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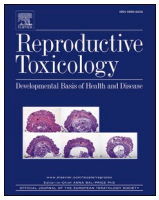

Review

A systematic review of bleomycin-induced gonadotoxicity: Mechanistic implications for male reproductive health and fertility

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ABSTRACT

Long-term cancer treatment complications in men include testicular dysfunction and infertility. Although various chemotherapies have been studied, there is limited evidence on their effects, especially for bleomycin. Despite its known lung toxicity, bleomycin's impact on male reproductive health is not well-researched. This systematic review aimed to evaluate bleomycin's effects on testicular function and fertility. A search of PubMed and Web of Science identified seven relevant animal studies on bleomycin's gonadotoxicity. The research, limited to animal models, shows that bleomycin significantly disrupts male reproductive health, including DNA damage in sperm, analogous to its effects on cancer cells, and notable histopathological changes in rodent testes. It reduces sperm quality and testosterone levels, correlating with Leydig cell degeneration and inflammatory responses, which further aligns with the drug's known capacity to induce lung inflammation. Due to the inherent limitations in extrapolating results from rodents to humans, further research, particularly in humans, is needed to confirm these findings, assess hormonal impacts, temporal patterns of effects (whether transient or permanent), and their impacts implications for offspring, as well explore potential mitigation strategies. These findings are a first step in raising awareness among clinicians about bleomycin's fertility risks and developing strategies for fertility preservation.

1. Introduction

Cancer represents a significant global health challenge, with an estimated 19.3 million new cases worldwide in 2020 [1]. Fortunately, advances in oncology treatments have led to a notable increase in overall cancer survival rates [2,3]. However, this progress has brought about a heightened awareness of the morbidity and long-term side effects faced by patients and survivors [4,5]. With a paramount focus on enhancing patient quality of life, novel approaches such as personalized medicine have emerged to address the limitations and adverse effects associated with conventional treatments. Nonetheless, chemotherapy remains a cornerstone in cancer therapy [4], targeting tumor cells, many of which are rapidly dividing, and consequently affecting processes like spermatogenesis [3,6].

For men of reproductive age or younger, preserving fertility post-cancer treatment is a primary concern [3]. Studies indicate that over half of male oncologic survivors express a desire to have biological children [7]. Testicular dysfunction, a common long-term side effect of cytotoxic chemotherapy, varies depending on the specific agent, dosage, patient's age, and stage of testicular maturity [5,8]. While reports of azoospermia and oligospermia following chemotherapy are prevalent [5,6,8], the resumption of spermatogenesis is unpredictable, and cases of severe and permanent infertility have been documented [7].

Many chemotherapy regimens involve combinations of multiple agents, making it difficult to determine their individual effects on fertility [9]. Bleomycin, a widely used anticancer agent, exemplifies this complexity.

Discovered in the late 1960s, bleomycin was among the earliest

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anticancer drugs developed [10]. Approved by the Food and Drug Administration (FDA) in 1975, bleomycin finds utility in the treatment of several cancers, including testicular cancer, squamous cell carcinomas, and malignant lymphomas [11]. Due to its efficacy and lack of myelosuppressive effects, bleomycin is included in several combination regimens for treating diseases such as Hodgkin's and non-Hodgkin's lymphoma (e.g., ABVD regimen) and testicular cancer (e.g., BEP regimen) [11,12].

Bleomycin exerts its cytotoxic effects primarily through DNA damage, facilitated by oxygen- and metal-ion-dependent mechanisms (Fig. 1) [11,13,14]. Additionally, it disrupts RNA synthesis, leading to impaired protein production [13,14]. Because of its large size and positive charge, bleomycin is administered parenterally and is quickly absorbed via intramuscular, subcutaneous, intraperitoneal (IP), or intrapleural routes [11]. It exhibits high bioavailability, with peak plasma concentrations typically attained within about one hour [11,12].

Bleomycin is metabolized by bleomycin hydrolase, which results in deamidation and inactivation reactions, rendering it inactive and nontoxic [11,13,15,16]. However, certain tissues, particularly the skin and lungs, accumulate bleomycin due to low levels and activity of bleomycin hydrolase, leading to tissue-specific toxicities [11,17]. While skin side effects are generally benign and resolve upon treatment cessation, bleomycin-induced pulmonary toxicity, notably pulmonary fibrosis, poses a significant risk, with mortality rates ranging from 3 % to 5 % [16,18].

Despite the reproductive toxicity associated with combination chemotherapies containing bleomycin [11,12], the individualized effects of bleomycin on fertility remain relatively understudied. Given its widespread use in men of reproductive age [11], further research is warranted to elucidate the specific impact of bleomycin exposure on testicular function and fertility. This comprehensive review aims to synthesize existing literature on this subject, providing valuable insights for future research endeavours and clinical considerations.

2. Methods

2.1. Search strategy

This review was conducted in accordance with the 2020 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. To identify original clinical and experimental studies examining the testicular damage induced by bleomycin, searches were conducted on PubMed and Web of Science up to May 23, 2023. The search strategy employed specific terms including "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 concepts, targeting titles and abstracts.

