyma is made up of convoluted seminiferous tubules separated by interstitial tissue and surrounded by a three-layered capsule (Creasy & Chapin, 2013). Furthermore, because seminiferous tubules make up the majority of the testicular volume (Sharma & Agarwal, 2011), the decrease in testis weight reported by Yaghutian Nezhad et al. (2021) is consistent with the decrease in seminiferous tubule diameters observed in the same study. Clusters of Leydig cells are found in the interstitium of the testes, near the seminiferous tubules. Sertoli cells, which are found on the periphery of those seminiferous tubules, are responsible for spermatogenesis. Spermatogenesis occurs from the peripheral tubule basal membrane, where Sertoli cells are found, to the lumen, where newly formed spermatozoa are found (Gurung et al., 2022; Sharma & Agarwal, 2011). Bleomycin was found in two studies (Thwaini, 2015; Yaghutian Nezhad et al., 2021) to be responsible for a decrease in the number of germ cells involved in the various stages of spermatogenesis per seminiferous tubule, namely spermatogonia, spermatocytes, and spermatids. Given that vacuoles frequently follow generalized germinal cell degeneration/atrophy, where they likely represent gaps left by missing germ cells, this decrease may be related to increased vacuolation of seminiferous tubules. This decrease in the number of cells filling the seminiferous tubules may also be associated with the previously mentioned decrease in tubule diameter, as well as with a detachment of the basal membrane (Thwaini, 2015).

Sertoli cells, which account for approximately 40% of the volume of the germinal epithelium (Creasy & Chapin, 2013; Sharma & Agarwal, 2011), are responsible for adult testicular volume and sperm production (Weinbauer et al., 2010), as well as defining compartments for adjacent germ line cells with their membranes (Creasy & Chapin, 2013; Shalet, 2009; Sharma & Agarwal, 2011). Despite non-significant changes in germinal epithelium thickness in bleomycin-treated animals, which are directly related to germ cell division, it was also found a significantly lower number of Sertoli cells per tubule (Yaghutian Nezhad et al., 2021). This finding may explain why there are fewer germ cells (Thwaini, 2015; Yaghutian Nezhad et al., 2021). Sertoli cells are also in charge of producing seminiferous tubular fluid. Therefore, changes in its functionality may also linked to the previously mentioned decreases in tubular diameter. Furthermore, all studies

shared one key metric, sperm count, and the findings were consistent in stating that bleomycin reduces sperm count (Amirshahi et al., 2014; Belhan et al., 2019; Ibrahim et al., 2023; Lu & Meistrich, 1979; Thwaini, 2015; Yaghutian Nezhad et al., 2021). This finding is compatible with previously reported structural changes and deterioration of the seminiferous tubules (Yaghutian Nezhad et al., 2021), decreased testis weight/body weight ratio (Carreau et al., 1988; Yaghutian Nezhad et al., 2021), and decrease in the number of germ line cells and Sertoli cells confirmed by Yaghutian Nezhad et al. (2021) and Thwaini (2015).

Significant expansion of the tunica albuginea, a structure primarily composed of collagens, was also observed. Given the nature of this tissue, this thickening could be the result of collagen accumulation (Reinert et al, 2013). In fact, a well-documented side effect of bleomycin is pulmonary fibrosis caused by increasing collagen production (Della Latta et al., 2015; Reinert et al, 2013). As a result, tunica albuginea's thickening could be caused by the same inflammatory process that occurs in the lungs. Aside from the previously described histological alterations in the normal structure of seminiferous tubules, severe necrosis was also described (Yaghutian Nezhad et al., 2021). Necrosis and disorganization of tubular contents are also signs of an inflammatory process (Lanning et al., 2002), implying that all of the changes may be linked. Bleomycin then appears to cause an inflammatory process that has a significant impact on the structure and organization of the seminiferous tubules, leading to changes in the rate of spermatogenesis.

Leydig cells, in turn, are in charge of testosterone production and secretion. The function of Leydig and Sertoli cells is regulated by the hormones LH and FSH, respectively (Ilacqua et.al., 2018; Sharma & Agarwal, 2011; Weinbauer et al., 2010), whose secretion is controlled by gonadotropin-releasing hormone, which is secreted by the hypothalamic-pituitary-testicular axis (Gurung et al., 2022). Thus, LH stimulates Leydig cells to synthesize and secrete androgens, specifically testosterone, whereas FSH stimulates Sertoli cell proliferation and thus promotes spermatogenesis (Ilacqua et.al., 2018; Shalet, 2009; Sharma & Agarwal, 2011). Histological analysis also revealed a greater degeneration of Leydig cells in bleomycin-treated animals (Thwaini, 2015), implying a decrease in testosterone levels, as reported in all hormonal studies (Amirshahi et al., 2014; Carreau et al., 1988; Ibrahim et al., 2023; Thwaini, 2015; Yaghutian Nezhad et al., 2021). Because testosterone acts on Sertoli cells to stimulate spermatogenesis, a decrease in its serum levels could also explain the reported decreased sperm count and number of germ line cells. Given this hormonal control, the degeneration of Leydig cells, as well as the reduced number of Sertoli cells and, consequently, germ line cells after

bleomycin exposure, could be linked to changes in LH and FSH secretion, respectively. Furthermore, because LH and testosterone levels are regulated by negative feedback, decreased testosterone levels and/or Leydig cell degeneration should cause the hypothalamus and anterior pituitary to secrete LH, resulting in an increase in its levels (Sharma & Agarwal, 2011). However, the results obtained for FSH and LH levels are inconsistent (Ibrahim et al., 2023; Thwaini, 2015; Yaghutian Nezhad et al., 2021), making it impossible to draw any conclusions or infer whether the cytotoxic action of bleomycin occurs at the level of the cells that release these hormones or at the level of hormonal control by the hypothalamic-pituitary axis (Vidal & Whitney, 2014). These contradictory findings on serum LH and FSH levels after bleomycin treatment could be attributed to differences in experimental design, such as the type of animal model used or the schedule or dosage of bleomycin treatment. More research is therefore needed to understand how bleomycin affects gonadotropin secretion, which is essential for testicular function.

In all studies compilated, when a seminal analysis was carried out, the sperm samples provided from cauda epididymis. Indeed, this is an excellent source because spermatozoa mature and become motile in this organ (ICH, 2005). Only three studies (Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021) evaluated all the conventional sperm parameters (sperm count, motility, and morphology). However, it is imperative that these parameters be examined in a seminal analysis, providing complete and comparative evidence. The techniques used to measure these parameters are well-established, but this analysis is highly subjective, so reporting the values should be standard practice to allow for analysis and comparison between articles of the respective values. Despite this, only three authors show the mean values of each parameter evaluated (Amirshahi et al., 2014; Belhan et al., 2019; Ibrahim et al., 2023).

Though never at levels below the WHO cut-offs, sperm vitality was reduced, which could be explained in part by bleomycin-induced structural changes in the seminiferous tubules or inflammatory process. This decreased sperm vitality following bleomycin treatment could also be linked to significantly lower percentages of total (Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021) and progressive sperm motility (Amirshahi et al., 2014; Yaghutian Nezhad et al., 2021). Moreover, normal sperm morphology was reduced in bleomycin-exposed animals (Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021). Morphological abnormalities can also lead to decreased vitality and motility because they can affect sperm survival and functionality. Furthermore, abnormal morphology is frequently caused by a defect in the spermatogenesis sequence (Asadi et.al., 2021), so this abnormality may be the result of the histological changes that have also been reported. It is also important to note that,

while the drug dosage and schedule of administration used in each study differ, making comparison of the results of each study difficult, it allows one to discuss whether the toxicity is immediate or appears after continuous administration. In fact, sperm count, vitality, total motility, and normal morphology all decreased immediately after a single bleomycin administration (Belhan et al., 2019; Lu & Meistrich, 1979). To determine the potential drug toxicity on spermatogenesis as well as its possible cumulative effect, future studies should evaluate sperm parameters after a single dose as well as repeated doses. Furthermore, it is suggested that analysis be performed a while after the end of treatments to determine the possibility of seminal parameter recovery. Like this, researchers will be able to determine whether the effects on spermatogenesis are temporary or permanent. Only Ibrahim et al. (2023) looked at sperm count, motility, and morphology after 60 days of treatment to see if they could recover. The duration of spermatogenesis in rodents is reportedly 63 days (ICH, 2005), and while 60 days were expected to be long enough to see some changes in these parameters in bleomycin-treated animals, no significant changes were observed. This finding not only objectifies bleomycin's long-lasting and possibly permanent effect but also reinforces bleomycin-induced structural changes in seminiferous tubules. Nonetheless, more research is needed to confirm bleomycin's long-term effect.

Conventional sperm quality parameters do not accurately predict male fertility (Wang & Swerdloff, 2014). Newer tests to determine sperm quality and function have been developed, and they have strong associations with male fertility. This comprehensive examination includes the evaluation of sperm DNA integrity (Bucar et.al., 2014; Sá et.al., 2015). The association between increased sperm DNA damage (Amirshahi et al., 2014) after bleomycin exposure and decreased mature chromatin condensation (Amirshahi et al., 2014; Yaghutian Nezhad et al., 2021) is based on the spermatogenesis process. In this sequence of events, sperm histones are replaced by protamines, which are approximately half the size of histones, promoting extreme compaction of DNA and protecting it from potential damage, in addition to its action during the checkpoint of spermatogenesis (Arafa et.al., 2020; Sharma & Agarwal, 2011). Incomplete sperm maturation results in a protamine deficiency, which increases the sperm's susceptibility to DNA damage (Sharma & Agarwal, 2011). Abnormal protamine expression is likewise described as reflecting a general spermatogenesis abnormality (Sharma & Agarwal, 2011), so these findings regarding the loss of correct chromatin condensation are consistent with alterations in spermatogenesis functionality reported in histological studies, as well as abnormal morphology also observed, which increase the

risk of apoptosis, DNA damage, and abnormal reactive ROS production (Asadi et.al., 2021).

Despite the testicular microenvironment's low oxygen tensions, this tissue is susceptible to OS due to the abundance of highly unsaturated fatty acids (especially 20:4 and 22:6) and the presence of potential ROS-generating systems (Kaur et.al., 2006). To counteract this threat, the testes have evolved a sophisticated array of antioxidant systems that include both enzymatic and non-enzymatic components. In terms of the enzymatic components of this defense system, the induction of OS in the testes results in the induction of mRNA species for SOD, GPx, and glutathione-S-transferase (GST) activities via the NF-B pathway (Kaur et.al., 2006). In brief, the major ROS generation and metabolism pathways are as follows: Superoxide can be produced by specialized enzymes such as xanthine or NADPH oxidases, or it can be produced as a byproduct of cellular metabolism, particularly the mitochondrial electron transport chain; then SOD converts superoxide to hydrogen peroxide (H_2O_2), which must be removed from the system as soon as possible; to accomplish this, CAT or peroxidases (predominant in the testes), such as GPxs, which use reduced glutathione (GSH) as an electron donor, are commonly used (Cecerska-Heryć et.al., 2021; Chiavaroli et.al., 2011). Ibrahim and colleagues (2023) investigated the levels of several antioxidant enzymes that protect against or mitigate ROS damage: SOD, which catalyzes the conversion of O_2^- into H_2O_2 and oxygen; CAT, which catalyzes the conversion of H_2O_2 into oxygen and water; and GPx, which is responsible for glutathione oxidation (Cecerska-Heryć et.al., 2021; Chiavaroli et.al., 2011). Bleomycin was discovered to inhibit the activity of these enzymes, which is consistent with the previously documented role of anticancer drugs, including bleomycin, in suppressing the cellular antioxidant system (Baetas et.al., 2019; Takeshima et.al., 2019). The dysregulation of this antioxidant system raises ROS levels. When the level of ROS exceeds this physiological limit, it can cause OS, which has a negative impact on sperm quality and fertility potential (Dutta et al., 2019).

The plasma membranes of spermatozoa contain a significant amount of lipids in the form of polyunsaturated fatty acids, making them an appealing target for oxidative damage. As a consequence, an increase in ROS levels can result in lipid peroxidation, which leads to the loss of nearly all of the lipid content of the membranes and abnormal sperm function (Dutta et al., 2019). One of the most studied and stable products of this lipid peroxidation is MDA. It has been used as a biochemical marker of OS, allowing the antioxidant/oxidant status to be monitored (Dutta et al., 2019; Chiavaroli et.al., 2011). According to three authors (Amirshahi et al., 2014; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021), bleomycin can reduce the amount of antioxidant enzymes, which causes an

increase in ROS generation and results in an OS status, which increases MDA levels. These findings indicate that bleomycin causes lipid peroxidation as a result of increased ROS production. Furthermore, in addition to reducing the levels of antioxidant enzymes, bleomycin's cytotoxicity involves the formation of ROS itself, as previously described (Brandt & Gerriets, 2023; Lazo, 1999), which leads to a higher OS status in testis and sperm in particular. This OS status and subsequent lipid peroxidation are linked to sperm functionality loss, as evidenced by a decrease in sperm motility (Amirshahi et al., 2014; Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021), as well as sperm plasma membrane rupture, which also explains the decrease in sperm vitality (Amirshahi et al., 2014; Belhan et al., 2019; Yaghutian Nezhad et al., 2021). Bleomycin's high ROS production could also be one of the causes of some observed histological changes (Thwaini, 2015; Yaghutian Nezhad et al., 2021), and sperm DNA fragmentation (Amirshahi et al., 2014).

