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The impact of minoxidil on male reproductive biology: a multi-approach study including systematic review, in vivo, and ex vivo experiments

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Doctor Scientiae

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Thesis submitted to the Animal Biology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Co-advisers: Janaina da Silva
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“Ora et labora et legere” (São Bento de Núrsia)

ABSTRACT

SANTANA, Francielle de Fátima Viana, D.Sc., Universidade Federal de Viçosa, February, 2025. **The impact of minoxidil on male reproductive biology: a multi-approach study including systematic review, in vivo, and ex vivo experiments**. Adviser: Sergio Luis Pinto da Matta. Co-advisers: Janaina da Silva and Séverine Mazaud-guittot.

Minoxidil is a widely used treatment for androgenetic alopecia (AGA), approved by the FDA for topical use in both men and women. It is often combined with finasteride, which targets androgen metabolism, to enhance therapeutic efficacy. While minoxidil is commonly used, its pharmacodynamic mechanisms are not fully understood. The prevailing theory is that minoxidil works through vasodilation and potentially by modulating androgen-associated enzymatic pathways. Recently, there has been growing interest in using low-dose oral minoxidil *off-label* as a monotherapy for alopecia. This approach has shown favorable clinical outcomes with fewer adverse effects than topical use or combination therapy with finasteride. However, the effects of minoxidil on male reproductive function and fertility remain insufficiently investigated. Given the increasing *off-label* use and potential endocrine-disrupting properties, it is critical to evaluate minoxidil's reproductive toxicity. This study aimed to examine the impact of minoxidil on male reproductive organs, specifically the testes and epididymis, using a multi-phase approach that included a systematic literature review, *in vivo* studies in mice, and *ex vivo* studies with human testicular tissues. The systematic review analyzed 31 studies, 27 of which focused on finasteride and 4 on minoxidil. Both drugs were identified as endocrine disruptors that induce oxidative stress and morphological changes in the male reproductive tract, particularly in murine models. Studies on finasteride were six times more prevalent than those on minoxidil, highlighting a research gap and the need for more studies on minoxidil. An *in vivo* study in male mice was conducted to assess the effects of oral minoxidil on testicular and epididymal morphology and function. Mice were given different doses of minoxidil or finasteride, and after 42 and 84 days, both drugs induced testicular damage, including seminiferous tubule vacuolization and reduced epithelial height. The most significant damage occurred at 5 mg/kg minoxidil. While both drugs caused epididymal changes, finasteride uniquely reduced sperm transit time and sperm count in the cauda epididymis. *Ex vivo* experiments with human testicular tissues revealed that minoxidil did not disrupt fetal testicular differentiation but affected adult testicular tissue by downregulating germ cell markers and steroidogenic enzymes, impairing Leydig cell function. These findings

suggest that minoxidil may disrupt testicular steroidogenesis and spermatogenesis, raising concerns about its reproductive safety. The study concludes that both minoxidil and finasteride pose risks to male reproductive health. Minoxidil primarily affects hormonal balance and testicular morphology, warranting further research and clinical trials to validate these findings and explore strategies to mitigate reproductive effects.

Keywords: Reproductive toxicology; Finasteride; Minoxidil; Androgenetic alopecia; Endocrine disruptors; Male fertility

RESUMO

SANTANA, Francielle de Fátima Viana, D.Sc., Universidade Federal de Viçosa, fevereiro de 2025. **Impacto do minoxidil na biologia reprodutiva masculina: um estudo multi-abordagem incluindo revisão sistemática, experimentos *in vivo* e ex vivo**. Orientador: Sergio Luis Pinto da Matta. Coorientadores: Janaina da Silva e Séverine Mazaud-guittot.

O minoxidil é amplamente utilizado no tratamento da alopecia androgenética (AGA), sendo aprovado pela FDA para uso tópico em homens e mulheres. Frequentemente, é combinado com finasterida, um fármaco que age no metabolismo dos andrógenos, para potencializar a eficácia terapêutica. Apesar de seu uso comum, os mecanismos farmacodinâmicos do minoxidil não são completamente compreendidos. A teoria predominante sugere que o minoxidil age por meio da vasodilatação e possivelmente pela modulação de vias enzimáticas associadas aos andrógenos. Recentemente, tem-se mostrado crescente interesse no uso *off-label* de minoxidil oral em doses baixas como monoterapia para alopecia, apresentando resultados clínicos favoráveis e menos efeitos adversos em comparação ao uso tópico ou à combinação com finasterida. No entanto, os efeitos do minoxidil sobre a função reprodutiva masculina e a fertilidade ainda não foram suficientemente investigados. Dado o crescente uso *off-label* e as possíveis propriedades de desregulação endócrina, é fundamental avaliar a toxicidade reprodutiva do minoxidil. O objetivo deste estudo foi analisar o impacto do minoxidil nos órgãos reprodutivos masculinos, especificamente nos testículos e epidídimos, utilizando uma abordagem multifásica que incluiu revisão sistemática da literatura, estudos experimentais *in vivo* em camundongos e investigações *ex vivo* com tecidos testiculares humanos. A revisão sistemática analisou 31 estudos, sendo 27 sobre finasterida e 4 sobre minoxidil. Ambos os fármacos foram identificados como desreguladores endócrinos, capazes de induzir estresse oxidativo e alterações morfológicas no trato reprodutivo masculino, especialmente em modelos murinos. Os estudos sobre finasterida foram seis vezes mais frequentes do que os sobre minoxidil, destacando uma lacuna na pesquisa e a necessidade de mais investigações sobre o minoxidil. O estudo *in vivo* com camundongos avaliou os efeitos do minoxidil oral na morfologia e função testicular e epididimal. Os camundongos receberam diferentes doses de minoxidil ou finasterida, e após 42 e 84 dias, ambos os fármacos induziram danos testiculares, incluindo vacuolização dos túbulos seminíferos e redução da altura epitelial. O maior dano foi observado com a dose de 5 mg/kg de minoxidil. Embora ambos os fármacos causassem alterações

epididimárias, a finasterida reduziu exclusivamente o tempo de trânsito dos espermatozoides e a contagem de espermatozoides na cauda do epidídimo. Experimentos *ex vivo* com tecidos testiculares humanos mostraram que o minoxidil não alterou a diferenciação testicular fetal, mas afetou os tecidos adultos, diminuindo a expressão de marcadores de células germinativas e enzimas esteroidogênicas, prejudicando a função das células de Leydig. Esses resultados indicam que o minoxidil pode prejudicar a esteroidogênese e espermatogênese testicular, suscitando preocupações sobre sua segurança reprodutiva. O estudo conclui que tanto o minoxidil quanto a finasterida impõem riscos à saúde reprodutiva masculina. O minoxidil afeta principalmente o equilíbrio hormonal e a morfologia testicular, sendo necessária mais pesquisa e ensaios clínicos para validar esses achados e explorar estratégias para mitigar seus efeitos reprodutivos.

Palavras-chave: Toxicologia reprodutiva; Finasterida; Minoxidil; Alopecia androgenética; Disruptores hormonais; Fertilidade masculina

SUMMARY

GENERAL INTRODUCTION

Alopecia

Hair is an important element in human life, not only because of its aesthetic character or the representation of social values, but mainly because of the protection it provides to the skin (DO NASCIMENTO et al., 2020). In this context, it is possible to mention a blockade against solar radiation and the reduction of friction in certain parts of the body (ALONSO et al. 2006). According to Alonso et al. (2006), hair in general also keeps mammals dry and warm, thus providing favorable conditions for adaption to the environment.

Occasionally, some atypical conditions may occur, interrupting or preventing hair growth, such as alopecia. Conceptually, Hunt and McHale (2005) point out that alopecia is a chronic inflammatory disease that affects the hair follicles, and may not be painful, despite the skin irritation and the physical problems caused by the loss of eyelashes and eyebrows. This study also mentioned that the etiology and subsequent development of alopecia are not fully understood, but it is an autoimmune disease that arises from a combination of genetic and environmental influences.

It is important to emphasize that there are several types of alopecia, but the most common, androgenetic alopecia (AGA)—also known as baldness—is primarily caused by genetic factors and is associated with androgen levels (HUNT & MCHALE, 2005). In the body, testosterone is converted into dihydrotestosterone (DHT) by the enzyme 5α-reductase (WILSON, 1996). The interaction of DHT with androgen receptors (ARs) in hair follicles triggers progressive follicular miniaturization, characterized by a shortened anagen phase (growth), prolonged telogen phase (shedding), and a gradual reduction in hair shaft diameter and length (HOFFMAN, 2002; LOLLI et al., 2017). Genetic factors play a crucial role in determining the distribution of ARs and the activity of 5α-reductase, explaining the hereditary nature of the condition (LOLLI et al., 2017). Elevated levels of 5α-reductase have been detected in patients with AGA (HO et al., 2024). Additionally, a study shown that dermal papilla cells from balding scalp hair follicles contain significantly more ARs than those from non-balding follicles (HIBBERTS et al., 1998).

It is estimated that AGA affects at least 50% of men of reproductive age (ALMOHANNA et al., 2018; GOMOLIN et al., 2020). The condition is more prevalent

among Caucasian men, followed by Asians and African Americans, and subsequently among Native American and Inuit populations (LOLLI et al., 2017; HO et al., 2024). However, a recent study conducted in the UK found a higher prevalence of AGA among Asian men compared to white men. Nonetheless, the study has some limitations, as the UK Biobank (UKBB) groups the Asian racial category as 81.1% South Asian and 18.9% from other Asian backgrounds, making it unclear which specific Asian subgroup is driving the observed AGA prevalence (KAMAL et al., 2024).

In women, female pattern hair loss (FPHL), previously referred to as androgenic alopecia (AGA), affects approximately 3–12% of the population (STARACE et al., 2019). FPHL is a common condition with two incidence peaks: one during reproductive years and another after menopause, suggesting a strong hormonal influence on its onset (BERTOLI et al., 2020). A study conducted in a Brazilian population found that FPHL affected 32.3% of adult women, with prevalence increasing from 8% among those aged 20–29 to significantly higher rates in older age groups (MÜLLER RAMOS et al., 2023). Prevalence of female pattern hair loss (FPHL) demonstrates significant geographic variation, with studies reporting diverse rates across populations. For instance, research in the United States showed a 19% overall prevalence in Caucasian women, escalating from 3% in their 30s to 32% in their 80s, while an Australian study indicated a 32% overall prevalence, with 13% observed in women in their third decade. Conversely, a large Taiwanese study revealed a comparatively lower overall occurrence of FPHL, though Caucasian women still exhibited higher rates than their Korean and Chinese counterparts, highlighting the influence of ethnicity and regional factors on FPHL prevalence (MÜLLER RAMOS & MIOT et al., 2015). The prevalence of AGA increases with age in both males and females (GAN & SINCLAIR, 2005), which also influences treatment adherence rates, ranging from 30% to 60%.

Approved treatments

The U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Agência Nacional de Vigilância Sanitária (ANVISA) in Brazil recommends the use of topical minoxidil and oral finasteride in appropriate concentrations to treat AGA. Appropriate because, both drugs were developed to treat other conditions. Minoxidil, for example, was developed in the 1970s for the treatment of hypertension and continues to serve that purpose, although it is also known for the topical treatment of baldness (PATEL et al., 2024). The hair growth potential of minoxidil

was demonstrated by observing patients using the drug to control hypertension. Since the 1980s, a specific minoxidil solution has been developed to treat hair loss in the male population (topical minoxidil). As a result, minoxidil is now available for the treatment of hypertension and baldness in both men and women (SUCHONWANIT et al., 2019). Although there is a consensus that topical minoxidil helps with hair growth, the exact logic behind how it works is not known. It was once taught that the process was solely due to the drug's ability to cause blood vessel dilation by opening potassium channels, meaning that when the drug is applied to the scalp, there is an improvement in blood flow and nutrition to the hair follicle. However, others vasodilators have been tested and have not shown the same hair growth phenomenon as topical minoxidil (ROSSI et al., 2012).

Regarding the handling of doses for hair disorders, Suchonwanit et al. (2019) demonstrate that the FDA recommends, two daily applications of 1 mL 5% MS (minoxidil solution) and half a capful of 5% MF (minoxidil foam) for androgenetic alopecia in men. Also, according to the studies, side effects were higher with the 5% MS applications (itching, in 4%; and headache, in 3% of patients). In the application of 5% MS and half a cap of 5% MF, erythema was reported in 3.9%; scaling/dryness in 2.8%; and folliculitis in 1.1%. The same symptoms were reported in the study by Rossi et al. (2012).

In addition to topical minoxidil, oral finasteride is considered one of the main therapeutic drugs for the treatment of androgenetic alopecia in men. According to Santos et al. (2017), it is an inhibitor of the enzyme 5α-reductase II, which is responsible for transforming testosterone into dihydrotestosterone (DHT). By reducing the levels of DHT (the hormone that causes hair loss), the possibility of an effective treatment for baldness increases. Like minoxidil, finasteride was not originally designed to solve problems hair growth problems. It was intended to treat the symptoms of benign prostatic hyperplasia, but as studies progressed, it was found that finasteride reduced hair loss and increased hair density and length, compared to place with daily oral administration of 1 mg for 12 months (ARCA et al., 2004). Because finasteride affects steroidogenic pathways, it may also affect fertility, as the process of spermatogenesis is highly controlled by reproductive hormones (RUSSELL et al., 1990). According to Santos et al. (2017) and Arca et al. (2004), the main effects of continuous use of the drug occur on libido *i.e* loss of sexual appetite and may also cause erectile dysfunction. Although more in-depth studies are needed, anxiety and depression can also be considered as side effects with prolonged use.

Minoxidil oral

As mentioned in the previous section, minoxidil has been used orally for the treatment of hypertension since the 1970s. For hair loss, the drug has been prepared for topical application, but some patients do not fully adapt to this form due to possible skin irritation (ROSSI et al., 2012). Similarly, finasteride for treatment of baldness also has negative side effects. Therefore, the *off-label* use of oral minoxidil provides a viable alternative for the patients. However, it is essential to monitor side effects, particularly concerning male reproductive health. The Australian Rodney Sinclair began to develop in-depth studies and concluded that minoxidil can be administered orally (*off-label*), as long as it is used in lower doses than for the treatment of high blood pressure.

The study by Sharma et al. (2020), also found that reducing the dose of oral minoxidil was effective in treating alopecia. Panchaprateep & Lueangarun (2020) emphasize that the limitation of oral minoxidil should be respected, especially due to the side effects caused by the standard dosage for hypertension (10-40mg). In this sense, considering the basic bibliography, the possible side effects are ankle swelling due to fluid retention, headache, dizziness, hives, and, especially hair growth in other regions of the body, which causes discomfort to the patients. Although the work of Perera and Sinclair (2017) indicates low blood pressure and pulse rate among the subjects, the results of the study by Panchaprateep & Lueangarun (2020) demonstrate that there is no significant change in either area. Sharma et al. (2020) also confirms that, the side effects in these two areas are minimal when the doses are administered appropriately for each case. Reports often focus on the appearance of facial or generalized hypertrichosis. By way of illustration, the table below shows the main doses of oral minoxidil used for hair treatment and their main side effects:

Review Doses adverse events

Review	**Doses**	**Adverse events**
Almohanna et al. (2018)	0.25 mg/day – 2.5 mg/day for 3 months.	Postural hypotension and hypertrichosis.
Beach et al. (2021)	1.25 mg/day for 3 months.	Hypotensive symptoms (dizziness), palpitations, ankle edema, hair loss, hypertrichosis, urticaria and paresthesia (arms and hands).
Gomolin et al. (2020) (Average across reviewed studies)	2.5 mg/day or 5 mg/day for 6 months	Hypertrichosis, lower limb edema and hair loss.

Jimenez-Cauhe et al. (2019)	2.5 mg/day or 5 mg/day between 6 to 12 months	Pedal edema
Jha et al. (2020)	1.25 mg/day for 6 months	Peripheral edema and hypertrichosis
Panchaprateep et al. (2020)	5 mg/ day for 6 months	Hypertrichosis and pedal edema.
Pirmez and Sala-Callo (2020)	2.5 mg/day and 5 mg/day for the period minimum of 6 months	Pedal edema, hair loss and hypertrichosis.
Sinclair (2017)	0.25 mg/day between 6 and 12 months	Urticaria
Sharma et al. (2020) (Average across reviewed studies)	0.25 mg to 5 mg between one and two daily doses for at least 6 months.	Postural hypotension and hypertrichosis.
Randolph and Tosti. (2021) (Average across reviewed studies)	2.5 mg/day to 5 mg/day for at the least 6 months.	Postural hypotension and hypertrichosis.

Source: Prepared by the author.

As observed in the table above, the minimum period of administration of the doses in the study in humans is 3 months, and may exceed 12, in addition to each being between 0.25 mg and 5 mg. The dosage in certain cases was once a day and in others, twice and regarding the side effects, hypertrichosis was the most related by the patients.