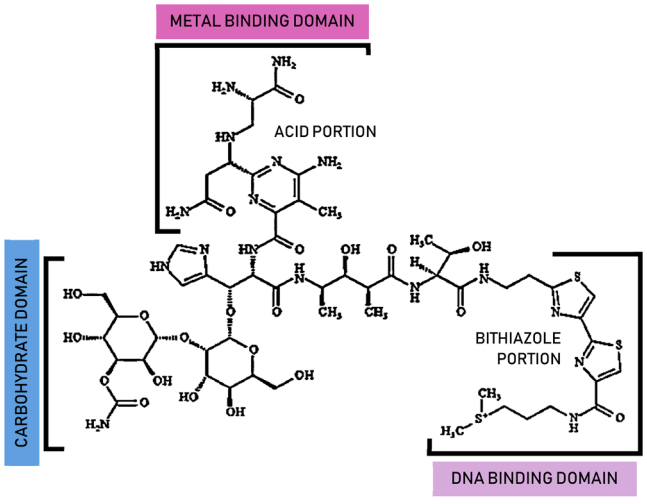

Fig. 1. Structure of bleomycin identifying main domains involved in DNA and metal binding.

Recognizing the limited availability of research addressing the direct impacts of bleomycin on the male reproductive system, Google Scholar was also included to enhance comprehensiveness and mitigate publication bias, as suggested by Gusenbauer and Haddaway [19]. Given the vast number of reports indexed by Google Scholar, the search strategy was refined to focus solely on titles, ensuring a more targeted and concise approach.

No restrictions were placed on the publication dates of the identified papers. Additionally, a comprehensive examination of relevant article bibliographies was conducted to identify any additional pertinent studies.

2.2. Selection process and the eligibility criteria (inclusion and exclusion criteria)

Following the removal of duplicates, full texts were subjected to rigorous scrutiny to ensure strict adherence to the eligibility criteria outlined below.

Inclusion Criteria:

- Articles focused on bleomycin-induced testicular damage, including studies that conduct histological analysis, sperm analysis, and assessments of reproductive hormones.
- Original articles encompassing both *in vivo* (animal), *in vitro* (laboratory), clinical trials, and human studies.

Exclusion Criteria:

- Review articles, as they do not contribute original data.
- Articles published in languages other than English, to ensure accessibility and uniformity in data interpretation.
- Studies exclusively investigating female fertility, as the focus of this review is on male reproductive health.
- Research involving combination therapy regimens where bleomycin is not studied in isolation, to avoid confounding effects from other drugs.

During the bibliographic screening of reference lists from the selected articles, one study [20] was identified that initially met all inclusion criteria. However, this study was ultimately excluded due to significant challenges in interpreting its results. Specifically, inconsistencies and gaps in the "Material and Methods" and "Results" sections compromised the reliability of its findings, precluding a robust reliable analysis.

In the end, a total of seven manuscripts met the inclusion criteria and were included in the final analysis, as depicted in the PRISMA diagram (Fig. 2).

2.3. Data extraction

The study's design, including sample size, whether it involved human or animal subjects, and details regarding the dosage and route of bleomycin administration, were meticulously collected and documented. Furthermore, specific reproductive outcomes, such as semen parameters and hormone levels, were compiled and recorded for analysis.