Aside from the OS, which causes DNA damage, aberrant apoptosis is a major cause of DNA damage (Yang et.al., 2018). In fact, some factors, such as ROS and drugs, activate the apoptotic cascade (Asadi et.al., 2021). Apoptosis is defined as a type of programmed cell death in which surplus, aged, or genetically damaged cells are eliminated. During spermatogenesis, germ cells die in the testes to supply the ideal germ cell ratio to Sertoli cells. Thus, excess cells produced during this process are killed by apoptosis, which is primarily dependent on the cooperation of Bcl-xL and Bax (Asadi et.al., 2021; Mostafa et.al., 2014). Both of these proteins are members of the B-cell lymphoma 2 (Bcl2) family, which is involved in the regulation of the apoptotic cascade, either inhibiting (Bcl2 and Bcl2L1) or promoting (Bax, Bak, and Bad) apoptosis (Mostafa et.al., 2014). Measuring apoptosis or the expression of genes associated with this process may thus be a powerful predictor of sperm quality and, as a result, male fertility (Asadi et.al., 2021). Yaghutian Nezhad used the remaining testis tissue to examine apoptotic gene expression, which revealed a decrease in Bcl2L1 expression caused by bleomycin, as well as non-significant changes in Bax expression (Yaghutian Nezhad et al., 2021). The reduction in this anti-apoptotic protein substantiates bleomycin-induced apoptosis. According to other studies (Mostafa et.al., 2014), this significant decrease in seminal Bcl2L1 is positively correlated with sperm count, motility, and normal morphology, all of which showed a significant decrease after bleomycin exposure. Additionally, Bcl2L1 is an apoptotic inhibitor that reduces ROS synthesis and consequently safeguards the cells (Asadi et.al., 2021). The reduction in apoptotic inhibitor gene expression is consistent with the induction of sperm DNA damage (Amirshahi et al., 2014) and OS (Amirshahi et al., 2014; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021), as well as seminiferous tubule

degeneration (Thwaini, 2015; Yaghutian Nezhad et al., 2021) caused by bleomycin treatment and a decrease in germ line cell number (Thwaini, 2015; Yaghutian Nezhad et al., 2021) via apoptosis.

2.6 | Conclusion

Bleomycin's potential reproductive toxicity has not been investigated or reported as thoroughly as its pulmonary toxicity. There have only been seven studies on the effect of bleomycin on male fertility, and all of them used animal models to infer the possible alterations in human reproduction. Furthermore, despite the scarcity of data on the effect of bleomycin alone on male fertility, the majority of current studies available and included in this review place a strong emphasis on evaluating the protective effects of various compounds and antioxidants on this bleomycin-induced toxicity, which remains poorly understood and undefined. It thus represents one of the major limitations for accumulating strong scientific evidence on how bleomycin alone affects testicular function and, as a result, male fertility potential.

Because there have been no human studies, conclusions must be drawn from existing animal studies. Keeping in mind the difficulties and ethical issues surrounding human *in vivo* studies, the truth is that, while rats and mice are the most commonly used animal models in experimental studies worldwide, these models have some limitations that affect how well the results can be translated to humans (Martinez, 2022). Unfortunately, the data gated by studies on rats and mice may differ from the results obtained in humans due to differences in study design (drug dose, route, and schedule of administration), but the genetic and physiologic differences between species are the most significant. The low number of spermatozoa per ejaculated compared to rats, for example, is one of the main differences between humans and other mammals in terms of reproductive issues, so the human sperm count is closer to the number that will produce maximum fertility (Working, 1988). Spermatogenesis is more efficient in mouse and rat models, resulting in higher fertility rates than in humans (Martinez, 2022; Working, 1988). Besides, in animal models, the percentage of motile and morphologically normal sperm is higher than in human sperm (Working, 1988). Because of these differences, comparing human and animal models is difficult and risky, and small changes in sperm parameters or testis histology in animal models may eventually be amplified in humans. This review identified a gap, noting that, in addition to more studies to back up the reported effects, human *in vivo* or *in vitro* studies are needed to understand the true impact of this chemotherapeutic agent on the human male reproductive system.

Human reproductive toxicity assessments are typically limited to assessing sperm quality, including sperm count, motility, and morphology, as well as new parameters such as DNA integrity or OS status. This is due to the difficulties in obtaining human testicular biopsies (Working, 1988). An ultrasound examination, on the other hand, can be addicted to the sperm analysis, providing information about testicular volume, for example, or blood tests to evaluate hormone levels, assisting in determining the impact of bleomycin on testicular function.

Whether these effects are temporary or permanent, how they affect seminal quality, fertility, and pregnancy rates, and whether they have any effect on offspring is also critical.

In conclusion, while the data gathered by the manuscripts included in this review demonstrated that bleomycin has a significant and negative effect on testicular function, as evidenced by changes in sperm parameters, testosterone levels, OS status, and testis histology, more research is required to provide substantial evidence about the effects of bleomycin isolated on male fertility. This evidence will be critical in providing oncologists with all of the information they need to transmit to oncologic patients, allowing them to make an informed and conscious decision about future reproductive potential and fertility preservation.

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Chapter III. Bleomycin *in vitro* exposure decreases markers of human male gamete competence

[Original Article]

This chapter is based on the following submitted manuscript:
Lobo de Almeida, A., Gonçalves, A., Barros, A., Sousa, M., Sá, R. (2023) Bleomycin *in vitro* exposure decreases markers of human male gamete competence

Abstract

Study question: Does exposure to bleomycin impact the *in vitro* reproductive competency of male human sperm?

Summary answer: The antitumor antibiotic bleomycin has a deleterious effect on human male gamete's reproductive competence that can potentially impact male fertility.

What is known already: Bleomycin is a chemotherapeutic agent used to treat several cancers that affect males of reproductive age; nevertheless, its effect on human reproductive potential alone has not been established before. The scarce data on this topic pertains to *in vivo* animal research and lends credence to the viability of this investigation.

Study design, size, duration: This study enrolled 45 participants of reproductive age between November 2022 and June 2023 undergoing clinical semen analysis. Participants with normozoospermic sperm parameters donated the remaining ejaculate for research.

Participants/materials, setting, methods: After clinical semen examination, sperm were extracted from seminal plasma in each participant's ejaculate and diluted to a concentration of $16x10^6$ sperm/mL. *In vitro* incubation was then performed in either sperm preparation medium (SPM) or supplemented with bleomycin (100 µg/mL). The outcomes of *in vitro* human sperm competence were compared between raw sperm, sperm incubated with SPM and the exposed group. Human sperm competence was assessed using several indicators, which included sperm motility, vitality, DNA and acrosome integrity, and mitochondrial membrane potential. Transmission electron microscopy was additionally employed to correlate the ultrastructural morphological information with functional assays.

Main results and the role of chance: The median age of the participants, who were all of reproductive age, was 36. The World Health Organization's criterion of normozoospermia was met by the raw sperm parameters. No participant had received a cancer diagnosis or had undergone chemotherapy, radiation therapy, or both prior to the current trial. When compared to raw and control sperm, bleomycin exposure for two hours *in vitro* significantly ($P < 0.001$) decreased sperm vitality, sperm motility, and sperm chromatin condensation. Additionally, bleomycin significantly ($P < 0.001$) increased sperm DNA fragmentation (sDNAfrag) and the proportion of sperm with low mitochondrial membrane potential (MMP) in comparison to raw and control sperm. Bleomycin can cause sperm DNA strand breaks that are both significantly ($P < 0.001$) and nonspecifically (Head staining pattern), but it also shows a preference for histone-rich regions (post-acrossomic region (PAR) staining pattern). Moreover, bleomycin significantly ($P < 0.05$) retarded the acrossomic response in comparison to control, while having no effect on the formation of

intracellular and extracellular reactive oxygen species. Bleomycin-induced changes in ultrastructural morphology support the functional ones.

Limitations, reasons for caution: This study was bequeathed with an *in vitro* design. The competence of the human male gamete that can potentially impact male fertility has been evaluated *in vitro*; however, its *in vivo* competence and clinical outcomes, which are essential for clinical translation, have not been investigated. Further studies with larger sample sizes, including prepubertal participants, are required to determine the exact mechanism by which bleomycin induces sDNAfrag, apoptosis, and oxidative stress. Furthermore, studies should be conducted to evaluate the potential long-term consequences on fertility and reproductive health, as well as on strategies to mitigate or counteract the effects of bleomycin.

Wider implications of the findings: Male gamete competency in humans is negatively impacted by bleomycin. Healthcare professionals should closely monitor and conduct additional research on the male gonadotoxicity of bleomycin in addition to its associated lung toxicity. In the meanwhile, it is recommended that all cancer patients receiving bleomycin-containing chemotherapy treatments be given a fertility preservation strategy.

Keywords: Bleomycin; Cancer; Human male gamete; Sperm parameters; Sperm DNA integrity; MMP; Apoptosis; Acrosome reaction; *In vitro* exposure; Fertility preservation.

3.1 | Introduction

In recent decades, society has assisted a boom in the oncology field with new and personalized therapies, early and optimized diagnosis, and reinforced public health education and awareness (Sung et al., 2021). As a result, some cancer diagnoses, once considered terminal, were converted into chronic conditions. These advances significantly and improved outcomes particularly in young cancer patients, whose 5-year overall survival rates currently reach 80% (Janssen et al., 2021).

The increased survival rates following cancer treatment emphasize how crucial it is to incorporate knowledge about how chemotherapy affects reproductive potential as well as fertility preservation measures into the overall management of these individuals.

Adolescents and young adults (AYAs; 15-39 years old) who have cancer represent distinct and complicated age group with particular disease biology and distribution (Barr, 2011; Trama et al., 2016). However, they frequently place a high value on their future fertility and family-building options (Xie et al., 2022; Yumura et al., 2023). Unfortunately, most cancer survivors endure long-term repercussions despite efforts to make therapeutic options with potent anti-tumor efficacy and minimal side effects. Gonadotoxicity linked to oncologic therapy is one such result, meaning that infertility is a common outcome in survivors (Xie et al., 2022; Yumura et al., 2023).

Chemotherapy regimens that combine bleomycin with other chemotherapy drugs to increase their effectiveness in treating certain types are often associated with an increased risk of gonadotoxicity. Bleomycin is often used to treat several types of cancer, including testicular cancer, in which its combination with etoposide and cisplatin (BEP regimen) is applied as standard therapy, providing a 5-year survival rate greater than 90% in the AYAs group (Al-Bader & Kilarkaje, 2015; Bieber et al., 2006; Khadivi et al., 2020).

BEP regimen (Bieber et al., 2006; Davis et al., 2023), cisplatin (Bieber, 2005), and etoposide (Baetas et al., 2019; Rabaça et al., 2020) gonadotoxicities are well established, while bleomycin's individual contribution to gonadotoxicity is uncertain. Few studies have investigated and described the deleterious effects of bleomycin on male reproductive function, but there are no reports in humans (Lobo de Almeida et al., 2023).

Bleomycin was isolated from cultures of *Streptomyces verticillus* in 1967, making it one of the first anticancer agents (Umezawa & Maeda, 1967). Since its approval in 1975 to treat testicular cancer, bleomycin has garnered significant clinical interest because of its wide range of actions (Brandt & Gerriets, 2022; O'Sullivan et al., 2003; Sikic et al., 1985).

Bleomycin cytotoxicity is reported to cause oxidative damage to DNA by an oxygen- and metal ion-dependent mechanism (Lazo, 2002; Reinert et al., 2013). Bleomycin's capacity to generate reactive oxygen species (ROS) is advantageous when used as a

chemotherapeutic agent, but it also plays a role in some of the adverse consequences, like pulmonary damage. The lungs are especially vulnerable to ROS, and in certain patients, the generation of ROS in lung tissue can lead to inflammation and lung damage, particularly pulmonary fibrosis, forcing treatment discontinuation. The decreased levels of bleomycin hydrolase, the enzyme that converts the antibiotic into a non-toxic form, in lung tissue increase this susceptibility (Aronson, 2016; Della Latta et al., 2015; Hay et al., 1991).

Given this, understanding the impact of bleomycin on fertility potential is essential for providing comprehensive care to cancer patients, especially those of reproductive age. Therefore, the current study was designed with the intention of assessing the effects of bleomycin on the quality, functionality and structure of human sperm *in vitro* while also investigating the underlying mechanisms of any effects. The ultimate goals are to improve the quality of sustainable oncology care, raise patient and healthcare professional awareness, and assist patients make educated decisions about preserving their fertility before beginning bleomycin anti-tumor treatments.