Male reproductive biology

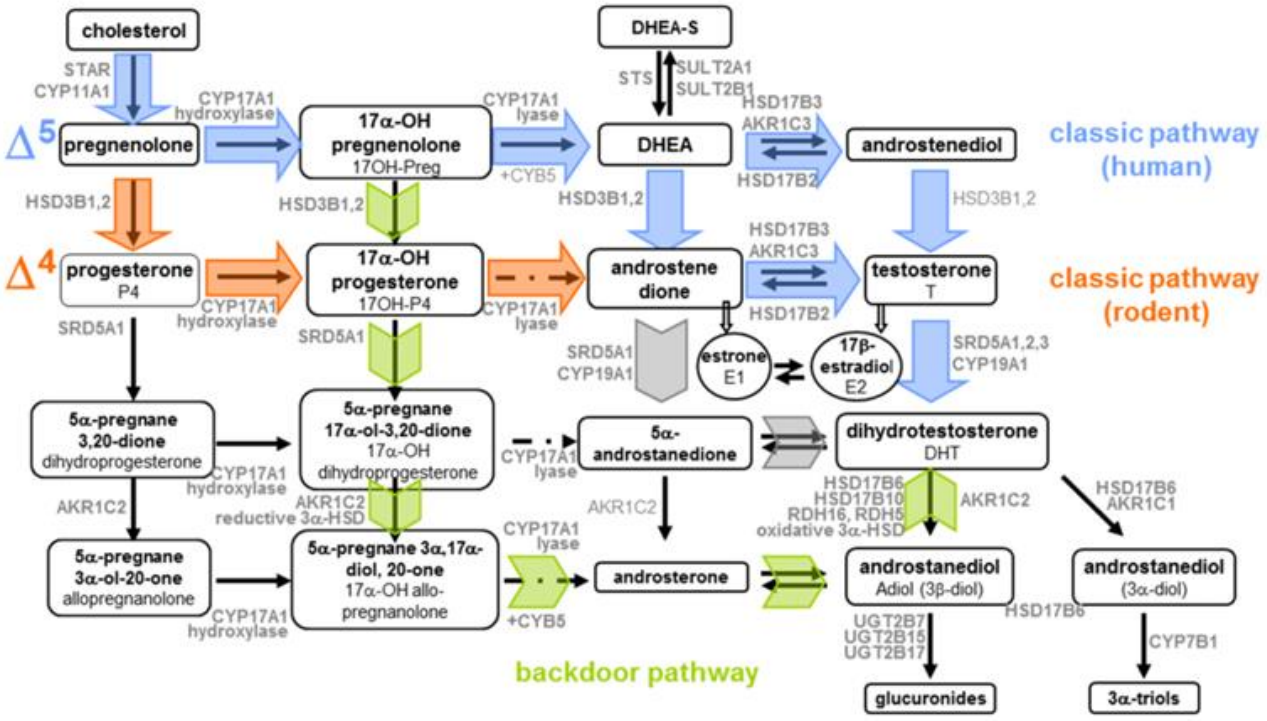
In males, the process of sexual development is complex and involves stages that start at beginning of fetal life and continue through puberty. Sexually dimorphic differentiation of the gonads occurs early in fetal life, around the sixth week of gestation in humans (MARTY et al, 2003) and between the 10th and 12th day of gestation in mouse (PETERS, 1970). The fetal testes begin to produce hormones that will influence the differentiation of other cells and masculinize tissues throughout the body so that they develop into male tissues and organs. The main male sex hormone, testosterone, which is produced by fetal testicular Leydig cells, acts through the androgen receptor (AR) in target peripheral tissues, to induce their male phenotype (MARTY et al, 2003; SVINGEN et al., 2018). In addition to testosterone, fetal Leydig cells produce a peptide hormone, insulin like-3 (INSL3) which is involved in the induction of testicular descent (PAYNE & HARDY, 2007).

During puberty, Leydig cells continue to produce testosterone, but at regulated levels controlled by the hypothalamic-pituitary axis and according to the needs of the body, such that they acquire different ultrastructural features (VILAR, 1970). Teerds & Huhtaniemi (2015), in their review article, shows that a significant difference between fetal and adult Leydig cells is that fetal Leydig cells are primarily activated by placental hCG rather than pituitary LH. This suggests that hCG is the primary hormonal stimulus

during the development of these cells. In contrast, in rodents, masculinization occurs normally during fetal life, even in the absence of pituitary LH, highlighting a key difference in hormonal regulation between species. Another difference between rodent and human Leydig cells is their response to environmental factors and drugs (HABERT et al., 2014; BEN MAAMAR et al., 2015).

Not only is testosterone production important in the testes, but also the conversion of cholesterol into other steroids such as 5α-dihydro-testosterone (DHT) and 17β-estradiol (E2). There are specific enzymes involved in this process that control each step of the conversion cascade. Teerds & Huhtaniemi (2015) clarify in their review that both fetuses and adults use the "classical" steroidogenic pathways, using either *de novo* synthesized cholesterol or lipoprotein-associated cholesterol from external sources to produce steroid hormones. Connan-Perrot et al., (2021) shows the preferred pathway with respect to species: rodents normally follow the Δ4 pathway, while humans follow the Δ5 pathway. Teerds & Huhtaniemi (2015) explain that these preferences are mainly related to two enzymes and their affinities, named CYP17A and 17β-hydroxysteroid dehydrogenase type 3 (HSD17B3). The figure by Connan-Perrot et al., (2021) (Fig. 1) shows that testosterone can be converted to 5α-DHT, which is critical for the masculinization of external male genitalia in humans and rodents. Previously, 5α-DHT was thought to be formed from testosterone through the action of 5α-reductase type 2 (SRD5A2), but since this enzyme is poorly expressed in fetal testes, the findings suggest that a significant portion of fetal 5α-DHT is derived from progesterone via an alternative "backdoor" pathway. Teerds & Huhtaniemi (2015) mentioned that the key enzymes in this pathway are the α-keto-reductases AKRC2 and AKRC4. Thus, it can be concluded that disruption of this pathway could lead to abnormal differentiation of the male genital tract, resulting in congenital malformations such as cryptorchidism, hypospadias, increased urogenital distance, and potentially infertility in adulthood (SKAKKEBAEK et al., 2001; SVINGEN et al., 2018)

Figure 1. Summary of the principal gonadal steroidogenic pathways. Steroid precursors and metabolites from the classic delta 4 (orange arrows) and delta 5 pathways (blue arrows) are shown together with the backdoor (alternative) pathway (green arrows).



Source: Connan-Perrot et al., (2021)

There are two other cell types in the developing testis that will give rise to the Sertoli cells and the germ cells. Together with the Leydig cells, they are essential for the spermatogenic process. The germ line is derived from the primary gonocyte, and during childhood a series of changes occur to form several intermediate types of spermatogonial cells. These developments are crucial for the establishment of a functional spermatogenic lineage, which ultimately enables the production of mature sperm (VILAR, 1970). Furthermore, according to Yao et al., (2015) the Sertoli cell lineage in the fetal testis begins to orchestrate the organization of testis cords, testis-specific vascular patterning, and the testis morphogenesis to compartmentalize the specific testis area for spermatogenesis and an interstitial for hormone production. In addition to these functions, fetal Sertoli cells also secrete anti-Mullerian hormone (AMH), which induces the regression of the Mullerian ducts, the precursors of the female reproductive tract (YAO et al., 2015). After birth, these cells continue to proliferate, providing physical support, junctional barriers, growth factors and hormones, and are responsible for the formation of a unique and essential environment, isolated from the immune system (GRISWOLD, 1998; OLIVEIRA & ALVES, 2015).

In addition to the testes, another organ plays an important role in adult fertility: the epididymis. It is an organ adjacent to the testis that is generally divided into four major

anatomical regions: initial segment, head (caput), body (corpus), and tail (cauda), as first described by Benoit (1926). It presents a single duct organ, highly convoluted and lined by a pseudostratified epithelium (De GRAVA KEMPINAS & KLINEFELTER, 2014) that connects the efferent ducts to the vas deferens (ROBAIRE, 2006). Six different cell types are found in the epithelium lining the epididymis: ciliated, basal, apical, halo, clear and main cells (CLELAND, 1957), which have different functions in the different segments of the organ. For example, in the initial part, the main cells are responsible for the reabsorption of water, ions and small organic molecules. In the head, however, they are involved in the secretion of proteins, and in the body, in the secretion of lipids in order to contribute to the modification of the sperm plasma membrane (De GRAVA KEMPINAS & KLINEFELTER, 2014). In the tail epithelium, there is a greater number of clear cells as they phagocytose cytoplasmic droplets and other luminal debris. In addition, there is evidence that these cells become abnormally large and full of lysosomes following conditions that disrupt the normal function of the testis and epididymis (ROBAIRE & HINTON, 2002). All these epithelial changes contribute to the maturation of the spermatozoa during their passage through the epididymis (ROBAIRE & HINTON, 2002), *i.e.* the spermatozoa leave the testis as immobile cells incapable of fertilizing the oocytes and, thanks to the epididymis, become mature cells capable of swimming and recognizing and fertilizing the oocytes (ROBAIRE, 2006). In addition to sperm maturation, another function of the epididymis is to store the spermatozoa until ejaculation and to protect them from the immunogenic reaction by a blood-epididymal barrier (De GRAVA KEMPINAS & KLINEFELTER, 2014).

Minoxidil's effects on the male reproductive system

Looking at the scientific research on the effects of minoxidil on male reproductive biology, there is no consensus on whether or not this drug acts via androgenic pathways. In this sense, the study by Sato et al. (1999), which considered the effect of minoxidil on testosterone metabolism by cultured dermal papilla cells from bald or non-bald scalp and dermal fibroblasts, showed that the drug has no antiandrogenic effect locally or systemically. Although the same study observed that minoxidil may alter enzymes that influence testosterone availability, such as 17bHSD (converts androstenedione to testosterone) and 5a-reductase (converts testosterone to dihydrotestosterone). Minoxidil increased 17b-hydroxysteroid dehydrogenase activity by almost 40% in dermal papilla cells from balding scalp, while the effect was less pronounced in dermal papilla cells from

non-balding scalp and in dermal fibroblasts. 5a-reductase activity was also slightly increased by minoxidil in dermal papilla cells from balding scalp but not in the other two groups (SATO et al., 1999).

Nuck et al. (1987) concluded that there is no antiandrogenic component to the mechanism of action of minoxidil in the hamster flank organ (the object of the study), and therefore, there is probably no antiandrogenic role in humans either (NUCK et al., 1987). However, Pekmezci and Türkglu (2017), who studied the *in vitro* expression levels of 5α-reductase type 2 (5α-R2) in a human keratinocyte cell line treated with minoxidil, demonstrated a significant downregulation of this gene expression. Other studies demonstrate the effects of minoxidil on the reproductive system in murine models. Mingsan et al. (2020) administered the drug at a dose of 20 mg once daily, for 21 days, and found an increase in the hormone estradiol and a decrease in testosterone in the serum of mice. Ozturk et al. (2020) administered minoxidil at a dose of 0.3 mg/kg intravenously for 30 seconds and demonstrated adverse effects on the testes of Wistar rats, such as morphological changes, including an increase in non-cohesive germ cells and compacted seminiferous tubules. An increase in stress markers such as MDA and myeloperoxidase (MPO) and in the total oxidative state of the tissue was also confirmed. Piner et al. (2002) found a decrease in serum testosterone in Charles River Wistar rats after oral administration of 15 mg/kg for 4 hours in the peripheral and spermatic veins of the testes. No study to date has elucidated the effect of minoxidil on the epididymis *in vivo* or on cells derived from the organ *in vitro.*

Finally, the FDA Adverse Event Reporting System (FAERS), a digital database run by the U.S. government that compiles reports of adverse events related to FDA-approved drugs, was analyzed by Wu et al. in 2016 and showed that minoxidil was associated with reproductive toxicity in both women and men. In any case, there are few studies evaluating testicular morphology after exposure to minoxidil, and thus more incentives are needed in this area to guide and ensure individualized recommendations for use.

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CHAPTER 1

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Comparative effects of finasteride and minoxidil on the male reproductive organs: A systematic review of *in vitro and in vivo* evidence

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Abstract

Finasteride and minoxidil are medicaments commonly prescribed for treating benign prostatic hyperplasia (BPA), hypertension, and/or androgenetic alopecia (AGA). The mechanism of action of finasteride is based on the interference in androgenic pathways, which may lead to fertility-related disorders in men. Minoxidil, however, can act in multiple ways, and there is no consensus that its use can adversely affect male fertility. Since finasteride and minoxidil could be risk factors for male fertility, we aimed to compare their impact on the two reproductive organs testis and epididymis of adult murine models, besides testis/epididymis-related cells, and describe the mechanism of action involved. For such, we used the PRISMA guideline. We included 31 original studies from a structured search on PubMed/MEDLINE, Scopus, and Web of Science databases. For *in vivo* studies, the bias analysis and the quality of the studies were assessed as described by SYRCLE (Systematic Review Centre for Laboratory Animal Experimentation). We concluded that finasteride and minoxidil act as hormone disruptors, causing oxidative stress and morphological changes mainly in the testis. Our results also revealed that finasteride treatment could be more harmful to male reproductive health because it was more associated with reproductive injuries, including damage to the epididymis, erectile dysfunction, decreased libido, and reduced semen

volume. Thus, this study contributes to the global understanding of the mechanisms by which medicaments used for alopecia might lead to male reproductive disorders. We hope that our critical analysis expedites clinical research and reduces methodological bias. The registration number on the Prospero platform is CRD42022313347.

Keywords: Male reproductive health; androgenetic alopecia; endocrine disruptors; oxidative stress, morphological alterations

1 INTRODUCTION

Finasteride and minoxidil are currently approved by the US Food Drug Administration (FDA) for treating benign prostatic hyperplasia (BPH) and hypertension, respectively. Furthermore, the FDA also recommends the combination of oral finasteride and topic minoxidil for treating male androgenetic alopecia (AGA) [1,2]. Nonetheless, growing evidence supports oral minoxidil use in low doses, which is more efficient [3,4]. In men, AGA reaches about 50% of them being the most common type of alopecia caused by genetic factors associated with an increased rate of conversion of testosterone to dihydrotestosterone (DHT), the most potent natural androgen, a process involving type II 5α-reductase [5,6]. DHT has been found to be the primary androgen involved in the pathogenesis of AGA by promoting follicular miniaturization [7], as well as, in the pathogenesis of BPH, by preventing the growth of epithelial cells in the prostate [8].

Finasteride is a synthetic anti-androgen that works by competitively inhibiting 5α-reductase, resulting in the inhibition of the conversion of testosterone to DHT, markedly suppressing serum DHT levels. Testosterone is the predominant androgen in the peripheral circulation, however, conversion to DHT is necessary for biological activity in some organs, such as liver, skin, seminal vesicles, and prostate [9]. Although this drug has been proven effective at reducing male pattern hair loss though the reduction of DHT levels, there has been debate over the adverse sexual side effects it could produce. Since testosterone production is controlled by a negative feedback loop [10], this medication could affect steroidogenic pathways in Leydig cells (LCs) or within the hypothalamic-pituitary-gonadal (HPG) axis by increasing the serum testosterone levels. Furthermore, histomorphological and functional damage in the dependent tissues of a normal rate of testosterone could lead to some adverse effects during the treatment. Studies have proved that finasteride impairs male fertility [11,12,13]. The adverse effects on male fertility

commonly related to this drug include decreased libido, erectile dysfunction, reduced ejaculatory volume, and temporary reduction in sperm count, which is caused by the imbalance of testosterone conversion [14,15,16].

Although minoxidil is also used to treat AGA, its mechanism of action is not well known. The process should occur through multiple pathways (vasodilatation, inflammatory action, and induction of the Wnt/b-catenin signaling pathway), including antiandrogen pathways [17]. In this partially unknown scenario, some authors point out that the hair-stimulating effect of minoxidil is not mediated by an antiandrogenic mechanism, such as finasteride since significant changes were not found in the testosterone levels in their experiments [18,19]. However, *in vitro* findings suggest that minoxidil increases the activity of enzymes from androgenic pathways, such as 17β-hydroxysteroid dehydrogenase (17β-HSD), involved in the last step of the testosterone synthesis, [19] and 5α-reductase enzyme [20], which could impair male fertility. Nonetheless, the literature regarding the minoxidil effects on male reproductive health is scarce despite the contemporary significance of this subject.

Wu et al. (2016) [21], who focused on understanding alopecia considering the side effects of minoxidil and finasteride for both women and men, assessed the FDA Adverse Event Reporting System (FAERS), a computerized information database established by the U.S. Government, which restores reports of adverse events for drug products approved by FDA. The authors retrieved events related to finasteride or minoxidil, from January 2004 to June 2014 and confirmed that finasteride was more associated with reproductive injuries by patients (sexual disorder, erectile dysfunction, decreased libido, and reduced semen volume) compared to minoxidil [21]. However, human studies that carried out an analysis of morphological, molecular, biochemical, or hormonal comparison of the effects of these two approved drugs on male reproductive organs have not yet been developed.

In this context, a systematic review of preclinical studies, *in vitro* and *in vivo*, is essential to understanding the main cellular pathways activated after finasteride and minoxidil exposure mainly in the testis and epididymis, since no study compared these two drug effects in these organs. Therefore, our results may provide new insights into male repro-toxicology, male reproductive health, and therapeutic alternatives for the treatment of alopecia. Thus, this study contributes to the global understanding of the mechanisms induced by medicaments used for alopecia, which might lead to male reproductive disorders.

2 METHODS

2.1 Focus question

The main question to be answered in this systematic review was: What is the impact of finasteride and minoxidil on the testis and epididymis based on preclinical model (*in vivo* and *in vitro*) evidence?