3. Results

3.1. Characteristics of the studies included in this systematic review

This systematic review identified seven studies that examined the

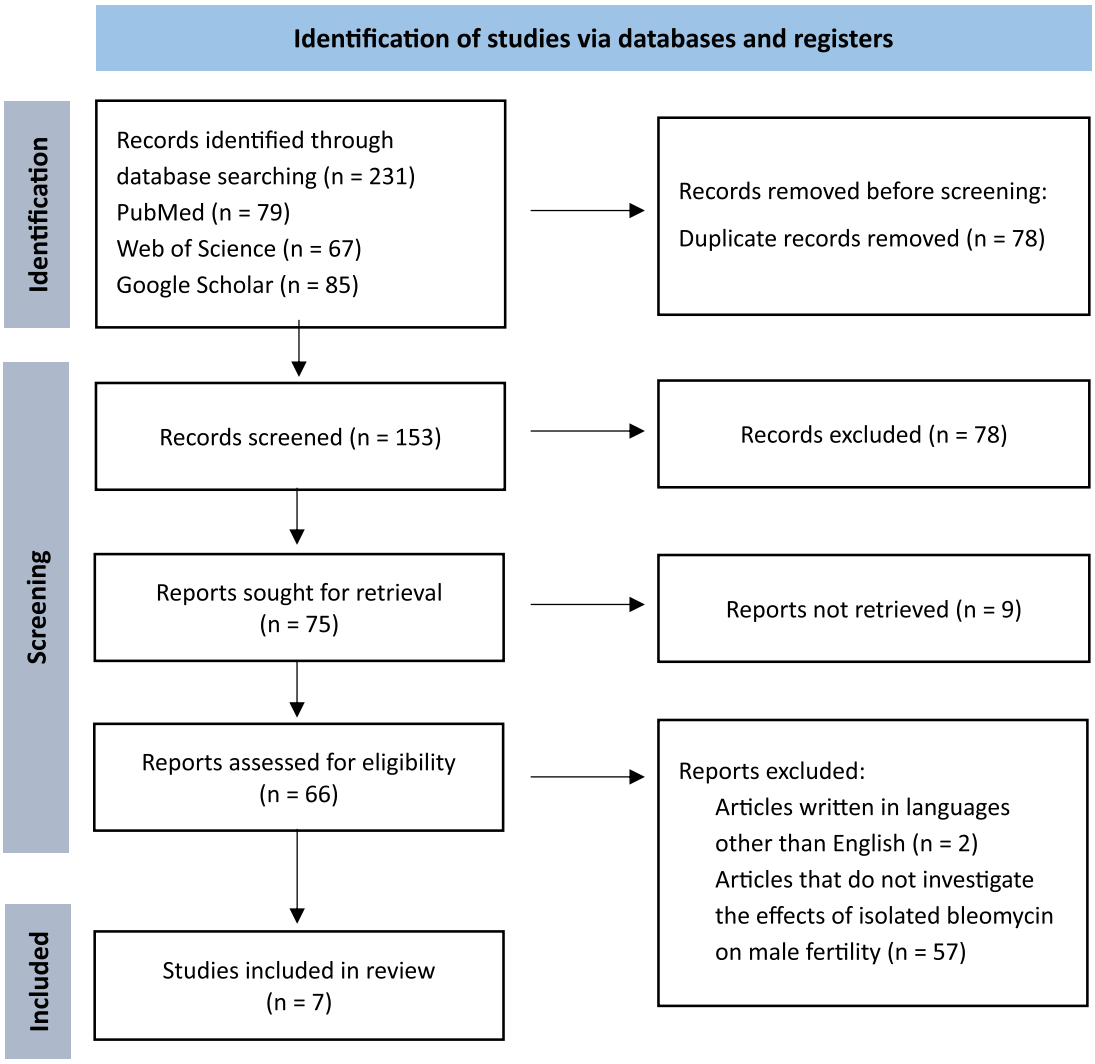

Fig. 2. PRISMA 2020 flow diagram of article search and selection strategy to assess the effects of isolated bleomycin on testicular and reproductive function.

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

Study	Design Animals	Control group	Experimental group	Schedule	Fertility Outcomes	Toxicity assessments
[21]	Male C3HHeB/FeJ mice	Saline IP	Bleomycin IP (> 1 mg/kg*)	Single dose	Sperm count Histopathology	29 days after injection 11 days after injection
[22]	Male Sprague-Dawley rats	Saline IP	Bleomycin IP (20 mg/kg)	Single dose	Hormone analysis Histopathology	On days 5, 10 and 30 after injection
[23]	10 male Wistar rats	Saline IP	Bleomycin IP (10 mg/kg)	2x/week/48 days	Semen analysis Hormonal analysis Antioxidant/oxidant status	After the 48 days of treatment
[24]	10 male Wistar rats	Saline IP	Bleomycin IP (15 mg/kg)	3x/week/8 weeks	Sperm count Hormonal analysis Histopathology	After the 8 weeks of treatment
[25]	8 male rats	Saline IT	Bleomycin IT (7.5 mg/kg)	Single dose	Semen analysis	14 days after injection
[26]	8 male Balb/c mice	Saline IP	Bleomycin IP (10 mg/kg)	Daily/35 days	Semen analysis Histopathology Antioxidant/oxidant status Genetic expression	After the 35 days of treatment
[27]	10 Albino male rats	Distilled water IP	Bleomycin IP (1.5 mg/kg**)	2x/week/3 weeks	Semen analysis Hormonal analysis Antioxidant/oxidant status	30 and 60 days after the treatment

*Bleomycin was administered as a single injection (0.01 mL/g body weight). When the drug concentrations were insufficient to reach the LD_{50}, larger injection volumes were used.
**The doses of bleomycin were expressed in units/kg, where 1 unit is approximately equal to 1 mg.
Abbreviations: IP, intraperitoneal administration; IT, intratracheal administration.

impact of bleomycin exposure on male fertility. Table 1 summarizes the design characteristics of these studies.

The rodent was the primary subject in all seven studies. Among the seven studies, five (71 %) utilized rats [22–25,27], while two (29 %) employed mice [21,26]. A variety of rat and mouse strains were employed. All studies, with the exception of one, included a control group that received saline solution injections [21–26] or distilled water [27] instead of bleomycin. Injection routes were predominantly IP in 6 studies [21–24,26,27] and intratracheal (IT) administration in one study [25]. Each study employed a consistent administration route for both experimental and control groups.

A diverse array of factors pertinent to male fertility constituted the outcomes under investigation. Sperm count, assessed in six of seven studies (with the exception of [22]), was the most frequently studied outcome. Additionally, four studies [23,25–27] conducted comprehensive sperm analyses, including parameters such as vitality, motility, and morphology. In addition, reproductive hormones (testosterone, LH, and FSH) were examined in the serum of animals in 5 studies [22–24,26,27], representing another extensively explored aspect. Furthermore, histopathological analyses were conducted in four studies [21,22,24,26] to discern potential morphological, anatomical, and cellular alterations induced by bleomycin in testicular tissue and related organs. Three studies [23,26,27] conducted additional analyses to evaluate the antioxidant/oxidant status of testicular tissue by measuring enzymes and specific markers. One study also investigated the gene expression of pro- and anti-apoptotic genes [26].

Moreover, five of the seven studies primarily aimed to evaluate the efficacy of potential protective agents in combination with bleomycin, with all tested compounds demonstrating positive outcomes (Table 2).

3.2. Histopathology

The testes play a critical role in male fertility however, due to their anatomical location, they are considered the most susceptible organ to external stressors [28]. Furthermore, the germinal epithelium of the testis is particularly vulnerable to the adverse effects of chemotherapy [6]. Table 3 presents the primary findings from the histopathological assessments conducted in four of the seven studies included in this review, which compared the exposed groups with their respective control counterparts.

Lu and Meistrich [21] conducted a pioneering study on the histopathology of testes following bleomycin exposure in 1979. They fixed the testes in Bouin's solution and stained them with periodic acid-Schiff-hematoxylin (PAS-H). Their examination revealed no discernible changes in interstitial tissue or tubule structure.