3.2 | Materials and Methods

3.2.1 | Ethical Issues

Patients who sought sperm analysis at the private clinic Centro de Genética da Reprodução Prof. Alberto Barros had their written informed consent and individual privacy ensured throughout the research, which was conducted in accordance with ethical principles.

In this study, surplus donor cells - freshly ejaculated sperm - were used instead of human or animal volunteers. This kind of activity is exempt from the rules of the Helsinki Declaration on Human Experimentation, which was updated in Fortaleza in 2013. The National Council on Medically Assisted Procreation's guidelines (CNPMA: https://www.cnpma.org.pt) and the National Law on Medically Assisted Procreation (Law No. 32/2006 amended by Law No. 48/2019: https://diariodarepublica.pt/dr/detalhe/lei/48-2019-122996204) do not require any further authorizations.

Under the Joint Ethics Committee CHUP/ICBAS permission numbers 704 2019/CE/P017 (266/CETI/ICBAS) and 2020-178 (139-DEFI/141-CE), human sperm samples were used in laboratory experiments.

3.2.2 | Participant selection and semen collection

After a three to five-day period of sexual abstinence, healthy participants who underwent spermiogram analysis at the aforementioned clinic had their sperm samples collected via masturbation in sterile receptacles. At a final concentration of $16x10^6$ sperm/mL, semen samples from 45 participants whose ejaculate volume was still present following clinical semen analysis were used. Prior to the current trial, no participant had received a cancer diagnosis or been subjected to radiation, chemotherapy, or both. Recently, the CNPMA disclosed that 276 sperm samples were cryopreserved due to oncologic illness (CNPMA, 2023) considering this value to be a good sample, hence the number of cases included in this study was deemed appropriate.

3.2.3 | Bleomycin dose determination

Bleomycin is frequently used with other chemotherapy medications, and the type and stage of the cancer can have an impact on how effective it is. Consequently, sperm cells were exposed to varying concentrations (0 - 800 µg/mL) of bleomycin in accordance with the study experimental conditions in a humidified incubator with 5% CO_2 for 2 hours, and the medication's concentration that inhibited 50% of cell viability was measured by trypan blue assay. This allowed for the determination of bleomycin's IC50 value.

3.2.4 | Experimental design and *in vitro* exposure

Each residual semen sample was centrifuged at 450 xg for 10 minutes after the clinical semen examination, and the supernatant was disposed of. Each pellet was then diluted to a final concentration of $16x10^6$ sperm/mL in sperm preparation medium (SPM; Origio, Jyllinge, Denmark). Three groups were created out of each semen sample: • prior to incubation (T0): sperm diluted solely in SPM; • control (CT): sperm diluted solely in SPM; and • experience (EXP): sperm diluted in SPM supplemented with bleomycin (100 µg/mL; cat#B8616). The samples from the CT and EXP groups were then incubated for two hours at 37°C in a humidified incubator with 5% CO_2, which corresponds to the exposure period determined by the half-life duration of bleomycin (O'Sullivan et al., 2003; Reinert et al., 2013). Both before (for the T0 group) and after the incubation period (for the CT and EXP groups), the following analyses were performed. Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Evaluations were conducted using optical Olympus CX21 (Olympus Corporation, Tokyo, Japan) and fluorescence Nikon Eclipse E400 (Nikon, Tokyo, Japan) microscopes.

3.2.5 | Conventional semen analysis

The World Health Organization (WHO) guidelines were followed in measuring sperm motility and vitality (WHO, 2021). Sperm motility was classified into four groups: total motility, progressive motility (which was further divided into slow (SlPM) and rapidly progressive motility (RPM)), *in situ* motility (or non-progressive motility), and null motility. Sperm vitality was measured using the eosin Y staining. For each parameter, two hundred sperm from each sample were aleatorily counted twice. Sperm morphology in the EXP group was not examined because abnormalities in this parameter are not expected to occur during the time of experimental exposure.

3.2.6 | Sperm chromatin condensation evaluation

The condensation of sperm chromatin was assessed using the previously described method with acidic aniline blue (AB) staining (Baetas et al., 2019). Briefly, sperm samples were spread on glass slides coated with 3-aminopropyltriethoxysilane and fixed for 30 minutes at room temperature (RT) using 3% glutaraldehyde in 0.2 M phosphate-buffered saline (PBS, pH 7.4). For five minutes at RT, the slides were dyed with 5% aqueous AB in 4% acetic acid (pH 3.5; VWR, Radnor, USA). A minimum of two hundred sperm with normal morphology were inspected on each slide. Consequently, percentages of sperm that remained unstained (showing protamine-rich mature chromatin) and those that stained dark blue (AB positive, indicating immature histone-rich nuclear chromatin), were calculated.

3.2.7 | Sperm DNA fragmentation determination

Sperm DNA fragmentation (sDNAfrag) was evaluated by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the *In-Situ* Cell Death Detection Fluorescein Kit (Roche, Mannheim, Germany), according to the manufacturer's instructions for the *in situ* technique. Succinctly, each sperm sample was spread on glass slides and fixed with 4% paraformaldehyde in PBS for 1 hour at RT. Slides were then washed in PBS and permeabilized for 2 minutes at 4°C with 0.1% Triton-X in 0.1% sodium citrate. After washing in PBS, the slides were incubated with 25 µL of TUNEL mixture for 1 hour at 37°C in a dark-moist chamber. Subsequently, the slides were washed in PBS and counterstained with 4',6'-diamino-2-fenil-indol (DAPI). A minimum of two hundred sperm with normal morphology were inspected on each slide. Each spermatozoon was then classified as either having DNA fragmentation (if it displayed green fluorescence) or not (DAPI staining only - blue fluorescence). As a percentage of all sperm counted, the number of sperm exhibiting green fluorescence (TUNEL-positive) was recorded.

Furthermore, four staining patterns were observed in accordance with the region stained: total nucleus staining (Head); post-acrosome region (PAR); acrosome vesicle region (AVR); and equatorial region (ER) (Sá et al., 2015).

3.2.8 | Oxidative status measurement

Oxidative damage to sperm DNA was evaluated by the luminol-based chemiluminescence assay using a 96-well white microplate (Costar, Kennebunk, USA) and a microplate reader (Biotek, Vermont, U.S.A). For each group's samples, four conditions were created for the measurement of integrated chemiluminescence: blank, negative control (C^-), positive control (C^+), and test. PBS was supplemented with 30% H_2O_2 for C^+, and 5 mM luminol solution in dimethyl sulfoxide (DMSO) was added to all conditions except C^-. The samples' chemiluminescence was expressed as relative light units (RLUs), and the results were generated automatically by the Gene5 Microplate Reader software.

3.2.9 | Mitochondrial membrane potential measurement

To measure the mitochondrial membrane potential (MMP), the fluorescent dye 5,5,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazoylcarbocyanine iodide (JC-1; cat#CS0390) was employed. At high MMP levels, JC-1 accumulates in the mitochondria, and forms aggregates that emit orange/red fluorescence, and at low MMP levels, it forms monomers that emit green fluorescence. The staining solution was prepared by mixing 25 µL of the JC-1 stock solution (1 mg/mL JC-1 in DMSO) in ultrapure water with JC-1 staining buffer 5x, from which the working solution was prepared. After fixing the sperm samples for one hour at RT using 4% paraformaldehyde in PBS, they were incubated for 20 minutes at RT using 1 mg/mL of JC-1 in PBS (pH 7.2) working solution. Following the incubation period, the slides underwent a PBS wash and a counterstain using mounting media containing DAPI. Two hundred morphologically normal sperm were counted for each slide, and the JC-1 ratio (high MMP: J-aggregates/low MMP: monomers) was calculated.

3.2.10 | Sperm acrosomal reaction evaluation

The peanut agglutinin (PNA) lectin from *Arachis hypogea*, a marker for the acrosomal region of human sperm, was used in two protocols to determine the acrosomal status: (1) lectin staining with biotinylated PNA (B-1075; Vector laboratories, Newark, CA, USA) and (2) fluorescence determination by fluorescein-5-isothiocyanate (FITC) - conjugated PNA (cat#L7381). For both tests, samples were spread onto microscope slides, and sperm were fixed for 1 hour at RT using 4% paraformaldehyde in PBS.

Next, for lectin staining, 3% hydrogen peroxide (H_2O_2) in methanol was used to block the endogenous peroxidase activity for 10 minutes at RT. After washing twice with PBS, the slides were incubated with serum-blocking solution (Histostain-Plus kit; Invitrogen, Paisley, UK) for 10 minutes at RT to block the non-specific reactions. The biotinylated PNA solution (1:30 in PBS) was then applied for 1 hour at RT. The slides were subsequently washed in PBS and immersed in streptavidin-peroxidase conjugate solution (Histostain-Plus kit; Invitrogen, Paisley, UK) for 30 minutes at RT. After washing twice with PBS, the slides were immersed for 2-5 minutes in a freshly prepared DAB solution containing 0.02% H_2O_2 to visualize the PNA binding sites (brown). Lastly, the slides were counterstained with hematoxylin, dehydrated, and mounted. The number of morphologically normal sperm with PNA staining was recorded as a proportion of all morphologically normal sperm (a minimum of two hundred).

For fluorescence determination, fixed sperm were then exposed to 0.005 M glycine in PBS and then permeabilized with 0.05% Tween-20 in PBS, both at RT. Then, in a moist camera overnight at 4°C, sperm were tagged with 25 µg/ml FITC-conjugated PNA, and finally counterstained with DAPI. The number of sperm emitting green fluorescence (acrosome-reacted cells) was recorded as a percentage of total counted normal sperm (blue-DAPI stained), after evaluation of a minimum of two hundred morphologically normal sperm.

The acrosome-reactive cells were separated into four PNA-labelling categories following each protocol: Class I - labeling of the whole acrosome, with labels uniformly dispersed across the acrosome region, regardless of label intensity; Class II - uneven acrosome labeling (patchy), with at least 20% of the acrosome cap without label; Class III - acrosome equatorial segment labeling only; and Class IV - no acrosome labeling.

3.2.11 | Transmission electron microscopy analysis

Transmission electron microscopy (TEM) was used to examine sperm samples at the ultrastructural level. Sperm samples were fixed for 2 hours at RT with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), then post-fixed with 2% osmium tetroxide for 2 hours at 4°C and dehydrated in a graded ethanol series. Sperm were then mixed with propylene oxide and embedded in epoxy resin. On an LKB-ultramicrotome (Leica Microsystems, Wetzlar, Germany), appropriate sperm fields were selected in 1 µm semithin sections and 50-70 nm ultrathin sections were cut with a diamond knife (Diatome, Pennsylvania, USA) and retrieved on copper grids (TAAB laboratories, Berks, UK). After contrasting with 3% aqueous uranyl acetate (BDH, Poole, UK) and Reynolds

lead citrate, sperm cells were observed in a JEOL 100CXII transmission electron microscope (JEOL, Tokyo, Japan) operated at 60 kV.

3.2.12 | Statistical analysis

Statistical analysis was performed using the SPSS software (IBM SPSS Statistics for Windows, Version 28.0, Armonk, NY: IBM Corp.). The mean ± standard deviation was used to present the results for all the sperm parameters. To evaluate group normality, the Kolmogorov-Smirnov test (N > 30) was employed, whereas Leven's test was utilized to evaluate variance homogeneity. Furthermore, for variables that followed a normal distribution, the Student's T test was utilized, and for variables that did not, the Wilcoxon signed-rank test was used to assess the difference between dependent groups. Additionally, a McNemar's test and a Pearson's Chi-squared test with Yates' continuity adjustment were solely used for TEM analysis. The threshold for statistical significance was set at P-value < 0.05.

3.3 | Results

3.3.1 | Participants and samples characterization at collection time

The samples used in this investigation were randomly selected. Table III. 1 provides a description of the participants' demographics and a description of the semen samples preliminary assessment. The subjects were of reproductive age (35.64 ± 5.56 years), with normal semen pH and viscosity and no round cells, leucocytes, or agglutination. The subjects' mean counts of sperm (96.39 ± 63.41x10^6/mL) at the time of collection, along with their mean percentages of total motility (59.36 ± 12.71%), progressive motility (45.87 ± 13.34%), vitality (75.51 ± 7.70%), normal morphology (4.51 ± 3.22%), and other parameters, were in line with the WHO's definition of normozoospermia (2021).

Table III. 1 Participants and sperm samples characterization at collection time.