2.2 Search strategy

This systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines [22]. Further information about the protocol for this systematic review was registered on PROSPERO - International Prospective Register of Systematic Reviews (CRD42022313347). Details about the Population, Interventions, Comparators, Outcome, and Study design (PICOS) are given in Table S1. A bibliographic search was performed using a two-step search: (1) direct search in electronic databases PubMed/MEDLINE (https://www.ncbi.nlm.nih.gov/pubmed), Scopus (https://www.scopus.com/home.uri), and Web of Science (https://www-periodicos-capes-gov-br.ez35.periodicos.capes.gov.br) completed on August 26th, 2021, at 08:43 am, and (2) indirect screening of reference lists from all the included studies identified in the direct search [23]. The keywords used as search filters were organized into four groups: animal models, organ (testis and epididymis), finasteride, and minoxidil, which were combined by Boolean connectors [AND] among them or [OR] between the two drugs. Search filters were initially created for the PubMed/MEDLINE database, using algorithms [MeSH Terms], which means indexed records, and [TIAB], which refers to papers recently published in an indexing process [24]. The same research descriptors were structured according to specific search algorithms required in Scopus and Web of Science databases. The search strategy is detailed in the supplementary materials (Table S2).

2.3 Eligibility Criteria

After the record identification through the databases, removal of duplicates, and initial screening, the full text of potentially relevant studies was retrieved and assessed by two independent researchers for eligibility for inclusion in the review.

Only studies that met the following eligibility criteria were selected: (i) *in vivo* studies that analyzed the finasteride or the minoxidil exposure effect on the testis and

epididymis of adult murine models; (ii) *in vitro* studies that analyzed the finasteride or the minoxidil exposure effect on the testis/epididymis-related cells.

Study exclusion was based on well-defined criteria, as follows: (i) Studies that used other organs/cell lines or evaluated female animals or other animal models, (ii) Studies containing exclusively non-relevant aspects for our systematic review, such as the exploration of the chemical structure of the enzyme 5α-reductase or the pharmacological profile of drugs, (iii) Studies that administrated only finasteride or minoxidil as a positive control, (iv) Studies lacking drug exposure, (v) Studies of behavior, (vi) Studies with treatment association, (vii) Secondary studies as reviews and (viii) Studies in a language other than English (ix) Studies *ex vivo*. The reference list of each included study was manually checked to identify additional studies. If a record was identified in this step, its reference list was also revised.

The kappa test was used for selection and data extraction (kappa= 0.870). Two reviewers (FFVS and AAL) independently conducted the literature search, removed duplicate articles, and examined titles and abstracts according to eligibility criteria. In case of discrepancies, a third reviewer (JS) decided whether the study met the inclusion criteria.

2.4 Data extraction and synthesis from in vivo and in vitro studies

Considering all included studies, data extraction according to our objectives was based on descriptive levels, as follows: (i) characteristics of publication: authors, publication year, and country; (ii) characteristics of the experimental animals or testis/epididymis-related cells: animal model, age, weight, number of animals, number of animals per group and number of groups; or the cellular type, cell lineage source, and culture medium; (iii) Intervention: drug, drug vehicle, drug dose, timing of exposure, route, and control compounds, (iv) comparison of primary outcomes and molecular pathways activated after finasteride and minoxidil exposure in preclinical models

2.5 Risk of bias assessment from in vivo studies

The quality of the included animal intervention studies was assessed using the criteria described in the risk of bias (RoB) tool of SYRCLE (Systematic Review Centre for Laboratory Animal Experimentation) [25]. The following types of bias described by the RoB tool were evaluated: selection bias (sequence generation, baseline characteristics, allocation concealment); performance bias (random housing and blinding of caregivers or investigators); detection bias (outcome assessment, blinding of outcome assessment);

attrition bias (incomplete outcome data); reporting bias (selective outcome reporting); and other sources of bias (the presence of ethics committee, which treatment the control group received and the omission of the route of administration). The items in the RoB tool were scored with "yes" (low risk of bias), "no" (high risk of bias), or "unclear" (indicating that the item was not adequately reported and, therefore, the risk of bias was unknown) [24]. The results of the risk of bias across the included studies were graphically expressed using the Review Manager 5.3 program from Cochrane Collaboration (RoB 2.0).

3 RESULTS

3.1 Included Studies and Characteristics of Publication

Our search strategy allowed for recovering 1367 records (PubMed/MEDLINE: 190, Scopus: 577, and Web of Science: 600). After removing 524 duplicates, 843 papers were screened by reading the title and abstract. Thus, 763 studies were excluded due to the absence of the established criteria (eligibility criteria). Eighty studies were assessed through the eligibility criteria, then 49 articles were excluded. Therefore, 31 records [26-56] were included in this systematic review. Out of these, 27 studies (24 *in vivo* and 3 *in vitro*) analyzed the finasteride effects and four studies (3 *in vivo* and 1 *in vitro*) examined the minoxidil effects on male reproductive organs (Fig. 1). No recovered study compared the two drugs.

The supplementary material contains the main characteristics of publication and experimental animal models or cell culture. The studies that administrated finasteride *in vivo* were published from 1991 to 2020 and were conducted in several countries, mainly the United States of America and Poland (25% each), followed by Brazil (12.5%), China, and Iraq (8.33% each). The three *in vitro* studies that investigated the effect of finasteride were published from 1993 to 1998 and were mainly conducted in the United States of America (66.66%). The three studies that administrated minoxidil *in vivo* were published from 2002 to 2020 and were from China, United Kingdom, and Turkey. The *in vitro* study that investigated the effect of minoxidil was published in 1982, and the country where the assay was performed was not informed.

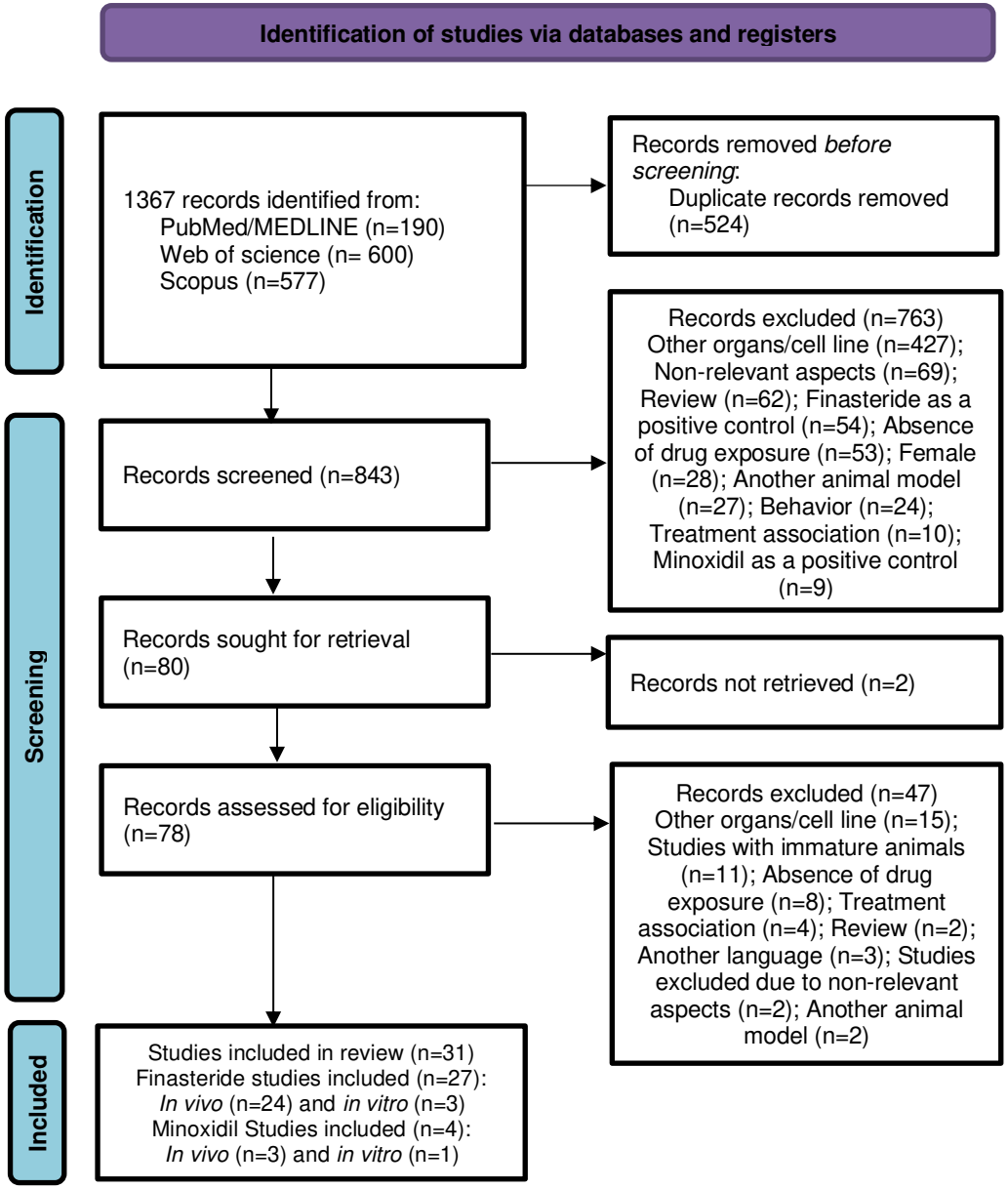

Fig 1. Flow diagram of the systematic review literature search results based on PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) (http://www.prisma-statement.org).

3.2 Preclinical studies: Characteristics of Experimental Design

Considering the animal models, the 24 studies that administrated finasteride *in vivo* mainly used rats (83.33%) [26,28-35,37-39,41-46,48,49], mostly Sprague Dawley and Wistar rats. The other finasteride studies used mice (12.5%) [27,36,40], such as Balb/c strains, except one study that used a wild rodent, the *Mesocricetus auratus* [47], as the he animal model. Regarding the three *in vivo* minoxidil studies, Wistar rats were the most used (66.66%) [51,52]. One study used KM mice [50] (Table S3).

The main experimental characteristics of the studies are described in Table S4. Concerning the finasteride exposure, almost 37.5% of the records did not inform how the drug was solubilized [31-34,36,44,46,47,49], while 20.83% of the studies dissolved it in methylcellulose [28,29,38,40,48], followed by 16.7% in water [27,30,37,39]. The lowest finasteride dose used was 0.01 mg/kg [30], and the highest dose was 250 mg/kg [40], both daily, by oral route. However, the vast finasteride doses used were 5 mg/kg (37.5% of the studies) [29,31-34,37,42,44,46], 20 mg/kg (12.5%)[35,46,48], and 1.5 [36,38], 10 [37,38], 25 [27,38], or 80 mg/kg [28,48] (8.33% each). The experiments that administrated finasteride lasted from 5 days to 10 months. Most studies assessed the effect of the drug administered daily (91.66%) [26-40,41-46,48,49], but two others offered only 3 [47] or 5 times/week [41] (4.16% each). The treatment was mainly supplied by oral route (75%) [27-34,37,39-44,46,48,49] , followed by subcutaneous [35,47] (12.5%) and intraperitoneal routes [38](4.16%). Regarding the control group, half of the studies with finasteride administered the vehicle for the control animals. However, some studies (12.5%) determined not to refrain from administering anything [42,43,49]. Around 33% of the studies did not specify if the animals received something as a placebo [31-34,36,44,46,47].

Concerning minoxidil, it was administered in a pure form [50, 51] or dissolved in sterile water [52]. The minoxidil doses varied from 0.3 mg/kg [51], 15 mg/kg [52], and 20 mg [50]. The experiments lasted from 30 seconds to 21 days. Two studies assessed the drug's effect as a single dose [51, 52], and one study administered the drug once a day for 21 days [50]. The minoxidil exposure was carried out by oral route (n=1) [52] or intravenously (n=1) [51]. One study did not mention the route used [50]. About the control group, most studies (n=2) administered the vehicle for the control animals [50, 52], and only one study did not administer anything [51].

Regarding the preclinical studies in *in vitro* models, as reported in Table S5, Leydig tumor cells (n=1) [53], the Sertoli cells[54], and cells of testicular parenchyma (n=1)[55] extracted from Crl:CD BR rats, were selected by the included studies that assessed the finasteride impact. No study used epididymis-derived cells. Regarding the culture medium, one study used Waymouth's MB 752/1[53], and one used the RPMI-1640 medium [55]. Only one study did not inform the culture medium used [54]. The only recovered *in vitro* study that investigated the effect of

minoxidil used rat testis cells from Sprague-Dawley rats [56]. The culture medium was not described.

As reported in Table S6, among the three *in vitro* studies that assessed the finasteride effect, one reported to have administered finasteride + 1 nM Dibutyryl-cAMP dissolved in ethanol 200-fold concentrated stock or, in the second assay, finasteride + 10 µg/ml 22OH Cholesterol [53]. The second study combined finasteride with conjugate T (1 mg/l) or T: BSA (10 mg/l), and, in the second assay, finasteride with DHT (1 mg/l) or saline solution [54]. The other study added human chorionic gonadotropin (hCG) (500 IU/ml stock solution) or 10 ml of distilled water in the finasteride solution [55]. The finasteride dose used by these *in vitro* studies ranged from 0.0001 to 1 mg/l, and the incubation period ranged from 20 min to 3 h at a temperature from 22 ºC to 37 ºC. The study that assessed the minoxidil effect administered this drug alone or in the minoxidil + hCG (0.1 or 10 mU) formulation, both in a medication concentration of 0.33 mg/l, 3.33 mg/l, and 33.3 mg/l. The incubation period was 90 min at 37 ºC [56].

3.3 Main outcomes: Comparison of the molecular pathways activated after finasteride and minoxidil exposure in preclinical models.

Through our search strategy, which had the same sensitivity for recovering studies that analyzed the effects of finasteride and minoxidil on male reproduction, we retrieved six times more records *in vivo* that examined finasteride than minoxidil. Despite the limited number of studies evaluating the minoxidil exposure effects, it was possible to compare the histomorphological, molecular, and hormonal effects of both drugs especially in testicular tissue. No recovered study with minoxidil considered epididymis in their evaluation, hence there is no possibility of comparison. *In vitro*, the possibility of comparison is even more limited than *in vivo* due to the reduced number of studies for both drugs. Firstly, only some studies in this field used testis-related cells, and no recovered study used epididymis-related cells. Furthermore, the finasteride and minoxidil included studies *in vitro* have only in common their hormonal level investigation. Therefore, the comparison between the two drugs was limited to hormonal aspects.

Main hormonal outcomes

Thirteen studies that analyzed finasteride action included in their investigation serum hormonal analyses (Fig. 2- left yellow box), 85% of which observed some hormonal alteration, mainly decreased serum DHT levels (7/8 studies) [29,32,33,38,42,43,45]. However, no study evaluates the DHT levels after minoxidil treatment. Decreased DHT levels after finasteride treatment are expected since finasteride is a 5α-reductase enzyme inhibitor. So far, it has been reported that the lack of this androgen could impact the adult testis, mainly the typical composition of the epididymal fluid crucial for sperm functionality. Three studies [28,39,48] extended their investigations to the effects of finasteride on sperm quality and fecundity (Fig. 2- green boxes). In

this approach, some studies revealed that, after finasteride exposure, the animals presented poor sperm quality, including decreased number, morphology, motility, and live spermatozoa associated with low DHT levels.

Finasteride records found increased serum testosterone levels (3/10 studies) [27,29,43]. However, 4/10 studies assessing the finasteride effect *in vivo* also identified a decrease of testosterone [30,32,33,42]. Also, some studies found a decrease in the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) [27,30] levels (Fig. 2 left yellow box). Regarding minoxidil, two studies *in vivo* [50, 52] reported that the treatment caused hormonal alterations, including decreased serum testosterone levels or decreased testosterone production in testis (Fig. 3, left yellow box). This result may be included in the mechanism of action of both drugs or even indicate lesions in LCs or interference with the HPG axis.

In the testis-related cells, one study *in vitro* [55] assessing finasteride's effect found a decrease in testosterone and estradiol (E2) levels (Fig. 2, right yellow box). The cells were treated with a very low finasteride dose (0.001, 0.01, and 0.1 mg/l). In the same way, there was a decrease in testosterone production *in vitro* when minoxidil was administered at doses of 0.33, 3.33 and 33.3 mg/l [56] (Fig. 3, right yellow box). An *in vitro* study with finasteride [53] strongly suggests that finasteride can decrease testosterone production by directly affecting LCs. The study demonstrated decreased activity of the side-chain cleavage of cholesterol enzyme involved in transforming pregnenolone into steroid hormones, such as testosterone or estrogen. However, although minoxidil treatment also led to decreased testicular and serum testosterone levels, it is unknown whether this drug affects LCs since no minoxidil study evaluated exclusively this cellular type.

Main histomorphological outcomes in the testis

The purple boxes in Figures 2 and 3 show the histomorphological alterations caused by the finasteride and minoxidil treatments, respectively. The histomorphological results revealed that both drug treatments cause detached germ cells, vacuolization, and degeneration of the seminiferous tubules, which are testis cell death signs (finasteride [27,31,33,34,37,40,41,42,43,47], minoxidil [51]). Most of finasteride studies indicated that finasteride might act as an endocrine disruptor and affect the synthesis and/or distribution of hormones and hormone deficiency leads to changes in morphology by programmed cell death exemplified by confirmed apoptosis and necrosis [27,37,43]. Although the exact mechanism cannot be elucidated for minoxidil, hormonal findings and also the histomorphological changes included increased noncohesive germ cells and packed seminiferous tubules give the first insights that after the administration of the drug, the lack of hormones lead to damage in the tissue morphology or cell death.