Recently, Yaghutian Nezhad et al. [26] conducted an extensive histopathological evaluation. Following testicular biopsy, fixation in Bouin's solution, and hematoxylin-eosin (HE) staining of 5 μm sections, they observed a significant increase in the thickness of the tunica albuginea. Although changes in germinal epithelium thickness were not statistically significant, a significant reduction in seminiferous tubule diameters, alteration in their normal structure, and evident signs of severe necrosis were observed. Furthermore, significant decreases in the numbers of spermatids, Sertoli cells, spermatogonia, and spermatocytes were noted, consistent with findings reported by Thwaini [24]. Thwaini's histological investigations, which entailed testicular removal, fixation in Bouin's solution, and HE staining of 5 μm paraffin sections, also revealed the presence of seminiferous tubule vacuolation, basal membrane detachment, and Leydig cell degeneration. In future studies, for a thorough histopathological assessment of compound toxicity, it is recommended to use the HE stain for its versatility and simplicity in analyzing testicular morphology in paraffin sections, which aids in cell identification and localization [29–31], and employ the PAS-H stain to obtain critical insights into the stages of spermatogenesis [30]. This will provide clear insights on the potential mechanistic actions of chemicals exposure.

Yaghutian Nezhad et al. [26] additionally reported a significantly lower testis weight/body weight ratio following bleomycin exposure, which contradicts Carreau's [22] conclusion of no changes in this parameter.

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

Study	Protective agent evaluated	Outcome
[23] [24] [25] [26] [27]	Royal jelly Valsartan Theophylline Thymoquinone Vitamin E and Selenium	Co-administration could effectively mitigate bleomycin's negative effects by inhibiting oxidative processes and restoring the antioxidant defense system

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

Study	Histopathology	
[21]	Interstitial tissue or structure of tubules	NC$^{\#}$
[22]	Testis weight/body weight ratio	NC*
[24]	Vacuolation of seminiferous tubules	↑$^{\#}$
	Detachment of basal membrane	↑$^{\#}$
	Number of spermatogonia and spermatocytes	↓$^{\#}$
	Degeneration of Leydig cells	↑$^{\#}$
[26]	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.

3.3. Semen analysis

Semen analysis represents the fundamental method for evaluating male (in)fertility. Consequently, six of the studies examining epididymal sperm were incorporated into this review (Table 4).

All studies assessing sperm via spermiogram employed a common metric, sperm count, which yielded consistent results. These studies demonstrated that bleomycin diminishes sperm count [21,23–27]. Sperm count was uniformly determined through microscopic observation using a hemocytometer counting chamber.

Amirshahi et al. [23], Belhan et al. [25], and Yaghutian Nezhad et al. [26] noted a decline in sperm vitality, assessed via eosin-Y and nigrosine stain.

Sperm morphology was evaluated in three studies using different staining techniques, including eosin-nigrosin [25], diff-quick [26], and eosin-Y [27]. The results demonstrated a consistent decrease in normal morphology across all studies.

Four of the reviewed articles [23,25–27] employed random field microscopy to assess sperm motility. Belhan's [25] and Ibrahim's [27] studies reported a significant decrease in total motility rate, while Amirshahi et al. [23] and Yaghutian Nezhad et al. [26] utilized the World Health Organization (WHO)'s four-category system, revealing a significant decrease in both rapidly ($\geq$ 25 m/s) and slowly ($<$ 25 m/s) progressive motility, and either a significant increase [26] or non-significant change [23] in non-progressive and immotile spermatozoa percentage.

A conventional semen analysis encompasses the aforementioned parameters. The standard and reference limits for human sperm analysis

Table 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	
[21]	Sperm count (fraction of untreated control)	↓#			
[23]	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 (%)	↓*			
[24]	Sperm count (sperm/mg of epididymis head)	↓*			
[25]	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]
[26]	Sperm count (x10^6/mL)	↓*			
	Vitality (%)	↓*			
	Motility (%)				
	Rapid progressive	↓*			
	Slow progressive	↓*			
	Non-progressive	↑*			
	Immotile	↑*			
	Normal morphology (%)	↓*			
	Mature chromatin condensation (%)	↓*			
[27]	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 [32].
b [33].

are defined by the WHO. Acknowledging interspecies physiological variances, a thorough comparison of results obtained by different authors was juxtaposed with WHO reference values for each seminal parameter relative to the manuscript's year of publication. However, this was only applied to three studies, as the remaining three [21,24,26] providing sperm analysis did not present quantitative results, only qualitative outcomes.

It is noteworthy that, aside from the decrease in some experimental group parameters compared to the control group, rat and mice studies reported quantitative results surpassing the WHO cutoff reference value for humans. The exception was Ibrahim's [27] finding, where animal sperm exhibited reduced total motility post-bleomycin exposure, falling below the WHO cutoff reference value [33]. Additionally, Ibrahim's study was the sole investigation evaluating the recovery of seminal parameters (sperm count, motility, and morphology) after 60 days of treatment. The study concluded that none of the assessed seminal parameters exhibited significant recovery after this period [27]. Hence, further studies on post-bleomycin recovery are warranted.

3.4. Sperm DNA damage and expression of apoptosis-related genes

Aberrant apoptosis is a major cause of DNA damage [34]. In fact, some factors, such as reactive oxygen species (ROS) and drugs, activate the apoptotic cascade [35]. 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 [35,36]. Both 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 [36].

As illustrated in Table 4, two studies also investigated sperm DNA integrity, encompassing the assessment of sperm DNA fragmentation [23] and the status of chromatin condensation [23,26].