Variable	*Mean ± Standard Deviation*
Age	35.64 ± 5.56
pH	7.79 ± 0.11
Volume (mL)	3.44 ± 1.75
Concentration (sperm x 10^6/mL)	96.39 ± 63.41
Vitality	75.51 ± 7.70
Hypo-osmotic swelling test (HOST) (%)	68.90 ± 10.15
Motility (%)	
Total	59.36 ± 12.71
Progressive	45.87 ± 13.34
In situ	13.49 ± 4.83
Null	40.64 ± 12.71
Normal morphology (%)	4.51 ± 3.22
Teratozoospermia index (%)	1.60 ± 0.15

3.3.2 | Determination of bleomycin's IC50 in human sperm

The sperm viability percentage at various bleomycin concentrations in comparison to a control without bleomycin allowed for the creation of a dose-response curve (Figure III. 1), from which the IC50 value (the concentration at which 50% inhibition occurred) was extrapolated. This value was 100 µg/mL, and it corresponded to the concentration of bleomycin that the sperm in the EXP group were exposed to.

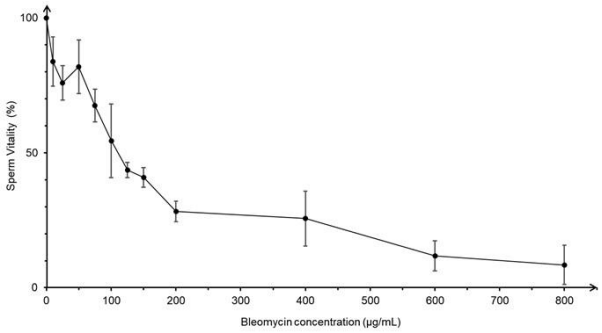

Figure III. 1 Dose-response curve of human sperm following exposure to various bleomycin concentrations (0, 10, 25, 50, 75, 100, 125, 150, 200, 400, 600, and 800 µg/mL). Sperm vitality is expressed as a percentage (%).

3.3.3 | Bleomycin' effect on human sperm parameters: vitality and motility

Figure III. 2 shows that EXP group displayed significantly ($P < 0.001$) lower mean values (33.89 ± 13.15%) for the percentage of sperm vitality than T0 (75.51 ± 7.70%) and CT (49.04 ± 15.56%) groups, while CT group also demonstrated significantly ($P < 0.001$) lower mean values for the percentage of sperm vitality than T0 group.

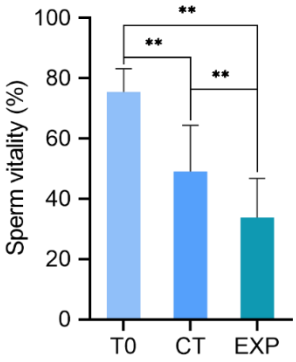

Figure III. 2 Bleomycin's impact on the vitality of human sperm. The results are given as the mean ± standard deviation (n=45). T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin. *P*-value < 0.05 was considered significant. Significantly different results (Student's T test): ** $P < 0.001$.

Figure III. 3 demonstrates that EXP group (26.33 ± 12.52%) showed significantly ($P < 0.001$) lower mean sperm motility than T0 (59.36 ± 12.71%) and CT (44.95 ± 16.73%) groups. In addition, EXP group showed significant changes in all sperm motility categories as compared to T0 and CT groups.

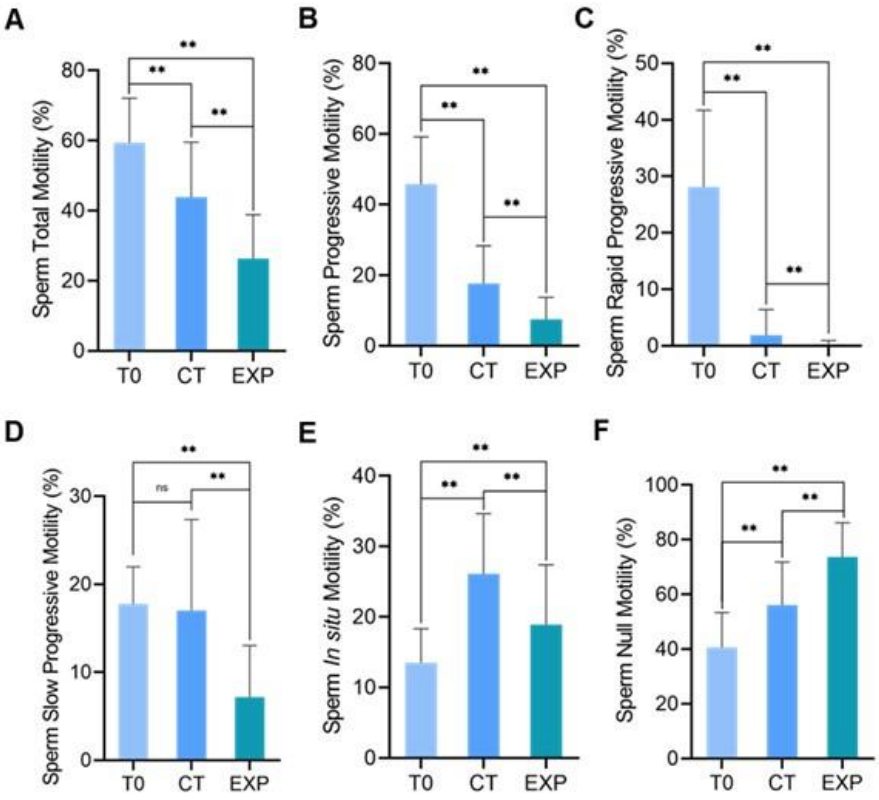

Figure III. 3 Bleomycin's impact on human sperm motility. A. Total motility; **B.** Progressive motility; **C**. Rapid progressive motility; **D**. Slow progressive motility; **E**. *In situ* motility; and **F**. Null motility. The results are expressed as mean ± standard deviation (n=45). T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin. *P*-value < 0.05 was considered significant. Significantly different results: ** $P < 0.001$.

After sperm were exposed to bleomycin, it was found that there was a rise in null motility and a substantial decrease in the mean percentage of total, progressive, RPM, SlPM, and *in situ* motility (Figure III. 3 and Table III. 2). Regarding the three main categories of motility, CT and EXP groups' mean percentage of motility was significantly increased in the sequence null > *in situ* > progressive (Table III. 2).

Table III. 2 Mean values of percentage of sperm motility categories and comparisons of these categories within and between study groups.

Study groups	Motility Categories					*P*-value (intragroup)
	PM (%)	RPM (%)	SIPM (%)	*In situ* (%)	Null (%)	
T0	45.87 ± 13.34	28.11 ± 13.59	17.76 ± 4.22	13.49 ± 4.83	40.64 ± 12.71	a (0.156), b, c (< 0.001)
CT	18.85 ± 13.38	1.87 ± 4.54	16.98 ± 10.35	26.10 ± 8.53	55.05 ± 16.73	a, b, c (< 0.001)
EXP	7.43 ± 6.19	0.26 ± 0.66	7.17 ± 5.87	18.90 ± 8.49	73.67 ± 12.52	a, b, c (< 0.001)
Statistical comparisons between groups (*P*-values)						
T0 vs. CT	< 0.001	< 0.001	0.391	< 0.001	< 0.001	
T0 vs. EXP	< 0.001	< 0.001	< 0.001	0.004	< 0.001	
CT vs. EXP	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	

T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin; PM, progressive motility; RPM, rapid progressive motility; SIPM, slow progressive motility.
The results are shown as mean ± standard deviation.
P-value < 0.05 was considered significant (Wilcoxon signed-rank test)
a. PM *vs In situ*; b. PM *vs* Null; c. *In situ vs* Null.

3.3.4 | Bleomycin's effect on human sperm DNA

A significantly (*P* < 0.001) lower mean percentage of sperm with condensed chromatin was found in EXP group (64.30 ± 9.74%) compared to CT (76.82 ± 8.68%) and T0 (80.77 ± 7.64%) groups. Additionally, a significant reduction in CT group as compared to the T0 group was seen (Figure III. 4).

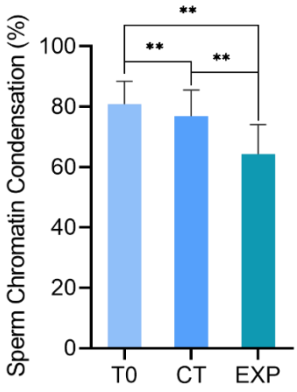

Figure III. 4 Bleomycin's effect on human sperm chromatin condensation. Results are expressed as mean ± standard deviation (n=45). T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin. *P*-value < 0.05 was considered significant. Significantly different results (Wilcoxon signed-rank test): ** *P* < 0.001.

By examining staining patterns, the TUNEL assay has been used to identify apoptotic sperm and the extent of DNA breakage brought on by exposure to bleomycin (Figure III. 5).

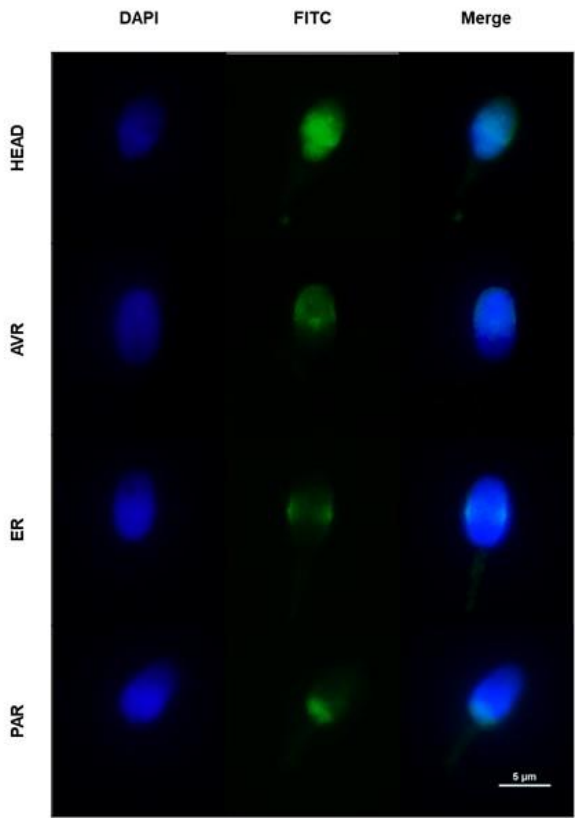

Figure III. 5 Patterns of sperm nuclear DNA fragmentation after exposure to bleomycin as detected by TUNEL labeling. Sperm exhibiting green fluorescence are positive; these include the following regions: HEAD in the head region, AVR in the acrosome vesicle region, ER in the equatorial region, and PAR in the post-acrosomal region. Observation under an x1000 total magnification fluorescent microscope.

When compared to T0 (9.78 ± 9.27%) and CT (12.50 ± 10.68%) groups, the mean percentage of sDNAfrag was significantly ($P < 0.001$) increased in EXP group (22.68 ± 13.42%) (Figure III. 6A). Regardless of the sDNAfrag staining pattern, this trend was likewise observed (Figure III. 6 B-E).

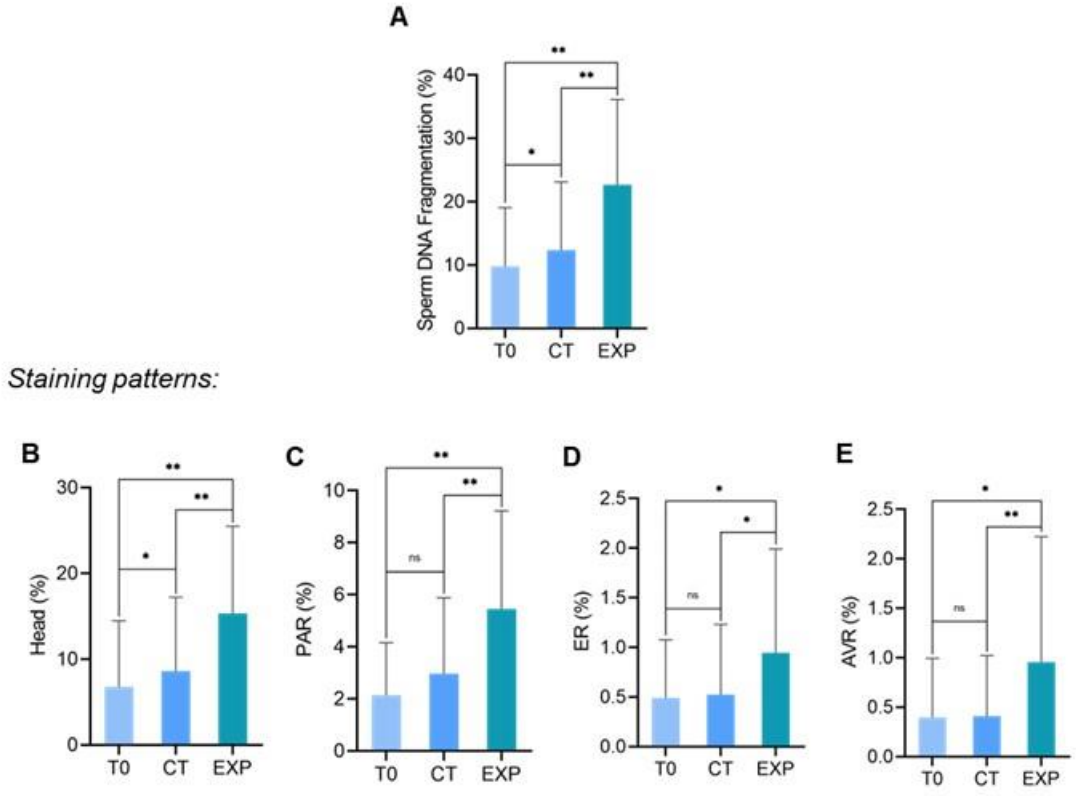

Figure III. 6 Bleomycin's effect on human sperm nuclear DNA integrity. Percentage of human sperm DNA fragmentation: **A.** total; **B.** on whole head (Head pattern); **C.** on post-acrosomal region (PAR pattern); **D.** on the equatorial region (ER pattern); **E.** on acrosome vesicle region (AVR pattern). Results are expressed as mean ± standard deviation (n=45). T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin. *P*-value < 0.05 was considered significant. Significantly different results: * $P < 0.05$ and ** $P < 0.001$. ns, not significant.