Our results show that finasteride treatment also affects the blood-testis barrier (BTB) proteins characterized by weakly immunostaining of N-cadherin and βcatenin, and occludin [34] (Fig. 2, right blue box). Furthermore, our results showed increased vinculin tight junction protein immunostaining in germs cells in different stages of the seminiferous epithelium cycle [34], probably altering the BTB. The BTB function is stimulated by testosterone, thus decreased testosterone levels after both drug exposure events visualized in this review may adversely affect the integrity of BTB.

Reactive and nitrosative oxygen species in testis

Comparing the biochemical results (left pink boxes Fig. 2 and 3), two studies [39,42] that exposed animals to finasteride and one [51] that investigated minoxidil visualized the occurrence of oxidative stress in the tissue, especially by the generation of the reactive oxygen species (ROS) and/or nitrosative oxygen species (RNS) and the increase of oxidative stress markers expression like malondialdehyde (MDA).

The decrease of antioxidant enzyme activity (such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx)) leads to an increase in the free radicals, especially peroxynitrite (ONOO*-) and hydroxyl radical (OH*), which lead to lipid peroxidation and reflected in the high levels of MDA characterizing the cell degeneration and can progress to cell death. Morphological testis cell death signs were visualized after both drugs administration. In addition, apoptosis was confirmed by one study using finasteride [27] that identified changes in genes and protein expression that led to apoptosis (Fig. 2, left bottom pink box). It was observed an increase in *p53, Casp3,* and *Bax* (pro-apoptotic marker) expressions and a decrease in *Bcl2* (anti-apoptotic marker) expression in spermatogenic cells. These alterations may be related to increased MDA levels. Indeed, one study that accessed the effect of finasteride on the testis [39] visualized an increase in MDA levels associated with reduction in the number, morphology, and motility of the spermatozoa. Furthermore, one Minoxidil study [51] also confirmed the association of high MDA levels and histomorphological alteration. However, although individual studies show that minoxidil hurts cells, in our review was not possible to conclude whether minoxidil could lead to apoptosis or decreased sperm quality, as observed after finasteride use, especially because of the high heterogeneity among the individual studies.

In addition, nitrosative stress could be another cause of BTB interruption. A study with finasteride [33] observed in the cytoplasm of germ cells and Sertoli cells (SC) (both components of the BTB) in different stages of the seminiferous epithelium cycle the immunoreaction for iNOS isoform (Fig. 2, top right blue boxes) that participates in the formation of RNS, which in testis is possibly associated with cell death or interferes with the morphology of the tight junction between

SCs. Once the SC is damaged, many histomorphological changes can be visualized because of its intrinsic relationship with germ cells. Nevertheless, a possible rupture in BTB after finasteride exposure could be caused by SC damage mediated by testosterone or nitrosative stress. We cannot conclude that minoxidil has the same mechanism as finasteride since there is not analyzes that identify this marker.

Imbalance redox in mitochondria and endoplasmic reticulum (ER)

Another interesting mechanism described by the individual studies included in this review was the cross-talk between mitochondrial oxidative stress and endoplasmatic reticulum stress (ERS) after the exposure. In our review, ERS and apoptosis markers, such as GRP-78, p-IRE1, and p-JNK54, were found in the testis when finasteride was administrated [43] (left bottom pink box Fig.2), showing the direct relationship between the activation of these organelles during finasteride exposure, especially by stimulating the release of calcium out of the endoplasmatic reticulum. This mechanism could cause cellular damage after drug exposure, and it could be relevant for further studies.

Main effects of finasteride in the epididymis

Although 13 studies examined finasteride effects on the epididymis, no recovered study with minoxidil considered this organ in their evaluations. Nine of these 13 studies found some epididymal alteration caused by the drug administration as biometric changes (Fig. 2 epididymis brown box). Through immunohistochemical analysis in the epididymis, some studies [32, 33] recognized differences in protein expressions after finasteride treatment as SOD and iNOS, the proteins described early related to ROS formation (Fig. 2 epididymis blue box). The finasteride molecular impact on epididymis was verified for only one included study, which found decreased glutathione peroxidase 5 (GPX5) transcript and increased extracellular superoxide dismutase (E-SOD) transcript in the caput epididymis region, both transcripts of antioxidant proteins involved in tissue defense against free radicals [32] (Fig. 2 epididymis pink box).

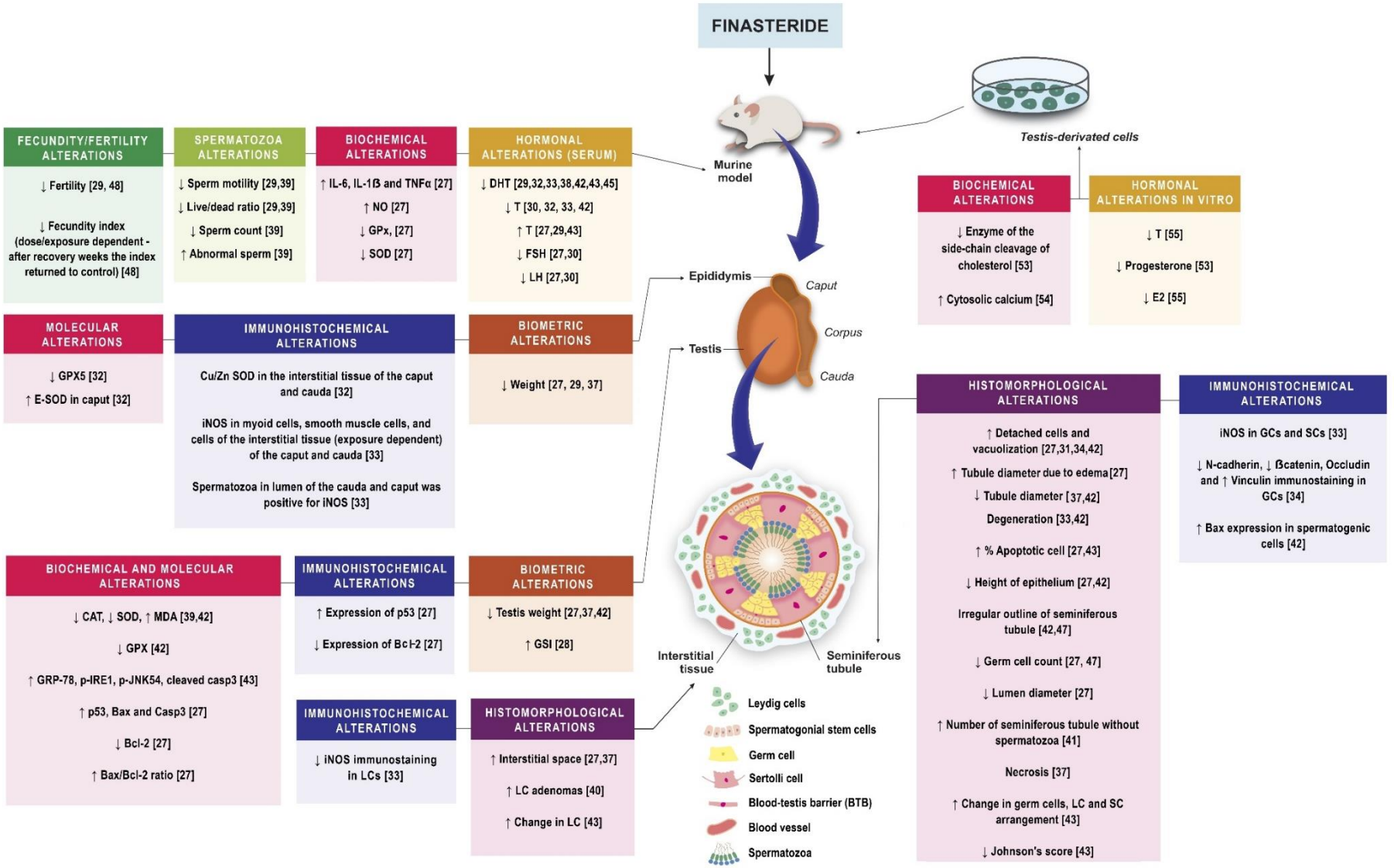

Fig. 2. Main effects of *in vivo* and *in vitro* finasteride treatment on the testis and epididymis of murine models. ↑ = upregulation/increase; ↓ = downregulation/decrease; Bax = Bcl2-associated X protein; Bcl2 = B cell leukemia/lymphoma 2 ; CAT = catalase; Casp3 = Caspase-3; DHT = dihydrotestosterone; E2 = estradiol; E-SOD = extracellular superoxide dismutase; FSH = follicle stimulating hormone; GSI = gonadossomatic index; GPx = glutathione peroxidase ; GPX5 = glutathione peroxidase 5; IL-6= Interleukin 6; IL-1β= Interleukin 1 beta; iNOS = inducible nitric oxide; LC = Leydig cells; LH = Luteinizing hormone; MDA = malondialdehyde; NO=Nitric oxide; p53= Tumor protein P53; SC = Sertoli cells; SOD = superoxide dismutase; T = testosterone; TNFα= Tumour Necrosis Factor alpha.

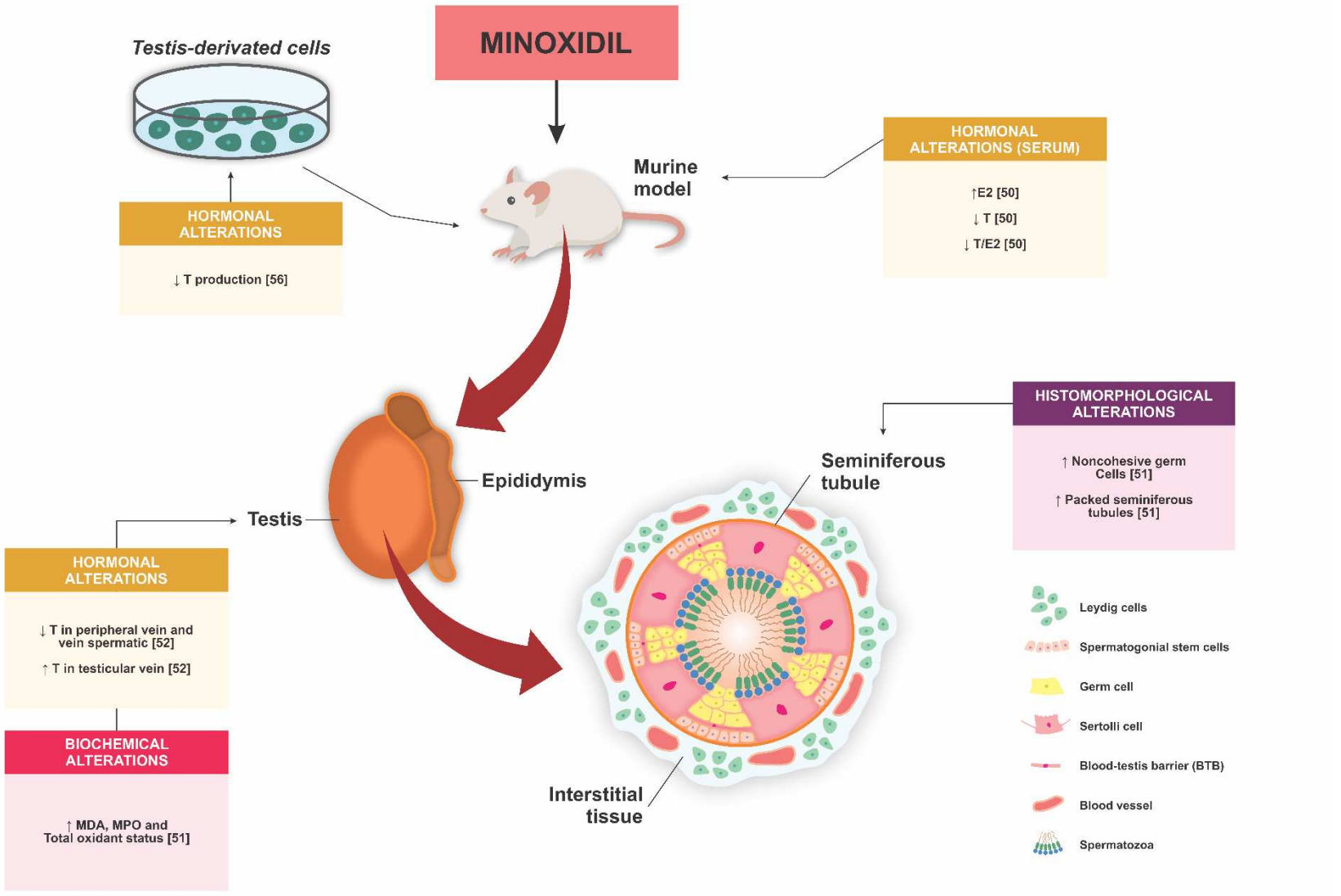

Fig. 3. Main effects of *in vivo* and *in vitro* minoxidil treatment in the testis of murine models. ↑ = increase; ↓ = decrease; E2= estradiol; T = testosterone; MDA = malondialdehyde; MPO = Myeloperoxidase.

3.4 Bias report from in vivo studies

The bias analysis based on the SYRCLE's tool is presented in Fig. 4a and 4b, which show the percentage of each risk of bias item across the included studies that assessed finasteride or minoxidil. The risk of bias analysis of individual studies is available in Supplemental files – S7. None of the studies fully met all the established criteria for any drugs. For those studies that administrated finasteride (n=24), none of the studies reported random sequence generation or random outcome assessment, which sets a high risk of bias. Regarding the blinding of participants and personnel (caregivers and investigators) and blinding of outcome assessment, just one study suggests that there was blinding (unclear risk of bias), while the others did not give this information and set a high risk of bias. Around 95.83% of the studies followed the methodology and presented the proposed results (low risk of bias), while 4.16% did not describe any methodology or have just informed that it was performed, which sets an unclear risk of bias.

Among the three studies that administrated minoxidil, allocation concealment, random sequence generation, blinding of participants and personnel (caregivers and investigators), blinding of outcome assessment, and random outcome assessment reveal a high risk of bias. All studies followed the methodology and presented the proposed results, thus presenting a low risk for reporting bias.

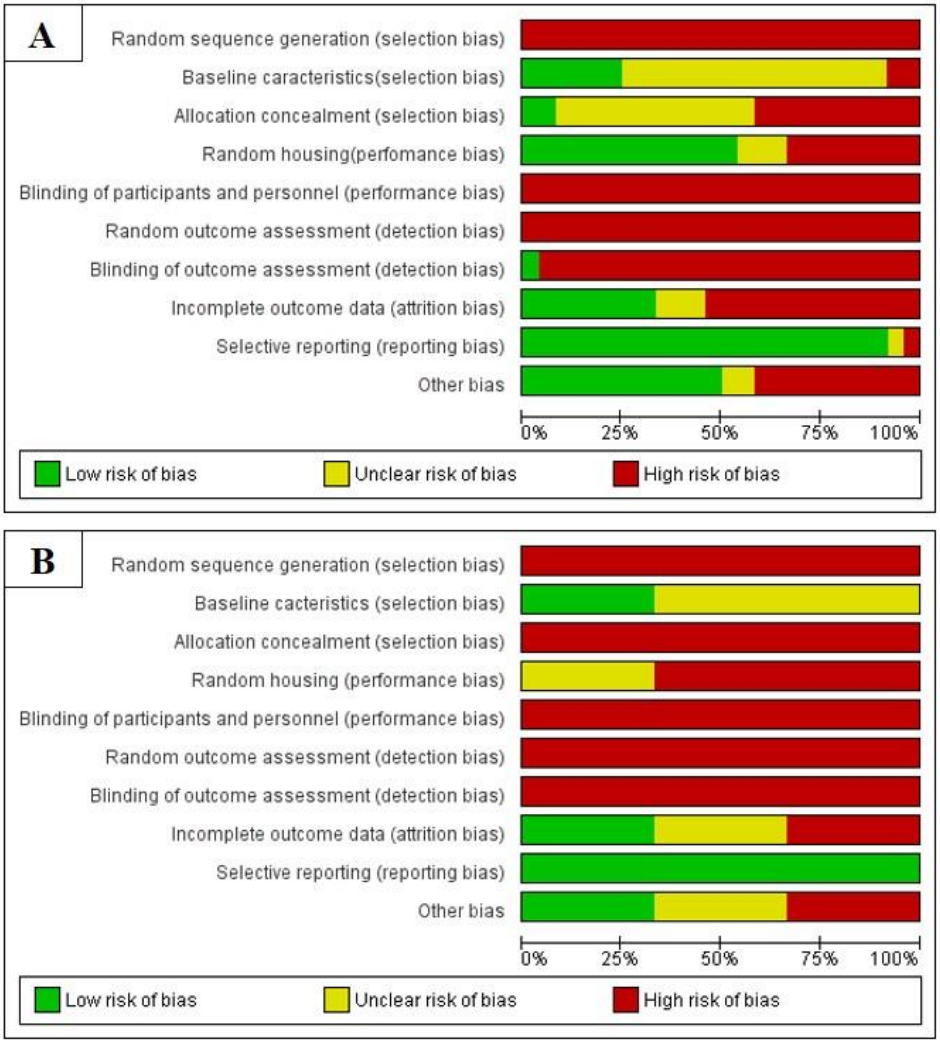

Fig. 4. Results of the risk of bias and methodological quality indicators for all included studies that evaluated the A- Finasteride and B- Minoxidil impact on testis and epididymis of murine models. The items of SYRCLE's RoB tool were scored with "yes" (low risk of bias), "no" (high risk of bias); or "unclear" (indicating that the item was not adequately reported and, therefore, the risk of bias was unknown.