Amirshahi et al. [23] employed the acridine orange test (AOT) and the aniline blue assay (AB), which revealed a notable increase in the percentage of sperm with DNA fragmentation and a decrease in normal sperm chromatin condensation, respectively. The findings regarding chromatin condensation by Amirshahi [23] agreed with those reported by Yaghutian Nezhad and colleagues [26] utilizing the same methodology. The link between increased sperm DNA damage [23] and reduced mature chromatin condensation [23,26] following bleomycin exposure is rooted in the spermatogenesis process. Incomplete sperm maturation heightens susceptibility to DNA damage [37]. Abnormal chromatin condensation status is also indicative of broader spermatogenesis abnormalities [37]. These findings regarding compromised chromatin condensation align with reported alterations in spermatogenesis function observed in histological studies, including abnormal morphology, which increases the likelihood of apoptosis.

Additionally, Yaghutian Nezhad et al. [26] investigated the expression of two distinct genes, Bcl2L1 and Bax, which respectively serve as an apoptosis inhibitor and activator of the intrinsic apoptotic signaling pathway. Real-time polymerase chain reaction was employed to assess the expression of apoptotic genes. The results demonstrated that the levels of Bax gene expression did not exhibit significant differences between the experimental and control groups, while Bcl2L1 expression significantly decreased in the presence of bleomycin.

Measuring apoptosis or the expression of genes associated with this process, as conducted by Yaghutian Nezhad et al. [26], may serve as a robust predictor of sperm quality and, consequently, male fertility [35]. The decrease in anti-apoptotic protein Bcl2L1 expression validates bleomycin-induced apoptosis. According to other studies [36], this notable reduction in seminal Bcl2L1 correlates positively with sperm count, motility, and normal morphology, all of which exhibited significant decline following bleomycin exposure. Additionally, Bcl2L1 acts as an inhibitor of apoptosis, reducing ROS synthesis and thereby protecting cells [35]. Therefore, the reduced expression of Bcl2L1 aligns with induced sperm DNA damage [23], as well as seminiferous tubule degeneration [24,26], and diminished germ cell numbers [24,26].

3.5. Reproductive hormone levels

The gonadotropin-releasing hormone (GnRH), released by the hypothalamic-pituitary-testicular axis, regulates the secretion of LH and FSH [38]. LH stimulates Leydig cells to synthesize and secrete androgens, specifically testosterone, whereas FSH promotes Sertoli cell proliferation, supporting spermatogenesis [37,39,40].

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

Table 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 (ng/mL)[a]	Mice reference range (ng/mL)[b]
			CT Mean ± SD	EXP Mean ± SD		
[22]	Testosterone (%)	↓*	100	27	NA	NA
[23]	Testosterone (ng/mL)	↓*	4.28 ± 0.23	2.47 ± 0.17	1.73 – 7.78	0.66 – 5.4
[24]	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	1.37 ± 0.02	-	-
	(ng/mL)		0.81 ± 0.02	0.40 ± 0.006	1.73 – 7.78	0.66 – 5.4
[26]	LH (IU/mL)	↑*				
	FSH (IU/mL)	↑*				
	Testosterone (ng/mL)	↓*				
[27]	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 [41].
b [42].

In both clinical diagnostic and research settings, hormonal status is primarily assessed using immunoassays, which entail an antigen-antibody reaction. These assays may include immunoradiometric assay (IRMA), as employed by Amirshahi et al. [23], Yaghutian Nezhad et al. [26], and Carreau et al. [22], and enzyme-linked immunosorbent assay (ELISA), utilized by Ibrahim et al. [27] and Thwaini [24].

Both testosterone and sperm are produced by the testes and regulated by LH and FSH. Consequently, to assess the impact of bleomycin exposure on reproductive hormone, testosterone serum levels were measured in all five studies. All reported a significant decrease in testosterone levels [22–24,26,27]. This is corroborated by histological analysis revealing heightened degeneration of Leydig cells in animals treated with bleomycin [24].

However, while testosterone serum levels exhibited concordance, LH and FSH serum levels did not. These hormonal levels were measured only in the three most recent manuscripts [24,26,27]. Thwaini's study [24] revealed that bleomycin did not induce significant changes in LH and FSH levels, suggesting resistance of endocrine cells to this cytotoxicity. In contrast, Yaghutian Nezhad et al. [26] observed an increase in both hormone serum levels, while Ibrahim et al. [27] reported a significant decrease. Ibrahim also evaluated serum hormone levels after 60 days of treatment, finding a decrease in testosterone and FSH levels and an increase in LH levels compared to the first 30 days of treatment [27].

Carreau and colleagues [22] conducted an *in vivo* assay to assess the interference of LH in testosterone levels and the recovery of testosterone levels after 5, 10, and 30 days of bleomycin injection. Contrary to Ibrahim's [27] findings, this experiment demonstrated that during the post-treatment recovery period, serum testosterone levels gradually recovered after 30 days, albeit incompletely. Furthermore, basal Leydig cell testosterone synthesis, as measured by crude testicular cell suspensions, decreased after 5 days and fully recovered after 30 days in both the treated and untreated groups. Additionally, under *in vitro* LH stimulation, testosterone production in the untreated group's cellular suspension was investigated. The results indicated that, under LH stimulation, testosterone levels decreased with an increase in bleomycin concentration, which did not occur in the absence of hormonal stimulation [27].

Analogous to sperm analysis and mindful of physiological differences between the species, hormone levels obtained were compared to human reference values (Table 5). Apart from the experimental group's FSH levels and the control group's LH levels in Ibrahim's [27] study, which fell below the minimum and exceeded the maximum of the range, respectively, all LH and FSH levels in the studies were within the human reference range. Regarding testosterone, reference ranges were established for both humans and mice. Testosterone levels in Amirshahi et al. [23] were within the normal range in comparison to both ranges. For the other studies [24,27], control testosterone levels were within the mice reference range, but only Ibrahim's results fell within the human reference range. The decrease in testosterone levels after bleomycin exposure placed them below the reference levels of both ranges [24,27].