Considering the sDNAfrag fluorescence patterns within each group, the mean percentage of sDNAfrag decreased significantly in the sequence Head > PAR > ER/AVR, even though the ER and AVR patterns did not differ statistically from one another (intragroup $P > 0.05$) (Table III. 3). Between all groups, only the Head pattern reported significant differences, namely, an increase. For the PAR, ER, and AVR, no significant differences were detected between T0 and CT groups, while between T0/CT and EXP groups, all these patterns showed a statistically significant increase (Table III. 3).

Table III. 3 Mean values of percentage of human sperm nuclear DNA fragmentation fluorescence patterns and comparisons of them within and between study groups.

Study groups	Staining patterns				*P*-value (intragroup)
	Head (%)	PAR (%)	ER (%)	AVR (%)	
T0	6.76 ± 7.71	2.14 ± 2.02	0.49 ± 0.59	0.40 ± 0.60	a, b, c, d, e (< 0.001), f (0.295)
CT	8.62 ± 8.62	2.97 ± 2.92	0.52 ± 0.71	0.41 ± 0.61	a, b, c, d, e (< 0.001), f (0.474)
EXP	15.33 ± 10.16	5.45 ± 3.76	0.94 ± 1.05	0.95 ± 1.27	a, b, c, d, e (< 0.001), f (0.937)

T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin; Head, head region; AVR, acrosome vesicle region; ER, equatorial region; PAR, post-acrosomal region.
The results are shown as mean ± standard deviation.
P-value < 0.05 was considered significant (Wilcoxon signed-rank test).
a. Head *vs* PAR; b. Head *vs* ER; c. Head *vs* AVR; d. PAR *vs* ER; e. PAR *vs* AVR; f. ER *vs* AVR.

3.3.5 | Bleomycin's effect on human sperm oxidative stress

Chemiluminescence assay used to measure extracellular and intracellular ROS did not detect any difference between all groups (Table III. 4).

Table III. 4 Mean values of ROS levels in samples of different study groups and comparisons between them.

Study groups	Mean ± Standard Deviation
T0	22.73 ± 34.35
CT	16.76 ± 25.75
EXP	17.13 ± 27.99
Statistical comparisons between groups (*P*-values)	
T0 vs. CT	0.270
CT vs. EXP	0.981
EXP vs. T0	0.266

T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin.
P-value < 0.05 was considered significant (Wilcoxon signed-rank test).

3.3.6 | Bleomycin's effect on human sperm MMP

Mitochondrial activity was detected by fluorescence of JC-1, and the results revealed both the presence of sperm with orange midpieces (active mitochondria, high MMP) and sperm with green midpieces or unstained (low MMP) (Figure III. 7).

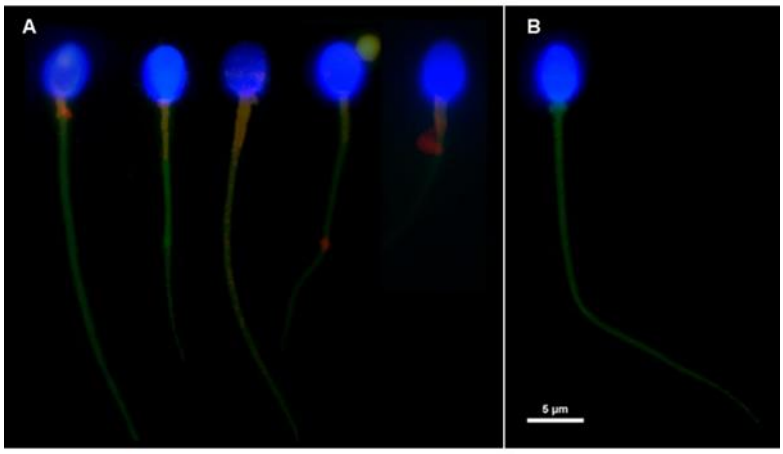

Figure III. 7 MMP in sperm exposed to bleomycin as detected by JC-1. A. Sperm that fluoresces orange or red in the middle pieces are associated with polarized mitochondria (high MMP); **B.** sperm that fluoresces only green in the middle pieces are associated with depolarized mitochondria (low MMP). Observation under an x1000 total magnification fluorescent microscope.

Bleomycin exposure increased significantly ($P < 0.001$) the proportion of sperm with low MMP (84.23 ± 8.04) when compared to T0 (92.29 ± 6.42%) and CT (91.16 ± 7.69%) groups, but this parameter was unaffected between T0 and CT groups (Figure III. 8).

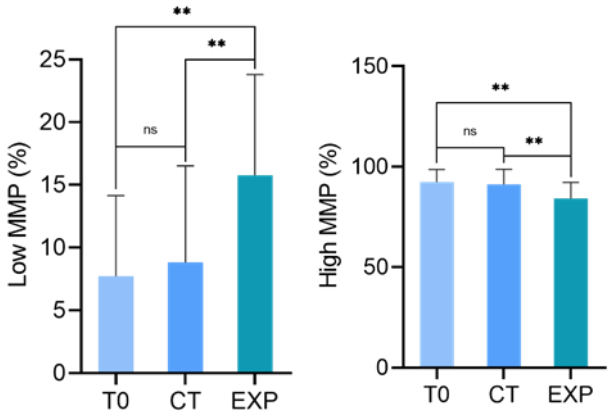

Figure III. 8 Bleomycin's effect on human sperm MMP. Percentage of human sperm with high and low MMP. Results are expressed as mean ± standard deviation (n=45). T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin. *P*-value < 0.05 was considered significant. Significantly different results (Wilcoxon signed-rank test): ** $P <$ 0.001. ns, not significant.

3.3.7 | Bleomycin's effect on human sperm acrosome status

To assess sperm acrosome status, fluorescence (Figure III. 9A) and cytochemistry (Figure III. 9B) assays were used to map the localization of PNA lectin.

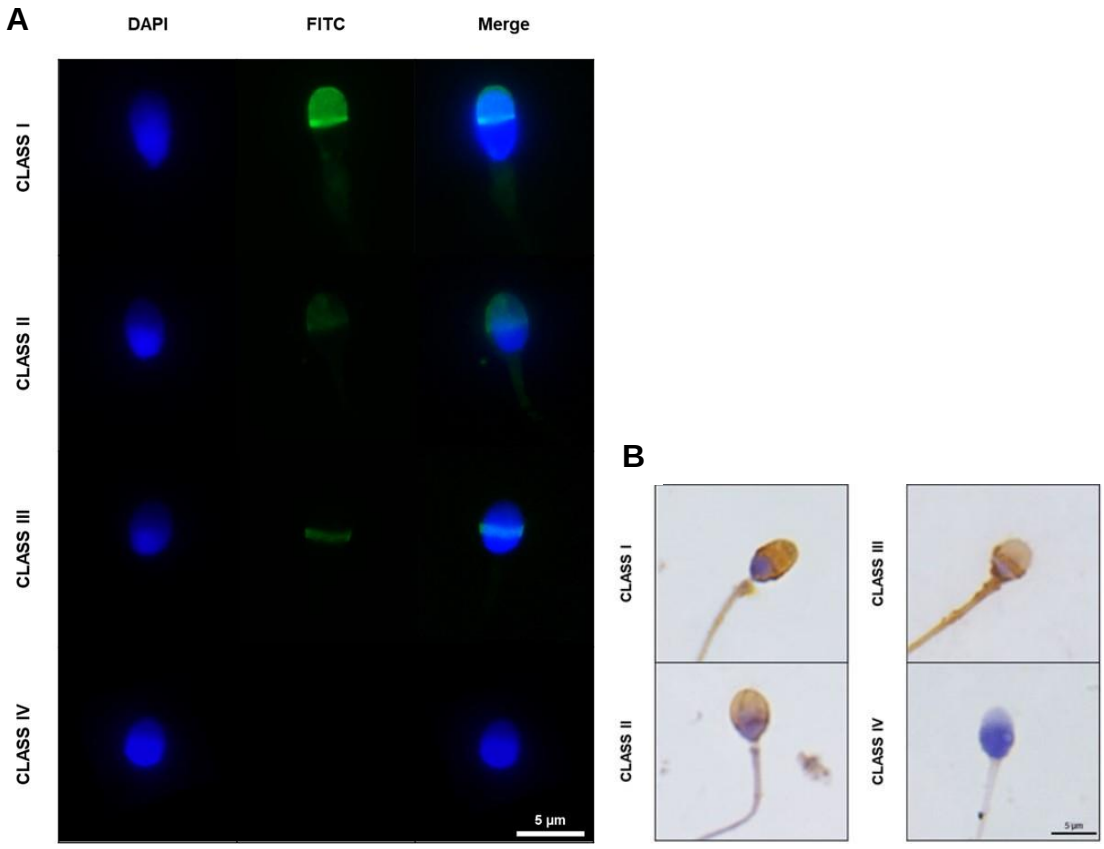

Figure III. 9 Patterns of acrosome-reacted sperm after exposure to bleomycin as detected by PNA FITC-fluorescence assay (A) and PNA-biotinylated staining assay (B). Sperm exhibiting complete acrosome staining (**Class I**), sporadic acrosome staining (**Class II**), acrosome staining limited to the equatorial segment (**Class III**), and acrosome staining absent (**Class IV**). Observation under an x1000 total magnification.

The acrosome reaction as detected by the fluorescence method is illustrated in Figure III. 10, which displays the differences between each study group for each class. Class I was significantly lower than the other classes within each group, while Class IV was significantly higher than the other classes. The Class II and Class III patterns did not statistically differ from one another, except in the CT (P = 0.006) (Table III. 5).

Staining patterns:

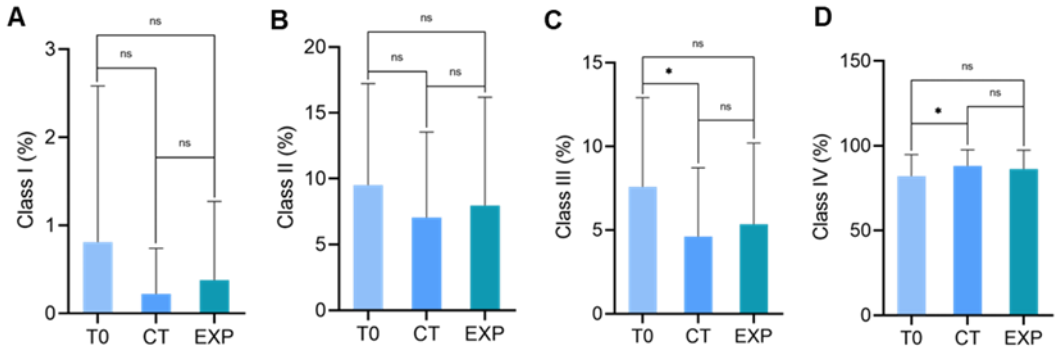

Figure III. 10 Bleomycin's effect on human sperm acrosome status as detected by PNA FITC-fluorescence assay. Percentage of human sperm acrosome reactive: **A.** Class I; **B.** Class II; **C.** Class III; **D.** Class IV. Results are expressed as mean ± standard deviation (n=45). T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin. *P*-value < 0.05 was considered significant. Significantly different results: * $P < 0.05$. ns, not significant.

Table III. 5 Mean values of percentage of sperm acrosome reacted staining classes and comparisons of them within and between study groups.