4 DISCUSSION

In our systematic review, we have assessed and compared for the first time the impact of two important medicaments, finasteride and minoxidil, both used to treat alopecia, on male reproduction. Two reproductive organs of adult murine models, testis and epididymis, in addition to testis/epididymis-related cells, were focused on this investigation. Our results demonstrate that both drugs can harm murine models' testis and testis-derived cell cultures. Furthermore, our outcomes demonstrated that finasteride

could damage the epididymis and decrease sperm quality. However, due to the lack of studies on minoxidil's effects on epididymis, it is impossible to determine if this drug affects this organ. Our results also reveal that both drugs could act through mechanisms involving hormonal disruptions and oxidative stress, which will lead to morphological changes that prevent the testis from functioning normally and result in poor sperm quality and decreased fertility.

In this review, the studies that access the effect of the drugs *in vivo* were performed mainly in the United States of America (1991-1998) and Poland (2004-2011) for finasteride; and China (2020), the United Kingdom (2002), and Turkey (2020) for minoxidil. Two out of three *in vitro* studies that investigated the effect of finasteride were conducted in the United States of America (1993 and 1998), and regarding the only study that evaluated minoxidil, the country where the assay was performed was not informed. These two medications were tested and approved by the FDA for the treatment of AGA in 1997 [1,2] and more recently (2016) by the European Medicines Agency (EMA) [57, 58]. In this period, although both drugs were already available for treating other conditions, they became widely used. Thus, this explains the number of preclinical studies performed in the USA and Poland before or at this period. Since the population has been widely exposed to the medication over the past years, reports to health agencies about the possible adverse effects of use are more consistent, and preclinical and clinical research must be performed to find new approaches to the therapy. In this way, new studies dated in mid-2020 have emerged in other continents, as exemplified by the studies included for minoxidil.

We recovered more articles that evaluated the effect of finasteride than minoxidil on the chosen organs and related cells. However, our search strategy had the same sensibility for recovering both studies. It is known that finasteride acts by inhibiting the transformation of testosterone into DHT [59]. Therefore, due to its effect on the androgenic metabolism, testis, the organ responsible for spermatogenesis and male sex hormone production [60], and epididymis, which plays a crucial role in spermatozoa maturation [61] have become the targets of a considerable number of studies on the adverse effects of finasteride. On the other hand, the FDA approved minoxidil in 1970 [62] for treating severe hypertension, a systemic disease unrelated to infertility [63]. Besides, since minoxidil is often used in combination with other medications, such as diuretics [64], and it may be challenging to relate secondary effects exclusively to this drug. In addition, some studies have demonstrated that although both drugs were effective

and safe in treating mild to severe AGA, treatment with oral finasteride was more common among this group by its efficiency, although related to more side effects [21].

Regarding the duration of the experiment that evaluated finasteride, 56 days was the most used, although several experiments varied from 5 days to 10 months. The information about the exposure time is crucial because it has already been shown that the duration of the cycle of the seminiferous epithelium is 56 days in rats [60] and that, on average, ten days of life for a rat in the fertile phase is equivalent to one human year [65] therefore is more than enough time to study the adverse effects in the stages of the cycle and also in the more prolonged exposure visualizing the impact of chronic exposure. Surprisingly, the studies that assessed the effect of minoxidil on the testis lasted only 30 seconds, 4 hours, or 21 days, aiming to evaluate the acute effect of the substance. The finasteride studies included in this review chose the oral route mainly because therapeutic agents are usually administrated this way to achieve systemic results. However, the minoxidil studies were divided into oral and intravenously injections. The most used doses were 5 or 20 mg/kg of finasteride. Doses of up to 80 mg/day in humans have been tolerated for treating BPH [66]; however, for AGA, the amount of 1 mg/day has already shown results for treating the condition [67]. Thus, the variation in doses can be essential to verify whether the adverse effects are dose-dependent. In the studies included here, the amount of minoxidil used were 0.3, 15, and 20 mg/kg. The authors did not clarify the reason for choosing these doses. Minoxidil is generally used topically, 1 mL of 2% solution formulation is applied twice daily in humans. However, more recently, doses ranging from 0.5 to 5 mg have been used orally in treating AGA [68], which justifies the administration chosen by one included study.

In our review, we observed that the majority of the included studies employed rats as their chosen experimental murine models, followed by mice. The rat is the animal model more characterized in the toxicology, developmental and reproductive physiology, and endocrinology research fields, mainly due to its sensitive response to interventions [69]. In this review, we also observed that some *in vitro* studies relied on rat-derived testis cells. Stocks (2004) [69] highlighted that many of the proposed *in vitro* tests require animal tissues or receptors derived from animal tissues. However, many authors emphasize that while *in vitro* models have certain limitations, such as absorption, metabolism, distribution, and excretion *in vitro* tests remain valuable because they allow the exclusion of side effects.

Although the studies included in this review describe the drop in testosterone and some consequences to the testicular tissue after finasteride administration, these studies did not clarify the mechanism involved. The HPG axis plays a crucial role in regulating fertility by synthesizing and secreting hormones [70]. The recovered results revealed testosterone reduction, changes LH and FSH levels after finasteride exposure, which, all together, indicate that the HPG axis could be disrupted after finasteride use. Regarding minoxidil, to the best of our knowledge, there is no evidence that this drug can affect the HPG axis. Hormone deficiency, as consequence of the disruption of HPG axis, can lead to alterations in reproductive organs, such as morphological alterations resulting in cell death, as confirmed by our findings.

According to our outcomes, another remarkable histomorphological damage is the impact on BTB, which possibly occurs through two distinct pathways i.e. mediated by hormones or by imbalance redox. It is already known that BTB plays a vital role in normal testis morphology as an immunological barrier to segregate the postmeiotic germ-cell antigens from the systemic circulation. It creates a unique microenvironment (called adluminal compartment) for germ-cell development and confers cell polarity, essential to spermatogenesis [71]. It is formed by proteins found in adherent or tight junctions (i.e., a-actinin, fimbrin, vinculin, spin, gelsolin, and zonula occludens-1), which have their expression modulated by testosterone [72]. Also, this hormone has been shown to control the cytoplasmic transit by stimulating protein related to endocytic pathways, such as clathrin or proteins involving in transcytosis (caveolin-1 and Rab11, for example) [73]. Therefore, the lack of the hormone allows the permeability of the BTB to toxicants, which leads to disorganization of the adluminal compartment. Thus, the decreased testosterone levels after both drug exposures visualized in this review, mediated by direct effects on Leydig cells or systemic pathways, could lead to damage in BTB.

Impacts on the BTB can also be triggered by free radicals' formation. Our findings confirm the presence of ROS, since the levels of MDA were high after both drug exposures, and the increased levels of peroxynitrite's precursors after finasteride treatment. The membranes from SC and germs cells are rich in polyenoic fatty acids prone to undergo peroxidative decomposition [74]. Lipid peroxidation caused by ROS can promote a degeneration process, and may contribute to cell death [75]. The control and regulation of cell death programmed events occur through Bcl2, Bax, and p53 proteins, which govern mitochondrial membrane permeability and release cytochrome c for the cytosol [76]. It either triggers the activation of the caspase cascade in the intrinsic

apoptotic pathway or amplifies extrinsic apoptotic signaling [77]. Another link between ROS and apoptotic events' occurrence could be through the crosstalk between mitochondria and the ER. When the calcium is forced out from ER by the oxidative stress, its reuptake could happen by mitochondria triggering cell death pathways. Once this happens in the seminiferous tubules, the BTB can be damaged, nonetheless, if the process described occurs in the intertubular space, it may affect the LCs and lead to a drop in testosterone.

In summary, we verified the medicaments relationship between i) the drop in testosterone (by the impact on LCs or HPG axis) and cell death; and the drop in testosterone and BTB damage; ii) the oxidative stress can also cause the same effects; iii) oxidative stress could be the precursor to the drop in testosterone as it can affect the LCs. These hypothetical pathways that we raised in this study, although they cannot be completely described in all their stages, give the first insights into the cellular mechanisms involved in the action of both drugs on the male reproductive system.

5 LIMITATIONS

Although the systematic review has been listed as a high-level study for evaluated studies in a blind way using specific tools [78], there are some limitations. After reviewing all possible pathways outlined in this review, the minoxidil mechanism of action is not well known. It has been supposed that the process could occur through multiple pathways (vasodilatation, anti-inflammatory agent, inducer of the Wnt/b-catenin signaling pathway) [17]. Therefore, could any of these other pathways affect fertility? We could not answer this question with our findings, but we look forward to further studies.

The bias analysis of the *in vivo* studies also demonstrated some underreported information. For studies that administered finasteride, the primary information not reported was random sequence generation, blinding of participants, and personal and random outcome assessment. For the studies with minoxidil, however, random sequence generation, allocation concealment, blinding of participants and personal, random outcome assessment, and blinding of outcome assessment were not reported. The significant heterogeneity of the data does not indicate that the researchers did not evaluate these parameters but that they have not included them in the reports, which characterizes a high risk of bias. In this case, the most significant limitation among the studies was the lack of information described by the researchers, which hindered the comparison among the studies. Because of this, our initial idea was to make a meta-analysis evaluation

impossible to complete. The lack of information in the methodology makes it difficult to replicate the work and diminishes the reliability of the research. Therefore, considering all limitations, we believe that researchers in the reproductive area can use our systematic review to improve the description of the methodology and results for a better reproduction and application of preclinical experiments results that may support clinical trials. Thus, our findings are essential for understanding the mechanism of action of finasteride and minoxidil, describing important points of bias. We hope to contribute to future studies, avoiding those elements of bias that impair the quality of the evidence.

6 CONCLUSION

We can conclude that finasteride and minoxidil harm the testis and testis-derivate cell cultures. Furthermore, our outcomes revealed that finasteride can damage the epididymis in murine models. It is apparent that both medications act as hormone disruptors, cause oxidative stress, and directly or indirectly affect the BTB in the testicular tissue, thus preventing it from functioning normally. *In vitro*, the comparison was limited to hormonal pathways. There is six times more male repro-toxicology research with finasteride than minoxidil *in vivo* and two times more *in vitro*. Therefore, further studies are needed to assess the effect of minoxidil on the male reproductive system, especially the testis and epididymis.

7 DECLARATIONS

7.1 Funding

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7.2 Conflict of Interest

The authors declare that they have no conflicts of interest

7.3 Author's contribution statement

FFVS and AAL: data curation; FFVS and JS: Conceptualization, investigation; FFVS, JS. RVG, JS and SLPM: Conceptualization, supervision.

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9 SUPPLEMENTARY MATERIAL

Table S1. PICOS (Population, Intervention, Comparison, Outcomes, Study design) statement

Parameter	Definition
Population	Any species of adult male murine models and any testis/epididymis-related cells
Interventions	Finasteride or Minoxidil
Comparison	Compare the Finasteride and Minoxidil effects in the testis and epididymis
Outcomes	Morphophysiological and molecular effects of Finasteride or Minoxidil on the testis and epididymis
Study design	Preclinical and *in vitro* studies will be included. Reviews, theses, and dissertations will be excluded

Table S2. Search filters used in PubMed, Scopus, and Web of Science databases.

DESCRIPTORS OF RESEARCH			
PUBMED			
LABORATORY ANIMALS	***ORGANS***	***MINOXIDIL***	***FINASTERIDE***
(”Mice”[MeSH Terms] OR “Rats”[MeSH Terms] OR Mice[Tiab] OR Mouse[TIAB] OR Rat[TIAB] OR Rats[TIAB] OR Hamster[TIAB] OR Murine model [TIAB] OR Animal model [TIAB])	(“testis”[MeSH Terms] OR “spermatogenesis”[MeSH Terms] OR “testosterone”[MeSH Terms] OR “spermatogonia”[MeSH Terms] OR “spermatocytes”[MeSH Terms] OR “spermatids”[MeSH Terms] OR “leydig cells”[MeSH Terms] OR “seminiferous tubules”[MeSH Terms] OR “blood-testis barrier”[MeSH Terms] OR “seminiferous epithelium”[MeSH Terms] OR “sertoli cells”[MeSH Terms] OR “spermatozoa”[MeSH] OR “semen”[MeSH] OR “epididymis”[MeSH Terms] OR testicle[TIAB] OR testicles[TIAB] OR testes[TIAB] OR spermatogenesis[TIAB] OR testosterone[TIAB] OR spermatogonia*[Tiab] OR spermatocyte*[TIAB] OR spermatid*[Tiab] OR “leydig cells”[TIAB] OR “seminiferous tubules”[TIAB] OR “blood-testis barrier”[TIAB] OR “seminiferous epithelium”[TIAB] OR “sertoli cells”[TIAB] OR spermatozoa[TIAB] OR semen[TIAB] OR “sperm quality”[TIAB] OR epididymis[TIAB])	(“minoxidil”[MeSH Terms] OR “Minoxidil”[MeSH Terms] OR Loniten[TIAB] OR Regaine[TIAB] OR Rogaine[TIAB] OR U 10858[TIAB])	("finasteride"[MeSH Terms] OR “Finasteride”[MeSH Terms] OR Proscar[TIAB] OR Propecia[TIAB] OR Finastid[TIAB])

Table S2 (*Continuation*). Search filters used in PubMed, Scopus, and Web of Science databases.

SCOPUS			
LABORATORY ANIMALS	***ORGANS***	***MINOXIDIL***	***FINASTERIDE***
(Mice OR Rats Mice OR Mouse OR Rat OR Rats OR Hamster OR "Murine model" OR "Animal model")	(Testis OR testicle OR testicles OR testes OR spermatogenesis OR testosterone OR spermatogonia* OR spermatocyte* OR spermatid* OR "leydig cells" OR "seminiferous tubules" OR "blood-testis barrier" OR "seminiferous epithelium" OR "sertoli cells" OR spermatozoa OR semen OR "sperm quality" OR epididymis)	(Minoxidil OR minoxidil OR Loniten OR Regaine OR Rogaine OR "U 10858" OR Aloxidil OR Foligain OR Kirkland)	(Finasteride OR Proscar OR Propecia OR Finastid)

Table S2 (*Continuation*). Search filters used in PubMed, Scopus, and Web of Science databases

WEB OF SCIENCE			
LABORATORY ANIMALS	***ORGANS***	***MINOXIDIL***	***FINASTERIDE***
(Mice OR Rats Mice OR Mouse OR Rat OR Rats OR Hamster OR "Murine model" OR "Animal model")	(Testis OR testicle OR testicles OR testes OR spermatogenesis OR testosterone OR spermatogonia* OR spermatocyte* OR spermatid* OR "leydig cells" OR "seminiferous tubules" OR "blood-testis barrier" OR "seminiferous epithelium" OR "sertoli cells" OR spermatozoa OR semen OR "sperm quality" OR epididymis)	(Minoxidil OR minoxidil OR Loniten OR Regaine OR Rogaine OR "U 10858" OR Aloxidil OR Foligain OR Kirkland)	(Finasteride OR Proscar OR Propeia OR Finastid)

Table S3. Characteristics of publication and experimental animals of the included studies that assessed the Finasteride or Minoxidil effects in the testis and/or epididymis of murine models.

Study	Country	Animal model	Age (days)	Weight (g)	Number of animals	Animals per group	Groups
Finasteride							
Bandivdekar AH., et al., 2000[26]	USA	Sprague Dawley rats	?	225-250	10	5	C=1 T=1
Chen X., et al., 2020[27]	China	Balb/c mice	?	28±3	12	6	C=1 T=1
Cukierski MA., et al.; 1991[28]	USA	Sprague Dawley rats	120	?	FE=80 SE=18	FE= C=40 T=40 SE= C=9 T=9	2
Garcia PV., et al., 2012[29]	Brazil	Sprague Dawley rats	60	?	75	25	C=1 T=2
Jaffat H. and Obaid FN., 2018[30]	Iraq	*Rattus Novergicus*	56-70	200-300	45	15	C=1 T=2
Kolasa A., et al., 2004[31]	Poland	Wistar rats	?	?	15	5	C=1 T=2
Kolasa A., et al., 2008[32]	Poland	Wistar rats	?	300-350	10	C=5 T=5	2
Kolasa A., et al., 2009[33]	Poland	Wistar rats	90	?	20	C=10 T=5	C=1 T=2
Kolasa A., et al., 2011[34]	Poland	Wistar rats	90	?	20	C=10 T=5	C=1 T=2
Lephart ED, 1995[35]	USA	Sprague Dawley rats	TE=54-68	?	TE=15	C=5 T=5	C=1 T=2
Mohebali, S., et al., 2020[36]	Iran	NMRI mice	?	?	60	12	C=2 T=3
Obaid FN e Jaffat HS., 2018[37]	Iraq	*Rattus novergicus*	56-70	200-300	45	5	C=1 T=2
O'Connor JC., et al., 1998[38]	USA	CRL:CD BR rats	63	?	15	3	C=1 T=4
Olayinka ET. And Adewole KE., 2020[39]	Nigeria	Wistar Rats	?	120-145	12	C=6 T=6	2

Table S3 (*Continuation*). Characteristics of publication and experimental animals of the included studies that assessed the Finasteride or Minoxidil effects in the testis and/or epididymis of murine models.