Because testosterone stimulates spermatogenesis by acting on Sertoli cells, a decrease in its serum levels could explain the reported reductions in sperm count and germ cell numbers. This hormonal control suggests that Leydig cells degeneration and decreased Sertoli cells numbers following bleomycin exposure may be associated with changes in LH and FSH secretion, respectively. Additionally, since LH and testosterone levels are regulated by negative feedback, decreased testosterone levels and/or Leydig cell degeneration should prompt the hypothalamus and anterior pituitary to increase LH secretion [37]. However, conflicting results regarding FSH and LH levels complicate drawing definitive conclusions about bleomycin's cytotoxic effects of bleomycin on the hypothalamus-pituitary axis [43]. Therefore, further research is required to comprehensively understand bleomycin's impact on hormonal regulation, while acknowledging potential discrepancies in experimental design that may contribute to conflicting findings.

3.6. Antioxidant/oxidant status in testicular function

Despite the low oxygen tensions in the testicular microenvironment, this tissue is susceptible to oxidative stress (OS) due to the abundance of highly unsaturated fatty acids (especially 20:4 and 22:6) and the presence of potential ROS-generating systems [44]. To counteract this threat, the testes have developed a sophisticated array of antioxidant systems that include both enzymatic and non-enzymatic components, such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD). These enzymes play crucial roles in protecting against or mitigating ROS damage: SOD catalyzes the conversion of superoxide radicals ($O^{2\cdot}$) into hydrogen peroxide (H_2O_2) and oxygen; CAT, converts H_2O_2 into oxygen and water; and GPx is responsible for glutathione oxidation [45,46]. Spermatozoa plasma membranes contain a significant amount of polyunsaturated fatty acids (PUFAs), making them susceptible to oxidative damage. Consequently, an elevated ROS levels can lead to lipid peroxidation, resulting in the depletion of membrane lipid content and impaired sperm function [47]. Malondialdehyde (MDA), a well-studied and stable product of lipid peroxidation, serves as a biochemical marker of OS, allowing for monitoring of antioxidant/oxidant status [46,47].

Three studies [23,26,27] delved into the impact of bleomycin on the antioxidant/oxidant status of the testes by quantifying levels of MDA (indicative of lipid peroxidation) and/or three antioxidant enzymes: CAT, GPx, and SOD (Table 6).

Amirshahi et al. [23] and Yaghutian Nezhad et al. [26] gauged MDA levels using standard methods based on the production of a pink pigment resulting from the reaction between MDA and thiobarbituric acid (TBA) [47]. Meanwhile, Ibrahim et al. [27] employed reference-specific biodiagnostic kits based on the Kjeldahl method [48]

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

Study	Antioxidant/oxidant status	
[23]	MDA (μmol/g tissue)	↑*
[26]	MDA (nmol/mg protein)	↑*
[27]	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.

to measure both MDA levels and the activity of the antioxidant proteins CAT, GPx, and SOD. This involved assessing organic nitrogen through a three-step process (digestion, distillation, and titration).

All studies observed bleomycin-induced OS in the testes, as evidenced by a significant increase in MDA levels [23,26,27], along with a significant decrease in the physiological levels of the antioxidant enzymes CAT, GPx, and SOD [27].

The findings indicate that bleomycin induces lipid peroxidation due to OS status. This OS status and resulting lipid peroxidation are associated with impaired sperm function, evidenced by decreased motility [23,25–27] and plasma membrane rupture leading to decreased vitality [23,25,26]. Bleomycin's high ROS production may also contribute to observed histological changes [24,26], sperm DNA fragmentation [23], and reduced Bcl2L1 expression [26]. Additionally, bleomycin's inhibition of antioxidant enzymes aligns with its known role in suppressing cellular antioxidant defenses, as seen with other anticancer drugs [49,50]. This dysregulation of these antioxidant systems can lead to OS, negatively impacting sperm quality and fertility potential [47].

4. Discussion

Bleomycin is a widely used anti-tumor agent, particularly among young male cancer patients [11]. Although it is often administered in combination with other drugs, understanding the specific contribution of bleomycin to gonadotoxicity is crucial. Despite its frequent use, there is a paucity of studies examining the isolated effects of bleomycin on male fertility. This systematic review synthesizes findings from seven studies that investigated bleomycin-induced testicular injury, revealing significant implications for various aspects of male reproductive health.

The reviewed studies consistently demonstrate that bleomycin adversely affects sperm parameters, including count, vitality, motility, and morphology. It also impacts sperm DNA integrity, hormone secretion, testicular histology, and the balance between antioxidants and oxidants within the testicular tissue of the studied rodent models. Specifically, the evidence suggests that bleomycin disrupts sperm chromatin condensation, testosterone production, and increases lipid peroxidation levels. While our review covers a broad spectrum of endpoints, several critical aspects remain underexplored due to the limitations in the existing studies. Sperm DNA integrity and antioxidant enzyme levels, for instance, were examined in only a single study, precluding definitive conclusions about bleomycin's specific impact on these factors.

Moreover, variations in study design, including dosage and injection schedule disparities, alongside differences in rodent strains utilized, contribute to discrepancies in reported outcomes, complicating the synthesis of conclusive findings. This heterogeneity highlights the need for standardized research protocols to enable more reliable comparisons and conclusive findings. Future research should address these gaps and explore the isolated effects of bleomycin on male fertility more comprehensively, contributing to a better understanding of its long-term implications for reproductive health.