Study groups	Staining classes				P-value (intragroup)
	Class I (%)	Class II (%)	Class III (%)	Class IV (%)	
T0	0.81 ± 1.77	9.51 ± 7.71	7.59 ± 5.32	82.10 ± 12.71	a, b, c, e, f (< 0.001) d (0.096)
CT	0.22 ± 0.52	7.06 ± 6.49	4.61 ± 4.11	88.11 ± 9.58	a, b, c, e, f (< 0.001) d (0.006)
EXP	0.38 ± 0.89	7.96 ± 8.24	5.34 ± 4.85	86.32 ± 11.15	a, b, c, e, f (< 0.001) d (0.063)
Statistical comparisons between groups (*P*-values)					
T0 vs. CT	0.053	0.055	0.002	0.002	
T0 vs. EXP	0.260	0.178	0.031	0.080	
CT vs. EXP	0.481	0.599	0.239	0.299	

T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin. The results are shown as mean ± standard deviation.
P-value < 0.05 was considered significant (Wilcoxon signed-rank test).
a. Class I *vs* Class II; b. Class I *vs* Class III; c. Class I *vs* Class IV; d. Class II *vs* Class III; e. Class II *vs* Class IV; f. Class III *vs* Class IV.

Figure III. 11 depicts the acrosome reaction as determined by the PNA biotinylated technique and displays the differences between each study group for each class. Based on the acrosome reaction' staining patterns, exposure to bleomycin causes a significant ($P = 0.047$) decrease in class IV in comparison to the CT group (Figure III. 11 A-D).

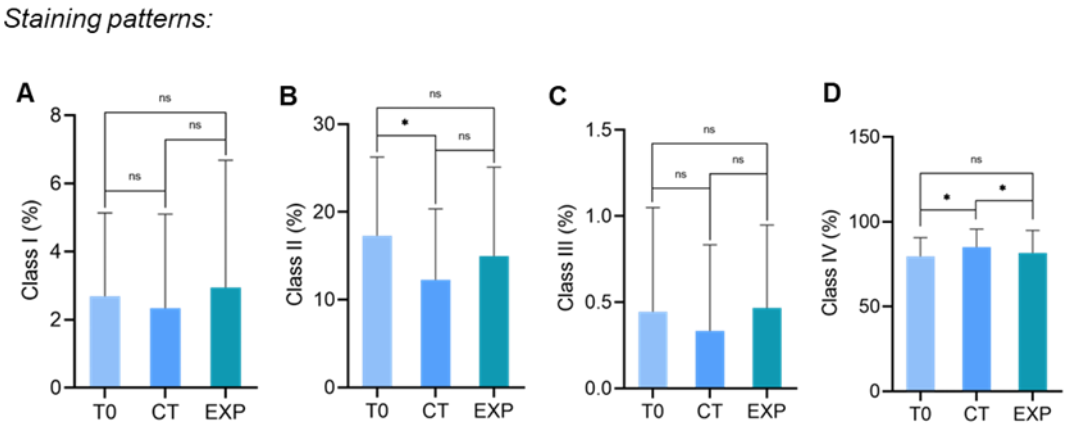

Figure III. 11 Bleomycin's effect on human sperm acrosome status as detected by PNA-biotinylated staining assay. Percentage of human sperm acrosome reactive: **A.** Class I; **B.** Class II; **C.** Class III; **D**. Class IV. Results are expressed as mean ± standard deviation (n=45). T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin. P-value < 0.05 was considered significant. Significantly different results: * *P* < 0.05. ns, not significant

All classes were statistically different from one another within each group (Table III. 6) and decreased significantly (*P* < 0.001) in the sequence Class IV > Class II > Class I > Class III.

Table III. 6 Mean values of percentage of sperm acrosome reacted staining classes and comparisons of them within and between study groups.

Study groups	Staining classes				P-value (intragroup)
	Class I (%)	Class II (%)	Class III (%)	Class IV (%)	
T0	2.69 ± 2.45	17.29 ± 8.98	0.44 ± 0.61	79.58 ± 11.08	a, b, c, d, e, f (< 0.001)
CT	2.33 ± 2.77	12.26 ± 8.09	0.33 ± 0.50	85.08 ± 10.69	a, b, c, d, e, f (< 0.001)
EXP	2.94 ± 3.73	14.96 ± 10.15	0.47 ± 0.48	81.63 ± 13.31	a, b, c, d, e, f (< 0.001)
Statistical comparisons between groups (*P*-values)					
T0 vs. CT	0.214	0.002	0.317	0.005	
T0 vs. EXP	0.788	0.114	0.784	0.194	
CT vs. EXP	0.128	0.055	0.108	0.047	

T0, at the time of sperm collection; CT, control without bleomycin; EXP; sperm exposed to bleomycin.
The results are shown as mean ± standard deviation.
P-value < 0.05 was considered significant (Wilcoxon signed-rank test).
a. Class I *vs* Class II; b. Class I *vs* Class III; c. Class I *vs* Class IV; d. Class II *vs* Class III; e. Class II *vs* Class IV; f. Class III *vs* Class IV.

3.3.8 | Bleomycin's effect on human sperm ultrastructure

Sperm responses to bleomycin exposure were also investigated at the ultrastructural level. Ultrastructural analysis provided by TEM revealed a number of morphological changes, which in combination with the other techniques performed in this study allow a deeper understanding of the structural and functional aspects of the *in vitro* effects of bleomycin on sperm. A heterogeneous combination of head and tail changes were found in varying percentages and most were shown to be individually and significantly more frequent in the EXP group compared to the T0 and CT groups.

Regarding chromatin condensation, a statistically significant ($P < 0.001$) higher percentage of sperm with non-condensed chromatin (Figure III. 12B) was observed in the EXP group compared to the CT group, which was also significantly higher than in the T0 group.

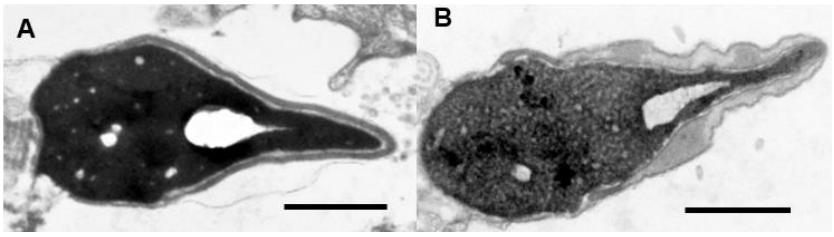

Figure III. 12 TEM micrographs of longitudinal sections of sperm with condensed (**A**) and uncondensed (**B**) chromatin. Bars = 0.5 µm.

A number of acrosomal alterations were also observed and are displayed in Figure III. 13. In comparison to the T0 and CT groups, the EXP group had a significantly higher frequency (*P-values* < 0.05) of absent, lifted and swollen acrosomes.

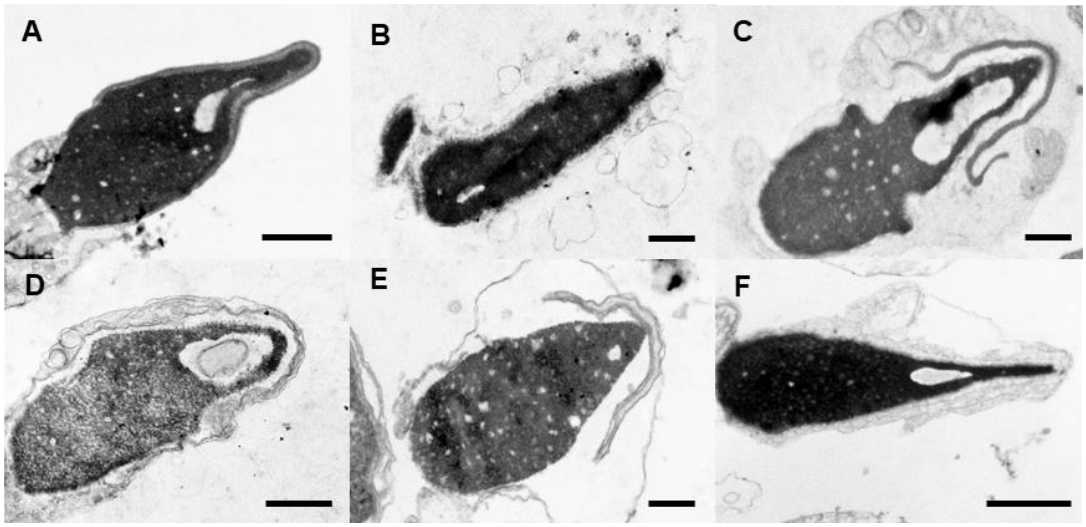

Figure III. 13 TEM micrographs of longitudinal sections of sperm with normal (**A**), absent (**B**), lifted (**C**-**E**) and swollen (**D**-**F**) acrosome. Bars = 0.5 µm.

Mitochondrial structural defects were also significantly increased in the EXP group compared to the CT group ($P < 0.001$), namely mitochondria with scarce cristae, without the characteristic inner mitochondrial membrane compartmentalization, and dilated, seemingly empty intermembrane spaces, as indicated by the arrow in Figure III. 14 (D-E). While there were no variations in the organization of the mitochondrial sheath between the CT and EXP groups, there was a significantly ($P < 0.001$) increase in the frequency of swelling of the mitochondrial sheath (Figure III. 14 C-E) in sperm of the EXP group than in the T0 and CT. These defects were frequently accompanied by excess of cytoplasmic residues, also known as residual bodies, in the sperm midpiece (Figure III. 14 A and E).

The frequency of these residual bodies was significantly higher in sperm from the EXP group when compared to the T0 ($P < 0.001$) and CT ($P = 0.016$) groups.

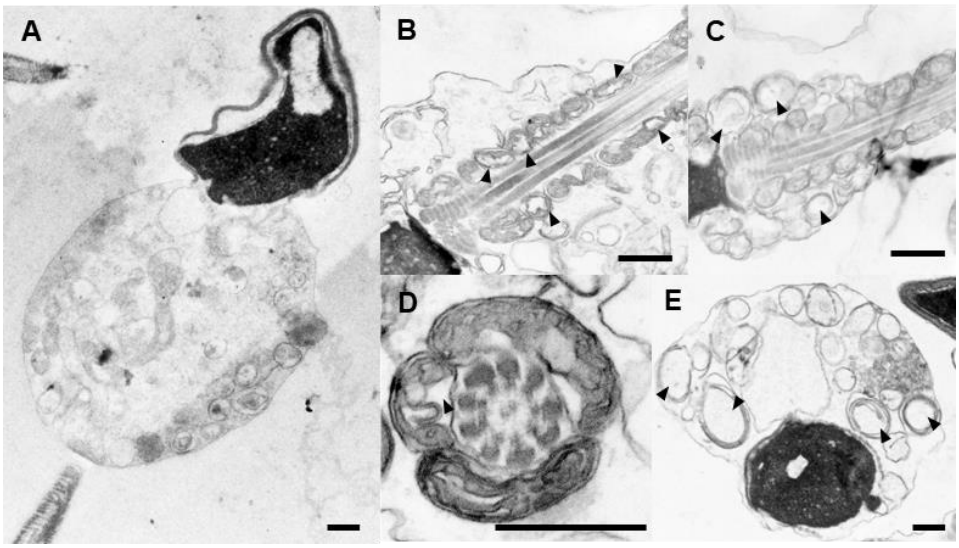

Figure III. 14 TEM micrographs of longitudinal (**A**-**C**) and transversal (**D** and **E**) sections of sperm exposed to bleomycin showing an empty mitochondrial matrix (arrows; **B**-**E**), swollen mitochondria (**C**-**E**), and an excess of cytoplasmic residues (**A** and **E**). Bars = 0.5 µm.

TEM analysis is the gold standard method for identifying flagellar structural defects, and the present study also allowed the observation of non-systematic or polymorphic flagellar defects affecting the axoneme and periaxonemal components (outer dense fibers (ODF) and the fibrous sheath spatial organization) in different percentages. When comparing the EXP and CT groups, alterations in the midpiece ($P < 0.001$), principal piece ($P < 0.001$), and endpiece ($P = 0.016$) axoneme structure were more frequent in the EXP group. Additionally, following bleomycin treatment, sperm were found to have a considerably larger percentage of swollen plasma membranes than the T0 ($P < 0.001$) and CT ($P = 0.004$) groups.

3.4 | Discussion

Bleomycin is an antitumor antibiotic highly effective in treating a variety of malignancies, such as Hodgkin's lymphoma, squamous cell carcinomas and most notably testicular cancer. Despite the fact that bleomycin use has been associated to skin reactions and lung damage, little is known about its gonadotoxicity.

As per the 2007 report by the US National Research Council (NRC) outlining a vision for toxicity testing (US NRC, 2007), an *in vitro* investigation was conducted in this study to evaluate the effect of bleomycin on human sperm potential.

This is the first investigation on human sperm, and its trustworthiness was ensured by the determination of an IC50 dose of bleomycin that matched the half-life of the medication in patients with normal renal function (Lazo, 2002; Scholar, 2007). To further ensure that the effects observed in the EXP group could only be attributed to bleomycin exposure, a T0 group and a CT group were included. Sperm parameters and DNA integrity were indeed shown to have drastically deteriorated during the incubation period. This is in line with the sperm quality alterations associated with the cancer itself that have been observed before initiation of testicular cancer therapy (Beaud et al., 2019; Hamano et al., 2017).