Study	Country	Animal model	Age (days)	Weight (g)	Number of animals	Animals per group	Groups
Prahalada S., et al., 1994[40]	USA	CRL:CD-1 (ICR) BR mice	SE=56 TE=84	SE=30-41 TE=29-40	SE= 90 TE=24	SE= C=30 T=15; 15; 30 TE= C=12 T=12	SE=4 TE=2
Rhoden EL., et al., 2002[41]	Brazil	Wistar rats	120	?	25	C=8 T=17	2
Shalaby AM., et al., 2020[42]	Kingdom of Saudi Arabia	Wistar rats	56-70	180-200	20	C=10 T=10	2
Soni KK., et al., 2017[43]	Republic of Korea	Sprague Dawley rats	70-84	300-320	20	C=10 T=10	2
Świder-Al-Amawi M., et a.l., 2007[44]	Poland	Wistar rats	?	300-350	15	C=10 T=5	2
Tobin VA and Canny BJ., 1997[45]	Australia	Sprague Dawley rats	?	250-350	20	C=10 T=10	2
Trybek G., et al., 2005[46]	Poland	Wistar rats	?	350-350	10	C=5 T=5	2
Vidigal DJA., et al., 2008[47]	Brazil	Mesocricetus auratus	365	?	20	C=10 T=10	2
Wise LD., et al., 1991[48]	USA	Sprague Dawley rats Crl:CD(SD) BR rats	FE=30-42 SE=42 TE=105	FE=75-150 SE=180-231 TE=435-535	FE=76-80 SE=30 TE=30	FE=19-20 SE=15 TE=15	FE= C=1 T=3 SE= C=1 T=1 TE= C=1 T=1
Zhang MG., et al., 2012[49]	China	Sprague Dawley rats	?	250-300	50	C=25 T=25	2

Table S3 (*Continuation*). Characteristics of publication and experimental animals of the included studies studies that assessed the Finasteride or Minoxidil effects in the testis and/or epididymis of murine models.

Study	Country	Animal model	Age (days)	Weight (g)	Number of animals	Animals per group	Groups
Minoxidil							
Mingsan, M., et al., 2020[50]	China	KM mice	?	18-22	20	10	C=1 T=1
Ozturk H., et al., 2020[51]	Turkey	Wistar Rats	90	240-280	21	7	C=1 T=2
Piner J., et al., 2002[52]	United Kingdom	Charles River Wistar rats	75-90	?	16	8	C=1 T=1

USA = United States of America; ? = Not informed; FE = First Experiment; SE = Second Experiment; TE = Third Experiment; C = Control; T = Treatment.

Table S4. Characteristics of exposure to Finasteride and Minoxidil of the included studies *in vivo from murine models*.

Study	Compound	Dose	Duration	Exposure	Route	Control
Finasteride						
Bandivdekar AH., et al., 2000[26]	Finasteride+ cottonseed oil 5% ethanol	4 mg/day	5 days	Daily	SC Injection	Castration
Chen X., et al., 2020[27]	Finasteride dissolved in 0.5 ml Distilled Water	25 mg/kg/day	35 days	Daily	Oral	Distilled water (0.5 ml/day)
Cukierski MA., et al.; 1991[28]	Finasteride in 0.5% aqueous methylcellulose	80 mg/kg/day	24 to 38 weeks	Daily	Oral	0.5% Aqueous methylcellulose (5ml/kg)
Garcia PV., et al., 2012[29]	Finasteride in methylcellulose	5 mg/kg	G1 and G2= 56 days G3= 56 days + 30 days of recovery	Daily	Oral	0.5% Aqueous methylcellulose 5mg/kg
Jaffat H. and Obaid FN., 2018[30]	Finasteride dissolved in Distilled Water	0.014 mg and 0.028 mg	8 weeks	Daily	Oral	0.014mg and 0.028mg Distilled water
Kolasa A., et al., 2004[31]	?	5 mg/kg	28 days and 56 days	Daily	Oral	?
Kolasa A., et al., 2008[32]	?	5 mg/kg	56 days	Daily	Oral	?
Kolasa A., et al., 2009[33]	?	5 mg/kg	28 days and 56 days	Daily	Oral	?
Kolasa A., et al., 2011[34]	?	5 mg/kg	28 days and 56 days	Daily	Oral	?

Table S4 (*Continuation*). Characteristics of exposure to Finasteride and Minoxidil of the included studies *in vivo from murine models*.

Study	Compound	Dose	Duration	Exposure	Route	Control
Lephart ED, 1995[35]	Finasteride suspended in oil (0.1-0.5ml)	20 mg/kg	2 weeks	Daily	SC Injection	0.1-0.5ml Oil
Mohebali, S., et al., 2020[36]	?	1.5 and 20 mg/kg	35 days	Daily	?	?
Obaid FN e Jaffat HS., 2018[37]	Finasteride dissolved in Distilled Water	5mg/kg or 10 mg/kg	8 weeks	Daily	Oral	5ml/kg or 10 ml/kg Distilled water
O'Connor JC., et al., 1998[38]	Finasteride dissolved in Methylcellulose	1.5, 10 and 25 mg/kg	15 days	Daily	IP Injection	0.25% Methylcellulose
Olayinka ET. And Adewole KE., 2020[39]	Finasteride dissolved in Distilled Water	3.13 mg/kg	14 days	Daily	Oral	1ml/kg Distilled water
Prahalada S., et al., 1994[40]	Finasteride dissolved in methylcellulose	FE=2.5, 25 and 50 mg/kg SE=0.25, 2.5 and 250 mg/kg TE=250 mg/kg	SE=5 weeks and 14 weeks TE=8 days	Daily	Oral	FE and SE=0.5% Methylcellulose TE=0.5% Methylcellulose+0.2ml/day sesame oil (SC)
Rhoden EL., et al., 2002[41]	Finasteride suspension in Saline solution (2ml)	2 mg/kg	10 months	5days/week	Oral	2 ml of Saline solution
Shalaby AM., et al., 2020[42]	Finasteride	5 mg/kg	8 weeks	Daily	Oral	No treatments or 0.5ml of distilled water
Soni KK., et al., 2017[43]	Finasteride suspension in Saline solution	1 mg/kg	90 days	Daily	Oral	No treatment

Table S4 (*Continuation*). Characteristics of exposure to Finasteride and Minoxidil of the included studies *in vivo from murine models*.

Study	Compound	Dose	Duration	Exposure	Route	Control
Świder-Al-Amawi M., et a.l., 2007[44]	?	5 mg/kg	56 days	Daily	Oral	?
Tobin VA and Canny BJ., 1997[45]	Finasteride in 0.2ml 80% triolene-ethanol	50 mg/kg	7 days	Daily	?	0.2ml 80% Triolene-ethanol
Trybek G., et al., 2005[46]	?	5 mg/kg	56 days	Daily	Oral	?
Vidigal DJA., et al., 2008[47]	?	0.0714 mg/kg	90 days	3 times/week	SC Injection	?
Wise LD., et al., 1991[48]	Finasteride dissolved in methylcellulose	FE=0,20,40,80 mg/kg SE=0, 80 mg/kg TE=0, 80 mg/kg	FE=27 weeks SE=14 weeks+ 6 recovery TE=32 weeks+ 6 recovery	Daily	Oral	0.5% Aqueous methylcellulose (5ml/kg)
Zhang MG., et al., 2012[49]	?	4.5 mg/kg	4 weeks	Daily	Oral	No treatment
Minoxidil						
Mingsan, M., et al., 2020[50]	Minoxidil	20 mg	21 days	Daily	?	Sterile water
Ozturk H., et al., 2020[51]	Torsion + Minoxidil	0.3 mg/kg	30 seconds	Single	Intravenously	No torsion
Piner J., et al., 2002[52]	Minoxidil+ GR40370D	15 mg/kg	4 hours	Single	Oral	750mg/kg GR40370D

? = Not informed; G1 = Group one; G2 = Group two; G3 = Group three; FE = First Experiment; SE = Second Experiment; TE = Third Experiment; SC = Subcutaneous; IP = Intraperitoneal.

Table S5. Characteristics of publication and experimental models of the included studies *in vitro* that assessed the Finasteride or Minoxidil effects extracted from murine models .

Study	Country	Cell type	Cell line	Source	Culture medium
Finasteride					
Freeman DA., et al., 1993[53]	USA	Leydig tumor cells	MA-10 cells	Rats	Waymouth's MB 752/1 with 20 nM HEPES, 1.2 g/l NAHCO3, 15% horse serum, ph 7.4
Gorczynska E. and Handelsman DJ., 1995[54]	Australia	Sertoli cells	NA	Wistar rats	?
Powlin SS., et al., 1998[55]	USA	Testicular parenchyma	NA	Crl:CD BR rats	5 ml of culture medium (RPMI-1640 medium, 10% FCS, 50 mg/ml soybean trypsin inhibitor)
Minoxidil					
Study	**Country**	**Cell type**	**Cell line**	**Source**	**Culture medium**
Parker, LN., et al., 1982[56]	?	Rat testis cells	NA	Sprague-Dawley	?

USA = United States of America; ? = Not informed; C = Control; T = Treatment; NA = Not applicable.

Table S6. Characteristics of exposure to Finasteride and Minoxidil in cells extracted from murine models of the included *in vitro* studies.

Study	Formulation administered	Dose	Assays	Incubation period
Finasteride				
Freeman DA., et al., 1993[53]	1 nM Dibutyryl-cAMP + Finasteride Finasteride +10 µg/ml 22OH Cholesterol	0.001,0.01, 0.1, 1 mg/l	1: 1 nM Dibutyryl-cAMP + Finasteride (1, 10, 100 or 1000 ng/ml) dissolved in ethanol 200-fold concentrated stock 2: Finasteride 1 µg/ml+ 1 nM Dibutyryl-cAMP Finasteride 1 µg/ml+22OH Cholesterol	1:? 2: 3h at 37ºC
Gorczynska E. and Handelsman DJ., 1995[54]	Finateride + T or T:BSA Finasteride + DHT	1 mg/l	1: Finasteride-pretreated Sertoli cells with conjugate T (1 mg / liter) or T:BSA (10 mg / liter containing the equivalent of 1 mg / liter of T) and only Sertoli treated with T or T:BSA 2: Finasteride-pretreated Sertoli cells with conjugate DHT (1 mg / liter) and only Sertoli treated with DHT	Finasteride: pre-incubation for 20 min at 22º
Powlin SS., et al., 1998[55]	Finasteride	0.0001, 0.001, 0.01, 0.1mg/l	1:10 ml of hCG (500 IU/ml stock solution) + Finasteride 2: 10 ml of distilled water for non-hCG stimulated controls)+ Finasteride	3 h at 34°C under vigorous shaking (175 rpm)
Minoxidil				
Parker, LN., et al., 1982[56]	Minoxidil	0.33 mg/l, 3.33 mg/l, 33.3 mg/l	1: Minoxidil alone 2: Minoxidil + hCG (0.1 or 10 mU)	90 min at 37ºC

? = Not informed; cAMP = Cyclic adenosine monophosphate; DHT = dihydrotestosterone; hCG = human chorionic gonadotropin.

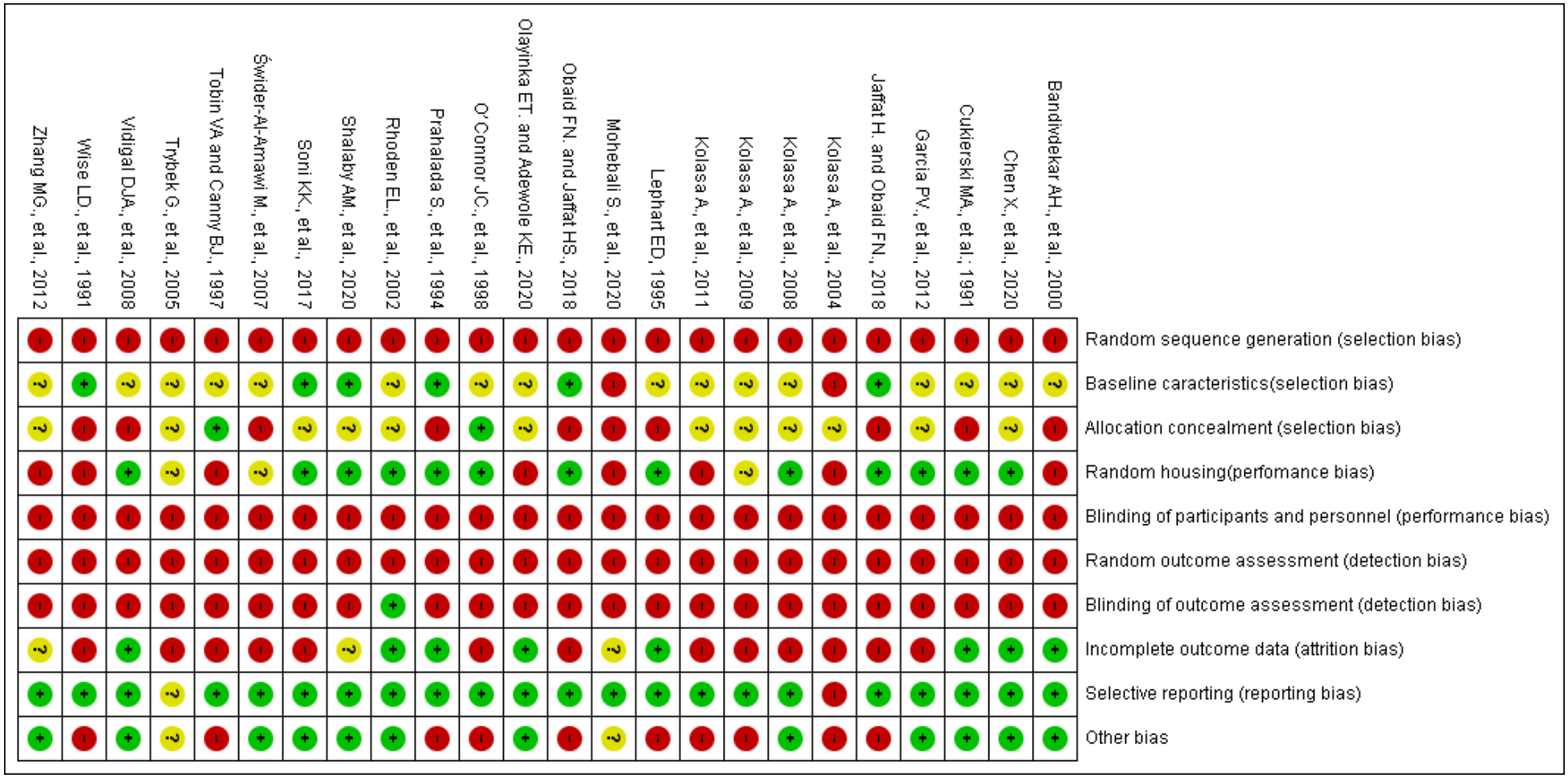

Fig. S1 Results of the risk of bias assessment for each included study that evaluated the Finasteride impact on the testis and epididymis of murine models in chronological order. Green = Low risk of bias; Yellow = Unclear risk of bias; Red = High risk of bias.

Fig. S2 Results of the risk of bias assessment for each included study that evaluated the Minoxidil impact on the testis of murine models in chronological order. Green = Low risk of bias; Yellow = Unclear risk of bias; Red = High risk of bias.

CHAPTER 2

Long-term oral exposure to finasteride and different doses of minoxidil induces testicular and epididymal alterations in adult Balb/c mice

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ABSTRACT: Oral finasteride and topical minoxidil are approved treatments for androgenetic alopecia, but low-dose of oral minoxidil, an *off-label* option, shows promising results. Despite minoxidil's vasodilatory and androgen-modulating effects, its impact on male reproductive health is poorly understood. Given its rising off-label use and potential reproductive impact, we conducted histopathological, hormonal, and oxidative stress analyses to characterize its effects on the testes and epididymides of adult Balb/c mice. A total of 120 mice were divided into six groups (n=20/group) receiving daily oral doses of distilled water (control), vehicle solution, 5 mg/kg finasteride, or minoxidil at 2.5, 5, or 7.5 mg/kg. Half of the animals were euthanized after 42 days, and the remaining half after 84 days of exposure. At 42 days, minoxidil and finasteride induced testicular structural changes, including vacuolization and reduced

seminiferous tubule proportion. These effects were accompanied by reduced estradiol levels and oxidative stress, potentially contributing to epithelial detachment and early signs of Leydig cell remodeling. After 84 days, minoxidil at 5 mg/kg caused the most prominent testicular damage, including reductions in epithelium height, and increased interstitial connective tissue, along with a trend toward Leydig cell hyperplasia. Epididymal alterations were observed with both drugs at both time points, although only finasteride significantly reduced sperm transit time (after 42 and 84 days) and cauda sperm count (after 84 days). Our findings revealed that at 42 days, both finasteride and minoxidil caused significant testicular structural damage, such as a decrease in the proportion of seminiferous tubules and an increase in the proportion of Leydig cells, possibly related to a non-significant hyperplasia, probably associated with a reduction in estradiol levels and/or oxidative stress. These findings suggest that minoxidil induced broader testicular alterations than finasteride, while finasteride more directly impaired epididymal function. These findings highlight potential risks associated with the long-term use of oral minoxidil, particularly at higher doses, and underscore the need for clinical studies investigating its effects on male reproductive function, especially in individuals of reproductive age.