In fact, dosage selection, informed by preliminary pharmacodynamic and pharmacokinetic studies, is crucial for the design of reproductive toxicity studies. However, several reviewed articles lacked clarity on this aspect, potentially contributing to the observed outcome disparities. Future research should prioritize the transparent reporting of dosage and administration schedules, supported by rigorous preliminary investigations, to enhance the reproducibility and interpretability of findings.

Route of administration is another key factor influencing study outcomes, ideally mirroring human usage patterns to maximize clinical relevance [51]. The majority of studies employed IP administration, with only one utilizing IT administration, both of which diverge from the preferred intravenous (IV) route in humans. Although IP administration is commonly used in rodent studies due to its simplicity and expediency [52], its clinical applicability is limited, raising doubts about its suitability for evaluating drug effects that are intended to mimic human exposure. Future studies should, therefore, prioritize IV administration, or, if this is not feasible, clearly justify the use of alternative routes and acknowledge the potential limitations in clinical translation.

Strain selection and sample size are additional factors that can significantly influence the robustness and comparability of studies. Some manuscripts omitted these crucial details, which hampers a thorough evaluation of the results' validity. To improve the comparability of future studies, harmonization of methodologies, including standardized histopathological analyses [31,53], is imperative. The adoption of universal guidelines outlining essential histopathological parameters for testicular toxicity evaluation would further enhance coherence and facilitate meaningful comparisons across studies.

Histological assessments revealed bleomycin-induced alterations in testicular architecture, characterized by decreased seminiferous tubule diameters, germ cell loss, Sertoli cell reduction, and tunica albuginea thickening. These changes correlate with diminished sperm parameters [54], reflecting compromised spermatogenesis and fertility potential. Furthermore, bleomycin-mediated Leydig cell degeneration likely contributes to decreased testosterone levels and disrupted hormonal regulation [55], necessitating further investigation to elucidate the underlying mechanisms and implications for male reproductive function.

In toxicological studies, testis weight and the testis weight/body weight ratio are key indicators of reproductive toxicity. Decreases in testis weight typically indicate germ cell loss and seminiferous tubule contraction, while increases may suggest elevated fluid content [30]. However, only two of the reviewed studies reported on this metric. Future human studies should employ non-invasive measurements, such as ultrasound, combined with hormone level assessments (testosterone, FSH, LH) to gain a comprehensive understanding of testicular function and reproductive toxicity. This approach will better inform clinical interventions aimed at mitigating adverse effects.

A particularly concerning finding is the significant thickening of the tunica albuginea, a fibrous capsule rich in structural collagen that surrounds the testis, observed in some studies, potentially indicating collagen accumulation parallel to bleomycin-induced pulmonary fibrosis [16,17]. This suggests a possible shared mechanism of collagen promotion, with implications for testicular function and structure. The observed necrosis and tubular disorganization further support the hypothesis that bleomycin triggers an inflammatory cascade [26,30], profoundly altering seminiferous tubule structure and impairing spermatogenesis. These insights emphasize the importance of further investigating inflammatory pathways in testicular toxicity research.

In addition to traditional sperm quality parameters, sperm DNA integrity warrants evaluation, given its significance in predicting male fertility [56]. Bleomycin exposure correlated with increased sperm DNA damage, accompanied by abnormalities in chromatin condensation, indicative of disrupted spermatogenesis. Oxidative stress, induced by bleomycin-mediated inhibition of antioxidant enzymes, coupled with lipid peroxidation, further compromises sperm function and viability, amplifying the drug's reproductive toxicity. Additionally, aberrant

apoptosis emerges as a significant contributor to DNA damage [35], triggered by factors such as ROS and drug exposure. These dysregulated apoptosis pathways, evidenced by altered expression of apoptotic regulators, coincide with impaired sperm parameters and histological abnormalities, underscoring the multifaceted impact of bleomycin on testicular health.

Regarding recovery, Ibrahim et al. [27] were the only researchers to examine the functional parameters 60 days' post treatment to assess potential recovery. Despite the anticipated recovery period aligning with the rodent spermatogenesis cycle (approximately 63 days) [29], no significant changes were detected in sperm count, motility, and morphology. Furthermore, Ibrahim's study evaluated serum hormone levels, finding a significant decrease in FSH and an increase in LH, with testosterone levels remaining unchanged [27]. This contrasts with Carreau and colleagues' findings [22], which showed a sharp decline in serum testosterone levels after 5 days, followed by a gradual, albeit incomplete, recovery by 30 days. Ibrahim's study also assessed the recovery of MDA and antioxidant enzymes (CAT, GPx, and SOD). While MDA levels remained unchanged, all tested antioxidant enzymes exhibited a decline [27], further indicating that the effects of bleomycin may persist beyond the treatment period. These findings underscore the need for more comprehensive and longitudinal research to fully understand the long-term effects and recovery potential following bleomycin treatment.

Finally, when interpreting findings from animal models for human reproductive health, it is crucial to consider species-specific differences in sperm characteristics and responses to toxicological agents. While rodent studies typically focus on sperm from the cauda epididymis, human studies analyze ejaculated sperm. The cauda epididymis plays a critical role in sperm maturation and storage, significantly influencing sperm quality and fertility outcomes. Therefore, recognizing these differences is essential for accurate extrapolation of findings to human contexts.