As sperm morphology and concentration are defined during the testicular stage of the male reproductive system, *in vitro* testing cannot predict changes in these parameters. In contrast, bleomycin therapy in *in vivo* animal studies was found to significantly reduce sperm count (Amirshahi et al., 2014; Belhan et al., 2019; Ibrahim et al., 2023; Lu & Meistrich, 1979; Thwaini, 2015; Yaghutian Nezhad et al., 2021) and normal sperm morphology (Belhan et al., 2019; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021).

The primary indicator of bleomycin cytotoxicity evaluated in the current study is sperm vitality, which was found to be significantly decreased in the bleomycin-exposed group. This finding agrees with those for mice (Yaghutian Nezhad et al., 2021) and rats (Amirshahi et al., 2014; Belhan et al., 2019).

Sperm motility is a prerequisite for male fertility and a crucial component of semen quality. When it is compromised, it is the primary cause of subfertility or infertility (Björndahl, 2010). The present study revealed that bleomycin considerably increases null motility while significantly reducing total and progressive sperm motility, unlike in T0 and CT groups where this reduction is counterbalanced by a notable increase in *in situ* motility. In fact, sperm samples exposed to bleomycin may be labeled as asthenozoospermic, a condition linked to a reduced capacity for fertilization (Agnihotri et al., 2016). A notable decline in both total (Belhan et al., 2019; Ibrahim et al., 2023) and progressive (Amirshahi et al., 2014; Yaghutian Nezhad et al., 2021) sperm motility was also documented in animal research. Notwithstanding, Amirshahi et al. (2014) reported no significant changes in the percentage of immotile and non-progressive sperm, while Yaghutian Nezhad et al. (2021) reported an increase in the percentage of immotile sperm and a decrease in the percentage of non-progressive motility.

Sperm vitality and motility have a well-established, significant positive correlation (Wang et al., 2003). It is probable that the observed decline in vitality as well as in motility are connected because the proportion of drop in both parameters is identical between the T0 and EXP groups. This suggests that most immobile sperm are actually dead cells.

In animal (Delbes et al., 2007; Hashemi et al., 2018; Kilarkaje et al., 2013; Moradi et al., 2021; Razavi-Azarkhiavi et al., 2014) as well as in human (Bujan et al., 2013; Ghezzi et al., 2016; O'Donovan, 2005) investigations, BEP regimen was also observed to reduce sperm vitality and motility. Furthermore, etoposide has been described as not altering sperm motility and vitality (Baetas et al., 2019; Rabaça et al., 2020). This could indicate that bleomycin and cisplatin are the only substances responsible for BEP's detrimental effect on sperm conventional parameters and, as a result, sperm quality.

Sperm motility is dependent on the adenosine triphosphate (ATP) generation. Two distinct processes, oxidative phosphorylation (OXPHOS) at the mitochondria and glycolysis, are used by sperm to create this molecule. However, it has been proposed that mitochondrial respiration provides the energy for sperm motility (Moscatelli et al., 2017; Paoli et al., 2011). Therefore, changes in mitochondrial functionality could be detrimental to sperm motility.

MMP is a key marker of mitochondrial functionality, revealing the energy status of the cell (Durairajanayagam et al., 2021), and a measure of sperm vitality (Espinoza et al., 2009; Moscatelli et al., 2017; Paoli et al., 2011). The results obtained in the present study showed that bleomycin induces a decrease in MMP. Therefore, the present investigation, in which bleomycin generated a decrease in MMP and sperm motility and vitality, verified the observed positive association between MMP and sperm motility and vitality (Paoli et al., 2011; Wang et al., 2003). These findings support previous research' conclusion that there is a significant correlation between sperm quality and mitochondrial functionality (Agnihotri et al., 2016; Durairajanayagam et al., 2021; Espinoza et al., 2009; Gallon et al., 2006; Moscatelli et al., 2017; Paoli et al., 2011). Moreover, sperm with high MMP are typically morphologically normal (Agnihotri et al., 2016; Durairajanayagam et al., 2021). This suggests that morphological defects can reveal crucial information regarding sperm functionality. Bleomycin may cause ultrastructural changes that can only be seen by TEM because it allows one to see inside the cell (Bragina & Bocharova, 2017; Moretti et al., 2016; Pesch et al., 2006), even though changes in sperm morphology are not anticipated to be visible by light microscopy following the incubation period of the current study.

In addition to functional changes in mitochondria, reduced motility may be associated with the aberrant morphology of the axoneme, dense fibers, and mitochondrial and fibrous sheaths (Barbagallo et al., 2020). Bleomycin caused several of these abnormalities in current investigation. A significant amount of cytoplasmic residual and aberrant mitochondrial morphology, such as enlarged sperm mitochondria virtually devoid of inner membrane compartmentalization and consequently a restricted quantity of cristae, were found. Upon closer examination of these anomalies, one may speculate that the

enlargement of mitochondria could be linked to dysfunctional mitochondria (low MMP), specifically as a consequence of mitochondrial fusion, a strategy used to assist damaged mitochondria in regaining functionality and adapting to energy requirements (Durairajanayagam et al., 2021; Huang et al., 2019). Additionally, the scarce of mitochondrial cristae and mitochondria without characteristic compartmentalization of the inner mitochondrial membrane (Bartoov et al., 1980) with dilated intermembrane spaces that were apparently empty (Pelliccione et al., 2011) may account for the swelling of mitochondria and appear to be linked to associated mitochondrial dysfunction, as the OXPHOS pathway's ATP synthesis takes place at the inner mitochondrial membrane's cristae (Durairajanayagam et al., 2021). The aberrant arrangement and misalignment of the mitochondrial sheath with respect to the head, as well as the abnormal spatial arrangement of ODF were also frequently observed in this study. These observations may contribute to abnormal patterns of motility (Moretti et al., 2016) that impede sperm penetration during fertilization (Zamboni, 1987). Not only were changes in the ultrastructure of the mitochondria observed, but there were also aberrant axonemal and flagellar structures; these observations may also be related to the sperm's reduced motility. The axoneme's dynein arms, which function as molecular motors for flagellum motion, are connected to the A-tubule of the nine peripheral microtubule duplets that surround a core pair of microtubules, producing a 9 + 2 arrangement (Gilpin et al., 2020). Men who are infertile due to low sperm motility and respiratory disorders frequently have changes in this structure, notably the absence of dynein arms and the presence of additional duplets (Pereira et al., 2017; Tournaye et al., 2017).

In contrast to reproductive toxicity, bleomycin pulmonary toxicity is well-researched as a serious and restricted side effect of this chemotherapy drug (Della Latta et al., 2015). Given that the axonemal structures of sperm flagellum and respiratory tract cilia are comparable (Gilpin et al., 2020), it would be interesting to compare the ultrastructural features of the bleomycin-induced defects in airway cilia reported in previous studies and the current work. Few publications have used ultrastructural analysis to see the morphological abnormalities in respiratory tract cilia brought on by bleomycin. It has been observed that bleomycin regularly modifies cilia, resulting in swelling, an excess of cytoplasmic matrix, and modifications to the number and arrangement of duplets in the cilia's axoneme (Sato et al., 1985; Sekiya et al., 1992). These anomalies were noted for the first time in the current bleomycin-induced study on human sperm. This demonstrates once more the link between the two systems, although further research is needed to elucidate this relationship.

Along with aiding in the understanding of the medication's mechanism of action, one goal of gonadotoxicity risk testing is to discover medications that may cause heritable damage (Santaballa et al.,2022)

Bleomycin's ability to induce DNA damage by single- and double-strand fragmentation and trigger apoptotic cell death is an essential mechanism of its anticancer action (Bolzán & Bianchi, 2018). Consistent with this and as previously reported in an *in vivo* animal study (Amirshahi et al., 2014), the TUNEL test employed in current work showed a significant increase in sDNAfrag after bleomycin exposure. Distinct TUNEL staining patterns were also identified, as these could indicate different phases of apoptosis, serve as a proxy for the effect of medication, and/or indicate sperm susceptibility (Sá et al., 2015). Bleomycin mostly caused Head pattern. Its deleterious potential is revealed by its inespecificity to regions with a larger concentration of protamines or coding genes, rather than only gene-poor and histone-rich regions (Nowicka-Bauer & Nixon, 2020; Sá et al., 2015).

The sperm's vulnerability to DNA fragmentation is affected by the sperm cell's lack of antioxidants and DNA repair systems, as well as the state of chromatin condensation (Zhang et al., 2019). In fact, oxidative stress, abortive apoptosis, and chromatin packaging are the three main cell mechanisms that induce damage to sperm DNA. These mechanisms can occur simultaneously or even be the cause of one another (Dutta et al., 2021; Muratori & De Geyter, 2018; Sakkas & Alvarez, 2010; Sharma & Agarwal, 2011). According to the current study, bleomycin significantly lowers the mean percentage of sperm with condensed chromatin, which is consistent with findings from *in vivo* animal experiments (Amirshahi et al., 2014; Yaghutian Nezhad et al., 2021). TEM ultrastructural investigation also confirmed this altered chromatin condensation. Chromatin remodeling happens when spermatids mature into sperm, a process in which protamines replace most the histones (Okada, 2022; Sharma & Agarwal, 2011). As a result, the current data clearly indicate that bleomycin has the ability to harm sperm chromatin and DNA rather than suggesting partial or inadequate histone-protamine replacement.

Sperm DNA integrity is not evaluated in standard semen analysis, hence even in sperm samples with normal semen analysis, sperm DNA damage may exist and be the root cause of many cases of idiopathic infertility (Avendaño et al., 2009). Furthermore, it adversely impacts the entire process of preimplantation embryonic development and pregnancy (Takeshima et al., 2019).

There has also been evidence of an increase in sDNAfrag and a decrease in chromatin condensation following therapy with a BEP regimen (Ghezzi et al., 2016; Khadivi et al., 2020; Maselli et al., 2012; O'Donovan, 2005; Razavi et al., 2020; Spermon

et al., 2006) and *in vitro* exposure to etoposide (Baetas et al., 2019; Rabaça et al., 2020). Moreover, two years after their oncologic medications ended, the majority of cancer survivors' sperm were shown to still have both sDNAfrag and chromatin abnormalities (Delessard et al., 2020). Prospective studies on cancer survivors in reproductive age showed that although sperm motility would increase 1 or 3 years' post-treatment, DNA damage can persist, or if it returned to normal values, chromatin compaction remains altered possibly due to changes in the expression of genes involved in chromatin remodeling. This is especially true in the case of testicular cancer and lymphomas, in which one's bleomycin is most frequently used (Beaud et al., 2019). Therefore, it is advised that reproductive AYAs receiving bleomycin-containing oncologic therapies also have their sDNAfrag and chromatin condensation studied.

Current results are consistent with Zhang et al.'s (2019) findings that decreased MMP is linked to changes in chromatin remodeling and, as a result, negatively associated with sDNAfrag. In fact, the current study's observations of decreased sperm MMP, motility, and loss of plasma membrane integrity along with elevated sperm DNA damage point to the possibility that bleomycin causes human sperm to initiate apoptosis. *In vivo* animal studies that showed a decrease in Bcl2l1 expression following bleomycin exposure support this notion (Yaghutian Nezhad et al., 2021). Bcl2l1 belongs to the Bcl-2 family of apoptotic inhibitors, which inhibits cytochrome c release (Kucuksayan et al., 2013). As a result, it induces cell apoptosis via the mitochondrial pathway, activating caspases-9 and -3 (Asadi et al., 2021). As a late indicator of apoptosis, caspase-3 activation alone is linked to an increase in DNA damage (Barroso et al., 2006). Therefore, it makes sense that the previously identified sDNAfrag patterns could actually correspond to various apoptotic stages (Sá et al., 2015). However, it is crucial to remember that this defense mechanism is not perfect, and as a result, genetic changes can be passed down to offspring (Aitken & Koppers, 2011). It is advised that conception be postponed throughout the treatment time and for up to two years following the conclusion of treatments due to these and other detrimental effects of chemotherapy on reproduction (Khadivi et al., 2020). A recent study using a rat model investigated the impact of pulmonary fibrosis on sperm quality and the function of the PI3K/Akt pathway (Wang et al., 2022). This route is engaged in multiple functions, such as supporting an anti-apoptotic action, controlling the activity of the Bcl-2 family, and apoptosis. Lung fibrosis-induced hypoxia blocked this signaling pathway, resulting in a drop in Bcl-2 generation and, ultimately, a loss in anti-apoptotic protection, or a promotion of apoptosis, which can ultimately lead to an infertile diagnosis (Wang et al., 2022).