1 INTRODUCTION

Alopecia, or hair loss, is a condition that causes aesthetic and psychological distress in patients and health concerns, as it can be an important sign of systemic diseases (ALMOHANNA et al., 2018). Androgenetic alopecia (AGA) is the most common type of non-scarring hair loss, affecting at least half of men of reproductive age (ALMOHANNA et al., 2018; GOMOLIN et al., 2020). The best-known therapies are oral finasteride and topical minoxidil. Although new treatment options have become available (e.g. low-level laser light, platelet-rich plasma, microneedling, and the use of oral minoxidil) (NESTOR et al., 2021). However, the use of these new therapies remains limited, because their efficacy needs to be confirmed and their cost is often high for most patients (GOMOLIN et al., 2020). Consequently, therapies with finasteride and minoxidil remain the most commonly used (DEVJANI et al., 2023).

Topical minoxidil, a drug approved by the United States Department of Health and Human Services, the FDA (Food and Drug Administration) to treat male AGA, is used in 2% to 5% formulations, and it acts causes vasodilation of dermal blood vessels and promote hair

growth (STOEHR et al., 2019). However, lack of efficacy and allergic contact dermatitis, are common barriers to this therapy (GOMOLIN et al., 2020). Oral finasteride was approved by the FDA in 1997 at a dosage of 1 mg for the treatment of AGA (DA SILVA & MICELI, 2018). It has demonstrated efficacy in alopecia by inhibiting the action the action of the enzyme 5α-reductase, responsible for metabolizing testosterone into dihydrotestosterone (DHT), thus reducing DHT levels in the body and consequently reducing hair loss (DA SILVA & MICELI, 2018). However, in addition to other side effects as examples, the inhibition of the enzyme 5α-reductase causes a disbalance of androgen levels in the body, which directly affect the male reproductive system (SANTOS et al., 2017), since adequate levels of testosterone maintain spermatogenesis in the testes and act on other organs in this system (RUSSELL & GRISWOLD, 1993).

Oral minoxidil is a possible option for patients with AGA who have experienced adverse effects after finasteride treatment or who have contact dermatitis when using topical minoxidil solution (PERERA & SINCLAIR, 2017). In addition, a recent retrospective review showed that low-dose minoxidil (2.5–5 mg once daily), administered as monotherapy or add-on therapy for 6–12 months, was effective and safe in male patients (PANCHAPRATEEP & LUEANGARUN, 2020). Although oral minoxidil (10- 40 mg/day) is approved for the treatment of hypertension (SICA & GEHR, 2001), oral minoxidil is not currently FDA-approved for hair loss, although it has been used at various doses (0.25 to 2.5 mg per day) in several clinical trials (BEACH et al., 2021; JHA et al., 2020, PIRMEZ & SALAS-CALLO, 2020; SINCLAIR, 2017). Jimenez-Cauhe et al. (2019) recently evaluated 41 men with AGA who received 2.5 or 5 mg of oral minoxidil daily for ≥6 months and found overall clinical improvement in 90.2% of patients. Adverse effects of hypertrichosis, lower limb edema, and falls were observed in 24.3%, 4.8%, and 2.4% of patients, respectively.

Male reproductive health is often overlooked in studies on dermatological treatments, despite evidence suggesting that therapies for alopecia may negatively impact the reproductive system (HUI et al., 2022). Despite this, there is a lack of consistent studies on its long-term effects on the male reproductive system, which justifies the need for in-depth investigations. Our team previously conducted a systematic review to summarize the existing evidence on the effects of exposure to finasteride or minoxidil on the two reproductive organs, the testis, which is the organ responsible for spermatogenesis and production of male sex hormones (RUSSELL et al., 1990), and the epididymis, which plays a crucial role in sperm maturation and quality

(FRANÇA et al., 2005), in adult murine models, as well as testis/epididymis-related cells (SANTANA et al., 2023*a*). In this review, it was well documented that finasteride acts as a hormone disruptor by inducing oxidative stress and morphological alterations in the testis and testis-related cells. The results further suggest that minoxidil may exert similar effects through comparable mechanistic pathways. Although, to the best of our knowledge, no studies have investigated the effect of minoxidil in the epididymis, our systematic review explored hypothetical pathways and provide initial insights into the cellular mechanisms involved in the effect of minoxidil on the male reproductive system. In our review, the potential mechanisms by which minoxidil may impair testicular function include: i) a reduction in testosterone levels, potentially due to direct effects on Leydig cells or disruption of the hypothalamic-pituitary-gonadal axis, leading to cell damage and compromise of the blood–testis barrier; and ii) oxidative stress, which may independently induce cellular damage or indirectly impair steroidogenesis by affecting Leydig cell viability. However, these mechanisms cannot be fully described in all their stages after minoxidil exposure due to the limited number of studies retrieved.

In this context, we aimed to evaluate and compare the effects of 42- and 84- day oral administration of finasteride (5 mg/kg) and minoxidil (2.5, 5, and 7.5 mg/kg) on the testis and epididymis of adult Balb/C mice. We focused on key reproductive parameters such as hormone levels, oxidative stress markers, and morphological changes. By investigating and characterizing the impact of both drugs in male murine models, the study seeks to provide a clearer understanding of their potential reproductive toxicity, mainly for the minoxidil. Our study will help elucidate the mechanisms underlying drug-induced alterations in male reproductive health and contribute to the development of safer therapeutic alternatives for androgenetic alopecia.

2 MATERIALS AND METHODS

2.1 Animals

Adult male Balb/C mice (n = 120, 75 days old) were provided by the Central Animal Facility of the Center of Biological and Health Sciences, *Universidade Federal de Viçosa* (UFV). The animals were housed in individual polypropylene cages under controlled temperature (21 ± 1ºC) and photoperiod (12–12 h light/dark). The animals received water and a standard rodent diet *ad libitum*. The research protocol followed the standards of the National

Council for the Control of Animal Experiments (CONCEA) and was approved by the Ethics Committee for the Use of Animals (CEUA-UFV, protocol 25/2022).

2.2 Experimental Design

Animals were randomly divided into 6 experimental groups (n=20 animals/group). Exposure to minoxidil was administered orally in daily doses. One group received 0.2 mL distilled water (control), one group received 0.2 mL vehicle (sodium laurel sulfate 1% and nipagin 0.16%), one group received 5 mg/kg of finasteride diluted in 0.2 mL vehicle solution, one group received 2.5 mg/kg of minoxidil diluted in 0.2 mL vehicle solution, one group received 5 mg/kg minoxidil diluted in 0.2 mL vehicle solution, and one group received 7.5 mg/kg minoxidil diluted in 0.2 mL vehicle solution. Ten animals per group were weighed and euthanized after 42 days of the treatment and the remaining ten mice were weighed euthanized after 84 days of the experiment. After euthanasia, the reproductive organs were collected and weighed and based in the ratio between the body weight and testicular, parenchymal and epididymal weights, the gonadosomatic (GSI), parenchymosomatic (PSI) and epididymosomatic (IES) indexes were calculated. The organs were fixed in Karnovsky solution (KARNOVSKY, 1965) for histology and frozen (-80°C) for other analyses. Oral minoxidil is approved for the treatment of hypertension, with standard doses ranging from 10 to 40 mg per day. However, lower oral doses such as 0.25 mg/day, 1.25 mg/day, 2.5 mg/day, and 5 mg/day are commonly used to treat AGA without causing serious side effects, which contributed to the choice of dose in this study, in which we evaluated whether there was testicular toxicity associated with its use. The finasteride dose reflects the concentration most commonly employed in murine model studies and, due to its well-documented effects on the male reproductive system (SANTANA et al., 2023*a*), statistical comparisons were conducted between the minoxidil and finasteride groups.

The 42-day period was chosen because it corresponds to the full duration of spermatogenesis in mice and therefore allows the observation of chronic changes (RUSSELL et al., 1990). Knowing that the approved treatment for AGA requires at least 4-6 months of use before improvement in baldness is observed (HO et al., 2012), we chose the 84-day exposure period, which corresponds to prolonged use of the drug.

2.3 Determination of testosterone and 17β-estradiol concentration

The serum concentrations of testosterone and 17β-estradiol were measured after 42 and 84 days of treatment using the ELISA kits commercially available for testosterone; (EIA-37K1A4, USA Diagnóstica) and estradiol (EIA-9140 (USA Diagnóstica)), according to the manufacturer's instructions.

2.4 Histological Processing

Testis and epididymis fragments for light microscopy were dehydrated in increasing concentrations of ethanol, embedded in 2-hydroxyethyl methacrylate (Historesin®, Leica), sectioned at 3µm thickness, and stained with toluidine blue/sodium borate 1%. Preparations were mounted with Entellan® (Merck, Frankfurt, Germany). Tissue images were captured on an Olympus AX-70 microscope and analyzed using ImageJ software.

2.5 Histopathology of testis and epididymis

For histopathological evaluation of the testes, 200 tubules from the histological sections per animal were recorded and classified as: usual (presence of organized germ layers in the seminiferous epithelium) or abnormal (presence vacuoles at the base and/or apex of the seminiferous epithelium, tubules with a detachment of germ cells, and tubules with only Sertoli cells and even tubules completely empty) as described by Johnsen (1970).

The histopathology of the epididymis was performed in longitudinal histological sections of the initial segment, caput, corpus, and cauda regions. We evaluated the morphology of the epithelium, observing the presence of epithelial vacuolization, cryptic epithelium, germ cells in the lumen, sperm within the lumen, and inflammatory infiltrates in the interstitium as already described (COIMBRA et al., 2023).

2.6 Testicular morphometry and stereology

The proportion and volume of the seminiferous tubules and their components – the seminiferous epithelium, the tunica propria, the lumen, and the intertubular compartment was estimated from 20 randomly digital images per animal for each experimental condition. A total of 5,320 points per animal were counting using a grid of 266 points, at 200X magnification, as already described (MOURO et al., 2019; SANTANA et al., 2023*b*). Briefly, the proportion of each component was determined by the ratio between the total number of counted points and the number of counted points at the component. Since the testicular density in mammals is

known to be close to 1 (JOHNSON et al., 1981), the volume of each component was calculated by multiplying the proportion of each component by the testicular parenchyma volume. Based on the body weight and seminiferous tubule volume, we calculated the tubulesomatic index (TSI). Based on body weight and seminiferous epithelium volume, we calculated the epithelium-somatic index (EPSI).

The mean tubular diameter per animal was obtained after measuring 20 random cross-sections of seminiferous tubules, without considering the stage of the seminiferous epithelium cycle. The height of the seminiferous epithelium was measured from the tunica propria to the tubular lumen in the same 20 random cross-sections of seminiferous tubules used to obtain the tubular diameter and considered, the mean of two diametrically opposite measurements. The diameter of the tubular lumen was obtained after subtracting the height of the epithelium from the diameter of the seminiferous tubule. The seminiferous tubule and lumen areas were calculated according to the following equation: area = $\pi.R^2$, where R is the tubular radius or the lumen radius, respectively. The epithelium area was obtained by subtracting the luminal area from the tubular area. The tubule epithelium ratio was calculated by dividing the tubule area by the epithelium area. Finally, the total length of the seminiferous tubules was estimated based on the volume occupied by the seminiferous tubules in the two testes and the mean of the seminiferous tubule area (MOURO et al., 2019; SANTANA et al., 2023*b*).

The proportion of intertubular elements in the testis was estimated by counting 1,000 points per animal using a reticulum of 540 points at 400X magnification, in fields randomly distributed in the histological preparations of each testis of the animals. Points were recorded on the components of the intertubular compartment, such as Leydig cells (nucleus and cytoplasm), blood vessels, lymphatic space, and connective tissue. The proportion of each component was determined by the ratio of the total number of points counted to the number of points counted in the component. The volume of each component was also calculated by multiplying the proportion of each intertubular component by the testicular parenchyma volume, as already described (MOURO et al. 2019; SANTANA et al., 2023*b*).

2.7 Morphometry and stereology of Leydig cells

The mean nuclear diameter of Leydig cells was measured at 400x magnification on 30 nuclei per animal. The nuclear volume of a single Leydig cell was obtained using the equation $4/3\ \pi R^3$, in which R is the nuclear diameter. The cytoplasmic volume of a single Leydig cell

was estimated by multiplying the cytoplasmic percentage by the nuclear volume and dividing it by the nuclear percentage. Finally, the single Leydig cell volume was calculated by adding the nuclear and cytoplasmic volumes (MOURO et al., 2019; SANTANA et al., 2023*b*).

The volume of Leydig cells in the testes was calculated by multiplying the proportion of Leydig cells in the testicular parenchyma by the weight of the parenchyma of both testes. The volume of Leydig cells per gram of testis was calculated by the ratio of the volume occupied by Leydig cells in the testes to the weight of the testes. The Leydigosomatic index (LSI) was calculated based on the volume of Leydig cells and body weight. The number of Leydig cells per testis was also estimated based on the individual Leydig cell volume and the total volume occupied by these cells in the testis. Finally, this value was divided by the gonadal weight to determine the number of Leydig cells per gram of testis, allowing comparisons between different species (MOURO et al., 2019; SANTANA et al., 2023*b*).

2.8 Oxidative status analyses

The activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST), along with the concentrations of nitric oxide (NO), malondialdehyde (MDA), and carbonyl protein (CP) content, were analyzed in the supernatant obtained from 100 mg of frozen testes and epididymis. The tissues were homogenized in potassium phosphate buffer (pH 7.4) and centrifuged at 12,000 rpm for 10 minutes at 4ºC.

As described in Santana et al. (2023*b*), SOD activity was assessed based on its capacity to catalyze the conversion of superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2), thus reducing the pyrogallol auto-oxidation rate (DIETERICH et al., 2000). CAT activity was evaluated following the methodology described by Goth (1991) and Korolyuk et al. (1988), with modifications by Hadwan & Abed (2016). This technique relies on the reaction between hydrogen peroxide and ammonium molybdate, producing a yellow-colored complex. GST activity was determined according to Habig et al. (1974) by detecting the formation of a conjugate between glutathione and 2,4-dinitrobenzene (CDNB).

Regarding oxidative and nitrosative stress markers, to quantify NO, the nitrite used as an indicator of nitric oxide synthesis and detected by the Griess reagent. Thus, 50 µL of the supernatant of the samples were added to microplates with an equal volume of Griess reagent and incubated at room temperature for 15 minutes. The absorbance was measured at 570 nm in a microplate scanning spectrophotometer. The concentration of nitric oxide in the samples was

determined using a standard curve with known concentrations of sodium nitrite and expressed in µM (RICART-JANÉ et al., 2002; DROGE, 2002). The MDA concentration was obtained by adding TBARS solution (15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 M hydrochloric acid) to the homogenate, followed by incubation in a water bath at 90°C for 40 minutes. Thiobarbituric acid-reactive substances are primarily products of lipid peroxidation, and MDA serves as a key indicator for assessing lipoperoxidation levels (BUEGE & AUST, 1978). The CP content was quantified using the method outlined by Levine et al. (1994), which involves the reaction between carbonyl groups and 2,4-dinitrophenylhydrazine (DNPH) to enhance specificity and sensitivity. Enzymatic activities of SOD and CAT, as well as the oxidative marker MDA, were normalized to total protein levels in testicular and epididymal tissue, determined by the method of Bradford (1976).

2.9 Sperm evaluation

Freshly dissected cauda from the left epididymides were sequentially sectioned in a Petri dish using a stainless-steel blade to obtain sperm-rich fluid. This fluid was diluted in 500 µL of Tris-citrate-fructose extender at 37°C, and aliquots were taken for analysis of sperm motility as described previously (GUIMARÃES-ERVILHA et al., 2024). Sperm motility was evaluated immediately post-collection under a bright field microscope at 400X magnification by adding 10 µl of epididymal fluid to 200 µl of tris-citrate-fructose. The spermatozoa were classified as either motile or nonmotile in three random fields in 2 histological slides of each animal and the total motility was expressed as percentage.

2.10 Daily sperm production, sperm number, and epididymal transit time

Testicular spermatids resistant to homogenization (stage 19 of spermatogenesis) and sperm from the caput/corpus and cauda epididymis were counted as described by Robb et al. (1978) and modified by Fernandes et al. (2007). For this purpose, frozen testes were decapsulated, weighed, and homogenized in 5 mL of 0.9% NaCl (Triton X-100 0.05%) for 3 minutes. It was then diluted 5 times (1:5) in the same solution and transferred to the Neubauer chamber, where the number of spermatids resistant to homogenization was counted. To determine daily sperm production, the number of spermatids per testis was divided by 4.84, which is the number of days that mature spermatids are present in the seminiferous epithelium of mice (ROBB et al., 1978, FERNANDES et al., 2007). Similarly, the caput/corpus and cauda-from the epididymis were used by homogenizing 200 mg of caput/corpus tissue and 100 mg of

cauda in 1 mL of 0.9% NaCl solution (Triton X-100 0.05%) for 3 minutes. The caput/corpus homogenate was then diluted 2-folds (1:2), while the cauda homogenate was diluted 1-fold (1:1). To calculate the transit time in each epididymis region, in days, the total number of sperms in each segment was divided by the daily sperm production (ROBB et al., 1978; FERNANDES et al., 2007).