Factors such as dosage, frequency of administration, and duration of treatment influence the severity of gonadotoxicity [58]. Specifically, it is important to determine whether there is a correlation between the treatment regimen and the extent of testicular damage. In studies that evaluated germ cell counts [24,26], significant decreases in the number of spermatogonia and spermatocytes were observed in rats and mice. However, these studies involved not only different rodent species different bleomycin treatment regimens: daily administration for 35 days in mice [26] and three times a week for 48 days in rats [24], although the dosage and route of administration were similar. Furthermore, in determining the testicular weight-to-body weight ratio, half of the dose of bleomycin but administered cumulatively daily for 35 days caused a significant reduction in this ratio in mice [26], however, no change was observed in rats that received only a single dose at double the concentration of each dose administered to mice [22]. MDA levels increased significantly regardless of species or treatment duration. Rats treated with bleomycin at 10 mg/kg IP twice weekly for 48 days [23] and mice treated with the same dosage and route of administration daily for 7 weeks (one week less than the rats) [26] both showed this increase. Testosterone levels also decreased significantly in all rodent species studied [22–24,26,27], with administered bleomycin doses ranging from 1.5 to 20 mg/kg and single-dose treatment regimen up to cumulative administration for 8 weeks, daily to twice a week. However, the effects on other reproductive hormones, LH and FSH, were inconsistent. Within the same dose and duration ranges, results varied: some studies observed significant changes, either increases or decreases [23,24], while others studies observed no change in the levels of these hormones [24,26,27]. Regarding seminal parameters, there was a significant decrease in sperm count [21,23–26], vitality [23–25], normal morphology [25–27], and mature chromatin condensation [23,26] in all the studies reviewed, with doses of bleomycin ranging from 1 to 15 mg/kg administered in a single dose for up to 8 weeks. However, motility results were less consistent [23,26]. While rapid and slow progressive motility decreased significantly in the two studies that assessed these parameters, the same was not true for non-progressive motility and immotile sperm. Non-progressive motility and immotile sperm were significantly decreased in mice (treated with 10 mg/kg bleomycin daily for 35 days) but showed no significant change in rats (treated with the same dose but administered twice a week for 48 days). Not only did the manuscripts reviewed not provide sufficient data on the results of the studies, they also varied widely, with, for example, doses and durations of treatment varying from a single dose to 8 weeks administered in different rodent species sometimes giving similar results, sometimes changes without being able to attribute the observed change specifically to a factor. The lack of consistency between studies challenges reaching definitive conclusions about the correlation between treatment regimens and the severity of adverse effects, specifically whether higher doses correlate with increased severity of gonadotoxicity. Information about recovery periods after treatment is also scarce. Understanding the duration required for recovery and its impact on gonadotoxicity would provide valuable information on the long-term effects of different treatment regimens. Overall, existing data indicate that bleomycin has a significant negative impact on testicular function in rodents. However, it is not yet clear whether these effects are the result of adverse reactions to bleomycin (side effects associated with normal therapeutic use) or toxicity (arising from higher doses or prolonged exposure). Recognizing these limitations, additional investigations with comprehensive reports are needed to clarify the relationship between bleomycin dosing regimens and resulting gonadotoxicity. Additionally, conducting follow-up analyses after treatment is crucial for evaluating the potential recovery of these parameters and long-term implications. Moreover, bleomycin-induced testicular injury encompasses a broad spectrum of adverse effects on male reproductive health, ranging from histological alterations to disruptions at the molecular level. A comprehensive understanding of these complex interactions requires integrated investigations that include multiple endpoints and mechanistic insights, which are essential for informing clinical practice and guiding therapeutic interventions.

5. Conclusion

Bleomycin's potential reproductive toxicity has not undergone the same depth of investigation as its pulmonary effects. With only seven studies examining its impact on male fertility, all conducted using animal models, our understanding of its effects on human reproduction remains limited. Surprisingly, despite the scarcity of data specifically on bleomycin's impact on male fertility, most studies focused on evaluating the protective effects of various compounds and antioxidants against bleomycin-induced reproductive toxicity. This emphasis on protective measures underscores the insufficient understanding of bleomycin's direct impact on testicular function and male fertility potential.

Given the absence of human studies, conclusions must be cautiously extrapolated from animal research [57]. While rats and mice are prevalent experimental models, their use poses limitations in translating results to humans due to differences in study design, genetic makeup, and physiological processes. Discrepancies between animal and human reproductive systems, such as lower sperm counts and less efficient spermatogenesis in humans compared to rodents, further complicate direct comparison and risk amplifying small effects observed in animal models. To address this gap, additional studies are necessary to corroborate these effects, alongside human in vivo or in vitro investigations, to accurately elucidate bleomycin's true impact on the male reproductive system.

Human reproductive toxicity assessments traditionally focus on sperm quality parameters, given the challenges in obtaining testicular biopsies. However, complementary approaches such as ultrasound examinations for testicular volume assessment and blood tests for hormone evaluation are crucial for a more comprehensive understanding of bleomycin's impact on testicular function. Future research should

prioritize investigating the duration and permanence of these effects, as well as their implications for seminal quality, fertility, pregnancy outcomes, and offspring health.

This review also underscores the variability in parameters and methodologies across reproductive toxicology studies, emphasizing the urgent need for standardized approaches to optimize the design and conclusions of future investigations. Researchers should aim to develop consistent methodologies that allow for accurate comparisons and more reliable conclusions. Future studies should also consider including human data, where feasible, to enhance the relevance of findings to clinical settings.

In conclusion, while existing animal data suggest a significant and negative impact of bleomycin on rodent testicular function, further research is imperative to establish robust evidence regarding its effects on human male fertility. This information is essential for oncologists and other healthcare professionals to provide comprehensive counseling to cancer patients, helping them make informed and conscious decisions about future reproductive potential and fertility preservation options.

CRediT authorship contribution statement

Rosália Sá: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mário Sousa:** Writing – review & editing, Funding acquisition. **Ana Fortuna:** Writing – review & editing. **Ana Lobo de Almeida:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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