Bleomycin-induced cellular damage, including DNA strand breaks, is known to be by a process that generates ROS (Allawzi et al., 2019.), like other chemotherapeutic agents (Mendes et al., 2022). This capacity to generate ROS is beneficial when used as a chemotherapeutic agent, but also contributes to its adverse effects, including pulmonary inflammation and lung fibrosis (Della Latta et al., 2015). Mitochondria are the key producers of ROS (Durairajanayagam et al., 2021). Even though ROS is a crucial mediator of sperm physiological processes like acrosome reaction, hyperactivation, and capacitation when present at normal levels, an increased rate of its production can result in oxidative stress (OS) (Dutta et al., 2021; Nowicka-Bauer & Nixon, 2020; X. Wang et al., 2003), and subfertility (Evans et al., 2021).

A chemiluminescence assay was conducted in light of available scientific data and, in contrast to expectations, bleomycin did not alter ROS levels in human sperm. The present results also do not support the hypothesis that sperm MMP breakdown leads to ROS overproduction (Zhang et al., 2019), or the studies performed on animal testicular tissue where malondialdehyde levels, a reliable indirect indicator of OS, were found to be significantly increased with bleomycin (Amirshahi et al., 2014; Ibrahim et al., 2023; Yaghutian Nezhad et al., 2021). This surprising outcome leads one to consider the sperm antioxidant system as an additional balance point.

The methodology used to assess the oxidant levels of sperm cells may be the cause of the current results, as it fails to account for antioxidant levels (Homa et al., 2019). Human sperm actually contain minimal amounts of antioxidants (Barbagallo et al., 2020), but they also contain high concentrations of glutathione (GSH), a vital antioxidant that scavenges a range of oxidants, including ROS (Brahmajosyula & Morimoto, 2023). Even though Ibrahim et al. (2023) reported a noteworthy reduction in other antioxidant molecule levels in rat testicular tissue following bleomycin action, studies on lung fibrosis (Arslan et al., 2002; Boyacı et al., 2006; Day et al., 2002; Razavi-Azarkhiavi et al., 2014; Tobwala et al., 2013) and testicular cancer (Cort et al., 2012) have more closely linked bleomycin to a depletion of GSH levels. Therefore, this data supports the theory that bleomycin may cause OS by a decrease in intracellular GSH content rather than a promotion of intracellular ROS production, as hypothesized by Day et al. (2002), despite the absence of studies that evaluated the bleomycin-induced OS on germ cells and, particularly in human sperm. Therefore, additional research assessing sperm antioxidant levels following bleomycin exposure is required to support or refute this theory.

Due to its detrimental effects on vitality, morphology, MMP, total and progressive motility, and early acrosomal reaction, OS is seen in low-quality semen samples (El-Taieb et al., 2015; Homa et al., 2019). Thus, regardless of the route, it appears that bleomycin

causes an OS condition in sperm, just like other chemotherapeutics do (Yang et al., 2018), which results in pathophysiological changes (Wang et al., 2003). Furthermore, antioxidant drugs or strategies should also be studied to mitigate these harmful effects of bleomycin OS.

Acrosome reaction is as a marker of human sperm fertility potential (Egeberg Palme et al., 2018). Following sperm capacitation, the acrosome reaction is brought on by contact between the spermatozoa and receptors in the oocyte's zona pellucida (ZP), which aids in fertilization. (El-Taieb et al., 2015; Okada, 2022; Sharma & Agarwal, 2011; Tello-Mora et al., 2018; Zhang et al., 2019).

Since there is no information available about how bleomycin affects sperm acrosome reaction, the current study employed different methods to assess the sperm acrosome status. In two of these techniques, the PNA was employed to bind to carbohydrates found on the acrosome's outer surface in unreacted sperm. Both optical and fluorescence microscopy were used to observe this binding. Reduced binding of PNA to the acrosome is the outcome of changes to the acrosomal membrane that take place during the acrosome reaction. Following bleomycin exposure, there were no statistically significant changes in the fluorescence assay. This could be explained by the marker's inespecificity, which has been seen on both acrosome-reacted and acrosome-intact sperm (D'Cruz & Haas, 1996).

The PNA-biotinylated test staining showed that bleomycin could significantly decrease successful acrosome reaction. The findings of the third technique used in the current study to assess acrosome reaction, which showed that the group exposed to bleomycin showed a significantly higher frequency of absent acrosomes as well as lifted and swollen acrosomes, may help to explain the increased binding of PNA-biotinylated to the acrosome. These lifted and swollen acrosomes may account for the observed decrease in overall acrosome reactivity in sperm exposed to bleomycin as compared to the experimental control group. However, upon examining the PNA-biotinylated binding staining patterns, it becomes evident that sperm exposed to bleomycin exhibit an increase in Class II at the expense of a decrease in Class IV when compared to the experimental control group. Since SPM aims to support sperm vitality, motility, and capacitation and does not trigger the acrosome reaction in the absence of the proper signals from the oocyte, the current results may suggest that bleomycin be involved in the acrosome reaction's retardation. It is interesting to note that acute myeloid leukemia mice have been shown to experience a spontaneous acrosome reaction in response to malignancy (Michailov et al., 2019). Further research on how bleomycin can potentially disrupt this intricate and highly regulated process is advised due to the paucity of direct information

currently available regarding the medication's effects on the acrosome reaction. It should be noted, however, that the effects may vary depending on bleomycin dosage, exposure duration, and particular experimental conditions, and that in some cases, the effects may be transient or reversible.

Nevertheless, the substantial rise in the Class IV staining pattern may be linked to a reduction in MMP. Following the activation of the acrosome reaction, there is a drop in the amount of ATP (Christen et al., 1983) and a decrease in the frequency of the sperm flagellar beat (Ishijima et al., 2002). These findings suggest that the sperm motility is decreased to aid in the fusion of gametes (Jabloűski et al., 2023).

3.5 | Conclusion

This is the first *in vitro* investigation of bleomycin's gonadotoxicity in humans. The current study's findings demonstrate that bleomycin-induced sperm quality loss is associated with significantly lower sperm motility and vitality as well as significantly higher DNA fragmentation and increased chromatin decondensed status. These findings were also consubstantiated by TEM analysis. In addition to these gamete indicators of reproductive competence, a markedly elevated level of mitochondrial dysfunction and spontaneous acrosome reaction, albeit at a slower pace, were also observed. Interestingly, the current data also showed for the first time a maintenance of the ROS levels following exposure to bleomycin, which contrasts with the well-documented induction of ROS overproduction by bleomycin.

The present findings support the limited *in vivo* animal research in part by validating the detrimental effects of bleomycin on male fertility. Nonetheless, it offers new evidence that makes it possible to suggest novel underlying mechanisms for bleomycin's actions. However, more research needs to be done. Specifically, GSH levels in sperm samples after bleomycin exposure should be examined, as should the expression of molecules like caspase-9 and -3 or others involved in mitochondrial apoptosis.

Like all *in vitro* research, this study has limitations, though. The translation of *in vitro* results to *in vivo* settings, particularly when it comes to the reproductive system, can be challenging and complex. The reproductive system is distinct in many respects because it is highly regulated and influenced by numerous factors. Cancer, as a complex and dynamic disease, introduces additional challenges when studying medications and treatments *in vitro* versus *in vivo*. Additional challenges include *in vivo* variability when influenced by lifestyle and environment, as well as ethical and regulatory constraints.

The current findings may, however, provide valuable insights both to develop further research and to develop effective interventions to preserve future reproductive potential

and outcomes of patients of reproductive age undergoing treatments with bleomycin. Once more, alert healthcare professionals to the need for patient-centered care that includes a fertility preservation approach. This approach respects the patient's individual needs and concerns and helps mitigate potential long-term health consequences. Moreover, giving the patient hope for the future gives him a sense of control over his family planning decisions.

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Chapter IV. Main Conclusions and Future Perspectives

This thesis investigated the effects of the chemotherapy drug bleomycin on male reproductive function *in vitro*. This was done in accordance with the US National Research Council's (NRC) 2007 report, which outlined a vision for toxicity testing (US NRC, 2007). A key component of this strategy is the increased use of high-throughput *in vitro* testing systems, which reduces the need for costlier and time-consuming toxicological studies that use experimental animal studies.

Cancer is a potentially life-threatening disability affecting millions of people across the globe, placing a huge burden on both the individual and society. The serendipitous discovery of anticancer treatments has revolutionized both our understanding and management of cancer. Still, many anticancer drugs lack adequate and sufficient safety data and information regarding effects on consequential comorbid conditions, despite their many short- and long-term benefits. As a result, important problems like reproductive toxicity persist. Moreover, the concomitant use of several anticancer drugs make it unclear what differences between chemotherapeutics are attributable to. This makes determining each drug's actual effectiveness and risk challenging.

The research on bleomycin-induced reproductive harm is scant and primarily focuses on investigations conducted on animals. In fact, the systematic literature review conducted for this thesis reveals that the only manuscripts that have been written on this assumption to yet have exclusively used *in vivo* rodent research to anticipate impacts on human male fertility. This not only contradicts the NRC's vision for toxicity testing (US NRC, 2007), but it also poses an increased risk to human health because it extrapolates findings from rodent toxicity testing to humans. The lowest level of unobserved adverse effect measured in the rodent model is typically divided by a safety factor of ten to account for the differences between rodents and humans and by a factor of tenfold additional uncertainty to account for inter-individual differences in order to define regulatory acceptance for human health (Dorne & Renwick, 2005). However, when assessing the effects of drugs on reproductive functions, this tacit assumption that morphogenetic and molecular mechanisms in rodent are translatable to human organogenesis is questionable because fundamental processes like cellular differentiation, mitosis and carcinogenesis, as well as endocrine processes, vary widely between species. When it comes to reproductive processes, these species differences are much more noticeable.

Analysis of the available data, however, indicates that bleomycin causes gonadotoxicity in rodents and has an impact on every aspect of testicular function, including the architecture of the seminiferous tubules and the hormones and cells involved in spermatogenesis, all of which are markedly altered (Lobo de Almeida et al., 2023a).

To fill the gap in the literature on human studies, the evaluation of the *in vitro* actions of bleomycin on human sperm function was pursued in this thesis. Human sperm

alterations can validly predict future human reproductive outcomes. Therefore, the *in vitro* model adopted in this study is not only in line with the NRC's vision because it considers the challenges of conducting *in vivo* studies in humans and the bleomycin therapeutic scheme in the vast majority of cases in concomitance with other drugs. The results of this thesis support the deleterious impact of bleomycin on conventional sperm parameters reported in rodent *in vivo* research. However, additional information was discovered, supporting the theory that bleomycin can trigger the acrosome response too soon and having a detrimental influence on the mitochondrial activity and DNA integrity of human sperm. The main study issue addressed can be answered by conventional sperm analysis, which is examined here for the first time in human samples (Lobo de Almeida et al., 2023b). However, these new results also raise additional questions and speculations regarding the mechanisms driving these impacts that need to be confirmed (Lobo de Almeida et al., 2023b).

This thesis' findings add significant scientific evidence, yet it is crucial to put into perspective the work that remains to be done to fully understand how bleomycin affects human fertility.

Therefore, in the future, it will be extremely important to clarify the role of OS and apoptosis in the negative impact induced by bleomycin on human sperm function through specific assays and it may even be possible to describe a new mechanism. Following the growing research and knowledge on the use of protective agents in combination with cytotoxic agents, it will be equally important, once the role of OS in the effect of bleomycin has been clarified, to study the effect of protective agents in reducing bleomycin-induced gonadotoxicity.

The results presented here for the first time and in detail regarding the ultrastructural analysis, suggest that a complete evaluation of the ultrastructural defects induced by bleomycin in the human sperm flagellum could highlight the multiorgan effect of bleomycin with emphasis on the association between the respiratory and reproductive, which ultimately could change the paradigm of management and study of these toxicities.

As suggested in Chapter II, it will also be important to comprehend how long bleomycin affects human sperm and whether those effects are transient or long-lasting. Acquiring a chronological comprehension of effects could also furnish instruments to propose a causal association between observable effects. Investigating the primary cause of each side effect of chemotherapy regimens that include bleomycin, like the BEP regimen, will also be important from an integrative standpoint.

In pharmacology, the study of how and why a particular molecule affects an individual is still in its infancy, but it has the potential to anticipate and improve patient reactions to treatment. By using biomarkers and genotypes, pharmacogenetics research

is changing the way cancer is managed. This is allowing for the early determination of whether a patient will benefit from anticancer medications to the point where the dangers outweigh the benefits. In reproductive medicine, the ability to classify the individuals reproductive potential based on changes in seminal parameters may allow to confirm whether these changes condition the effects of bleomycin, even though the genetic origins of infertility are still mostly unknown.

The need to holistically contemplate the psychosocial needs of the oncological patients makes this *in vitro* study on human sperm an example for future *in vitro* studies in context of pharma-toxicology research. It also offers new insights into building strong evidence that will assist oncologists and cancer patients in discussing the risks associated with chemotherapy regimens, including bleomycin, and in making an informed and more thoughtful decision regarding fertility preservation before initiating treatments. Thus, the present work enables a better and informed patients' protection in the present, envisioning their future.

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