2.11 Statistical Analysis

Statistical analyses were performed comparing vehicle, finasteride, and the three doses of minoxidil with the negative control group (water) and the three doses of minoxidil groups with the finasteride group. The data study was assessed for normality using the Shapiro-Wilk test. Nonparametric data were compared using the Kruskal-Wallis test followed by Dunn test. The GraphPad Prism software v8.0.1 (GraphPad Software, USA) was used, considering a significance level of p-value ≤ 0.05.

3 RESULTS

3.1 *Minoxidil and finasteride decreased serum 17β-estradiol levels but did not affect the testosterone levels after 42 days of treatment*

At 42 days, minoxidil at dose of 2.5 mg/kg decreased in 22.64% the serum 17β-estradiol levels compared to the water control group. At this time point, treatment with finasteride and minoxidil did not significantly change serum testosterone levels compared to the water control. However, minoxidil at 5 mg/kg significantly increased testosterone (2.58%) and estradiol (11.55%) levels compared to the finasteride-treated group. After 84 days, the exposure to finasteride and minoxidil did not affect serum testosterone or 17β-estradiol levels **(Table 1)**.

Table 1. Hormonal parameters of male Balb/c mice after long-term exposure to finasteride and minoxidil.

	Control	Vehicle	Fin 5mg/Kg	Min 2.5mg/Kg	Min 5.0mg/Kg	Min 7.5mg/Kg
42 days						
17β-Estradiol (pg/MI)	76.52 ± 3.64	37.26 ± 14.66	20.49 ± 7.22	17.30 ± 5.03*	65.14 ± 4.48#	41.4355 ± 16.29
Testosterone (pg/MI)	2.43 ± 1.26	4.01± 1.97	0.60 ± 0.25	2.20 ± 1.24	8.87 ± 2.39#	4.22 ± 1.46
84 days						
17β-Estradiol (pg/MI)	55.10 ± 15.10	37.26 ± 14.66	25.27 ± 7.17	30.96 ± 13.45	65.63 ± 17.89	8.90 ± 4.35
Testosterone (pg/MI)	3.66 ± 2.14	0.71 ± 0.44	2.79 ± 1.26	2.17 ± 0.97	3.81 ± 1.50	0.35 ± 0.21

Fin – Finasteride, Min – Minoxidil. Data expressed as mean ± SEM. (*) shows significantly different values compared to control ($p \leq 0.05$) and (#) shows significantly different values compared to finasteride using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group

3.2 *Effect of long-term exposure to minoxidil on testicular and epididymal weights*

Exposure to minoxidil or finasteride for 42 or 84 days did not affect relative testicular weight (gonadosomatic index - GSI), relative testicular parenchyma weight (parenchymosomatic index - PSI), and relative epididymal weight (epididymosomatic index - ESI) compared to the water control group. The other biometric parameters, such as body, absolute testis, and absolute epididymis weights, did not change after the treatments **(Supplementary Table 1 and 2)**.

3.3 Long-term exposure to finasteride and minoxidil induced histopathological changes in the testis and epididymis

The presence of rare immature germ cells in the lumen of the seminiferous tubules was observed after exposure to minoxidil at the doses of 2.5 mg/kg and 7.5 mg/kg for 42 days and after exposure to minoxidil at the doses of 5 mg/kg and 7.5 mg/kg for 84 days. In addition, initiation of a vacuolation process was observed in the groups exposed to finasteride and the two highest doses of minoxidil (5 mg/kg and 7.5 mg/kg) after 42 days. This initial vacuolation process was also observed after 84 days of exposure to finasteride and all doses of minoxidil tested **(Figure 1)**.

Detached cells were also found in the epididymal lumen of all animals after 42 days **(Table 2 and Figure 2)** and 84 days **(Table 3 and Figure 3)** of exposure. After 42 days of treatment, epithelial vacuolization was observed in the epididymal corpus region of animals treated with finasteride and the highest dose of minoxidil. However, after 84 days, corpus epithelial vacuolization was observed only after treatment with the highest dose of minoxidil.

In addition, hypertrophy of clear cells was observed in the cauda region of the epididymis after administration of finasteride and all doses of minoxidil for 42 days. However, after 84 days of exposure, hypertrophy of clear cells was observed in the corpus region in animals treated with the vehicle, minoxidil 2.5 mg/kg, and minoxidil 5 mg/kg, and in the caput epididymis region in animals exposed to the lowest dose of minoxidil. Cryptic epithelium (i.e., irregular epithelial folds, loss of cellular cohesion, or atypical morphology that made it difficult to clearly identify the normal structure) was observed in the epididymis cauda after 84 days of exposure to vehicle and minoxidil at doses of 2.5 mg/kg and 7.5 mg/kg. Semi-quantitative data from the histopathological analyses of the epididymis are shown in the **Supplementary Table 3** (42 days) and **Supplementary Table 4** (84 days).

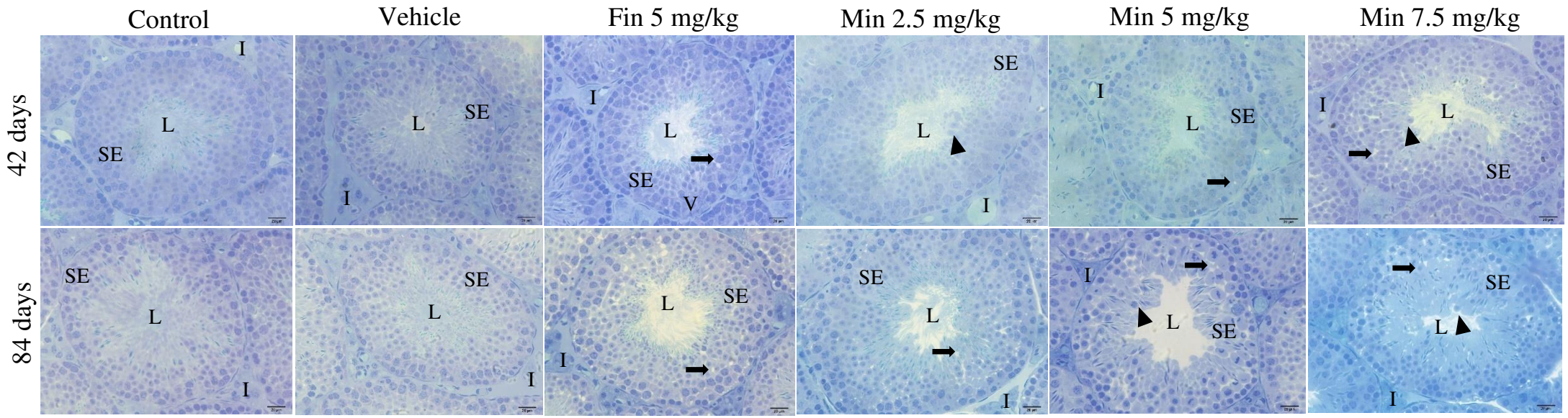

Figure 1. Representative testicular photomicrographs of Balb/C mice orally exposed to finasteride and minoxidil for 42 and 84 days. L – Lumen, SE - Seminiferous epithelium, I – Intertubular space, Arrow – Vacuoles, Arrowhead – Immature germs cells in the lumen. 3 µm section stained with toluidine blue. Scale bars 20 µM

Table 2. Main histopathological findings in the epididymis after exposure to finasteride and minoxidil for 42 days

	Initial segment	Caput Region	Corpus Region	Cauda Region
Control	None	None	Detached cells in the lumen	Detached cells in the lumen
Vehicle	None	Detached cells in the lumen	Detached cells in the lumen	Detached cells in the lumen
Finateride 5 mg/kg	None	Detached cells in the lumen	Detached cells in the lumen Epithelial vacuolization	Detached cells in the lumen Epithelial vacuolization Hypertrophy of clear cells
Minoxidil 2.5 mg/kg	None	Sperm's reduction in the lumen	Detached cells in the lumen	Detached cells in the lumen Hypertrophy of clear cells
Minoxidil 5 mg/kg	None	None	Detached cells in the lumen	Hypertrophyy of clear cells
Minoxidil 7.5 mg/kg	None	Detached cells in the lumen	Detached cells in the lumen Epithelial vacuolization	Detached cells in the lumen

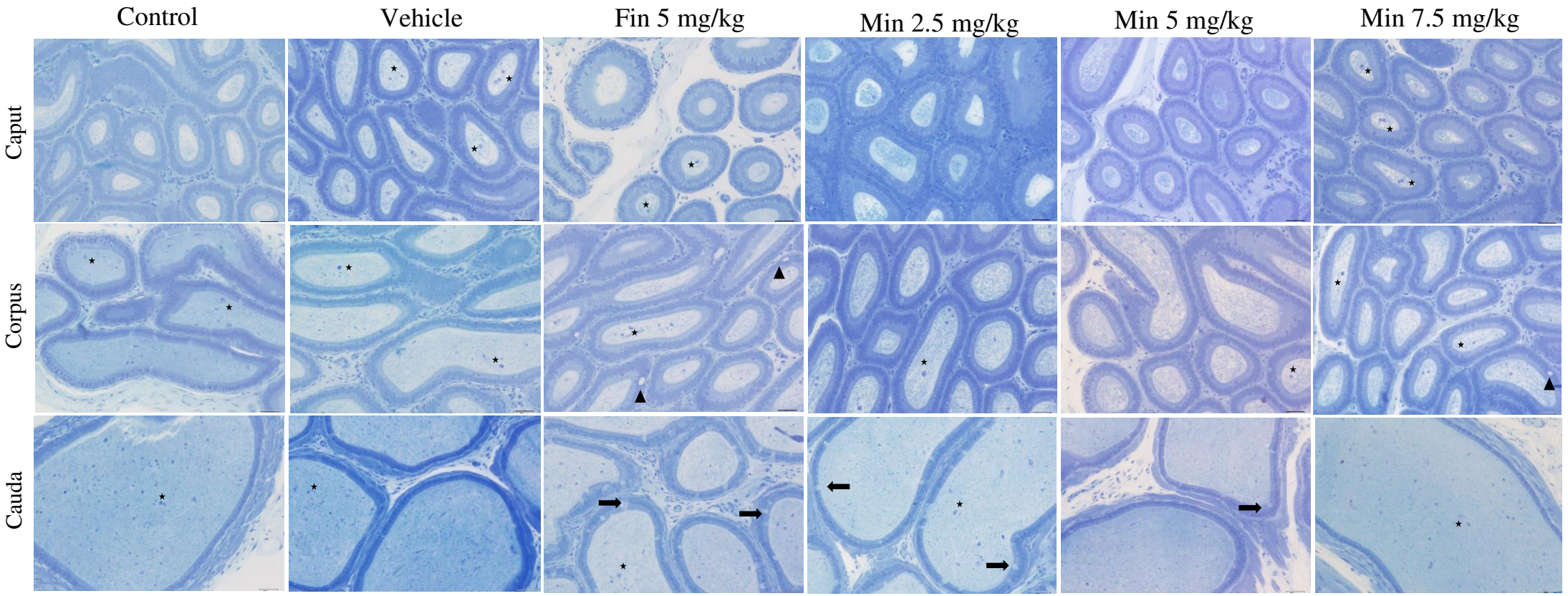

Figure 2. Representative epididymis photomicrographs of Balb/C mice orally exposed to finasteride and minoxidil for 42 days. Fin – Finasteride, Min – Minoxidil, Star – Cells in the lumen, arrowhead – vacuoles, arrow – Hypertrophy of clear cells. 3 μm section stained with toluidine blue. Scale bars 20 μM

Table 3. Main histopathological findings in the epididymis after exposure to finasteride and minoxidil for 84 days

	Initial segment	Caput Region	Corpus Region	Cauda Region
Control	None	Detached cells in the lumen	Detached cells in the lumen	Detached cells in the lumen
Vehicle	None	Detached cells in the lumen	Detached cells in the lumen Hypertrophy of clear cells	Cryptic epithelium
Finateride 5 mg/kg	None	Detached cells in the lumen	Detached cells in the lumen	Detached cells in the lumen
Minoxidil 2.5 mg/kg	None	Detached cells in the lumen Hypertrophy of clear cells	Hypertophy of clear cells	Cryptic epithelium
Minoxidil 5 mg/kg	None	Detached cells in the lumen	Detached cells in the lumen Hypertrophy of clear cells	Detached cells in the lumen
Minoxidil 7.5 mg/kg	None	Detached cells in the lumen	Detached cells in the lumen Epithelial vacuolization	Detached cells in the lumen Cryptic epithelium

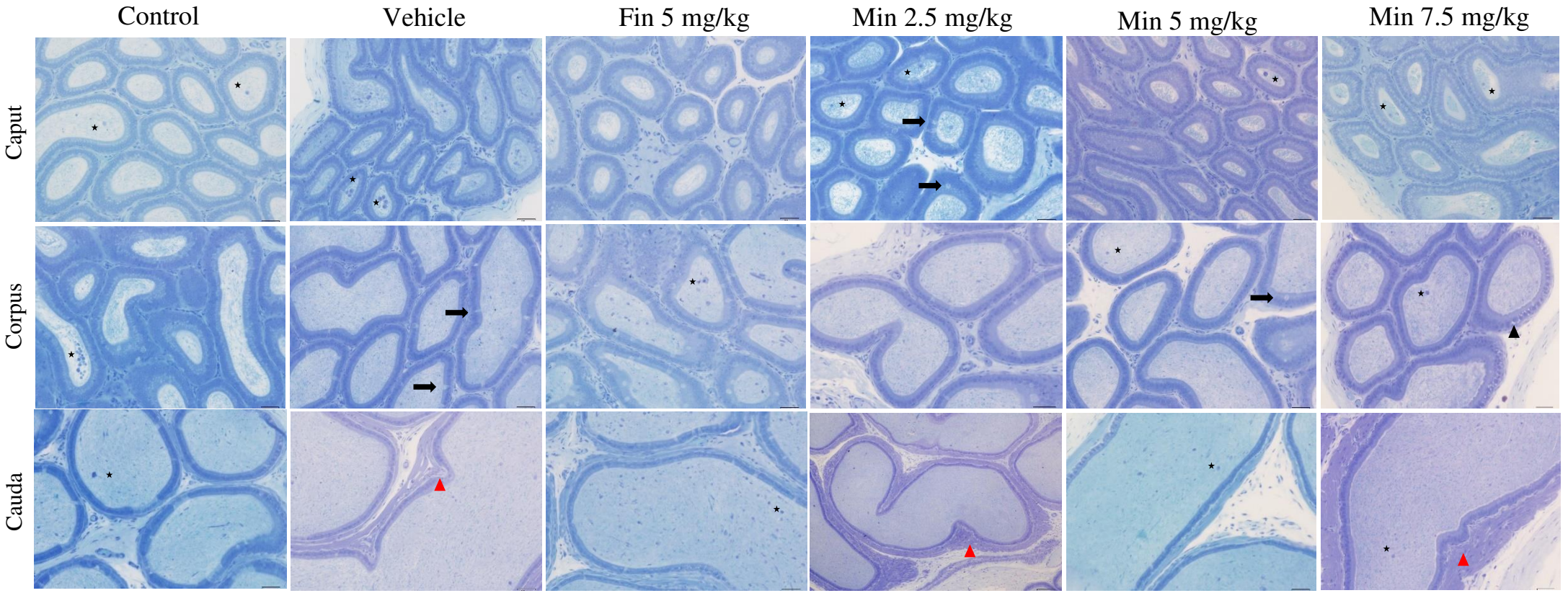

Figure 3. Representative epididymis photomicrographs of Balb/C mice orally exposed to finasteride and minoxidil for 84 days. Fin – finasteride, Min – minoxidil, Star – cells in the lumen, black arrowhead – vacuoles, red arrowhead – cryptic epithelium, arrow – Hypertrophy of clear cells. 3 µm section stained with toluidine blue. Scale bars 20 µM

3.4 *Histomorphometry and stereology revealed several testicular alterations after long-term exposure to finasteride and minoxidil*

3.4.1 Proportion and volume of testicular components

We performed morphometric analyses of the testicular tissue to detect possible changes in testicular morphology. First, we determined the proportions and volumes of the seminiferous tubules, seminiferous epithelium, lumen, tunica propria, and intertubular tissue. No significant changes were observed in the volumes of these components after drug treatments compared to the control group **(Supplementary Figure 1)**.

After 42 days of exposure, a decrease (9.52%) in the percentage of tubules was observed in the group treated with minoxidil 2.5 mg/kg and an increase (47.62%) in the percentage of intertubule in the finasteride group compared to the water control. Compared to finasteride, treatment with minoxidil at 5 mg/kg resulted in a 17.21% increase in lumen proportion, while the 7.5 mg/kg dose led to a 28.09% reduction in intertubular compartment proportion. The proportions of seminiferous epithelium, lumen, and tunica propria did not change significantly after 42 days of exposure to the medicaments compared with water control **(Figure 4)**.

After 84 days of exposure, the percentage of seminiferous tubules decreased in the minoxidil 5 mg/kg (7.69%) and 7.5 mg/kg (4.54%) groups compared to the water control. The percentage of seminiferous epithelium decreased in the finasteride group (7.01%) and the minoxidil 5 mg/kg group (5.96%). The percentage of lumen increased after exposure to finasteride (95.44%). Compared to finasteride, there was a decrease in the percentage of seminiferous tubule (5.30%) and the percentage of lumen (37.50%) in the minoxidil 5mg/kg group. The proportions of the tunica propria did not change significantly after 84 days of exposure to the medicaments compared with water control **(Figure 4)**.

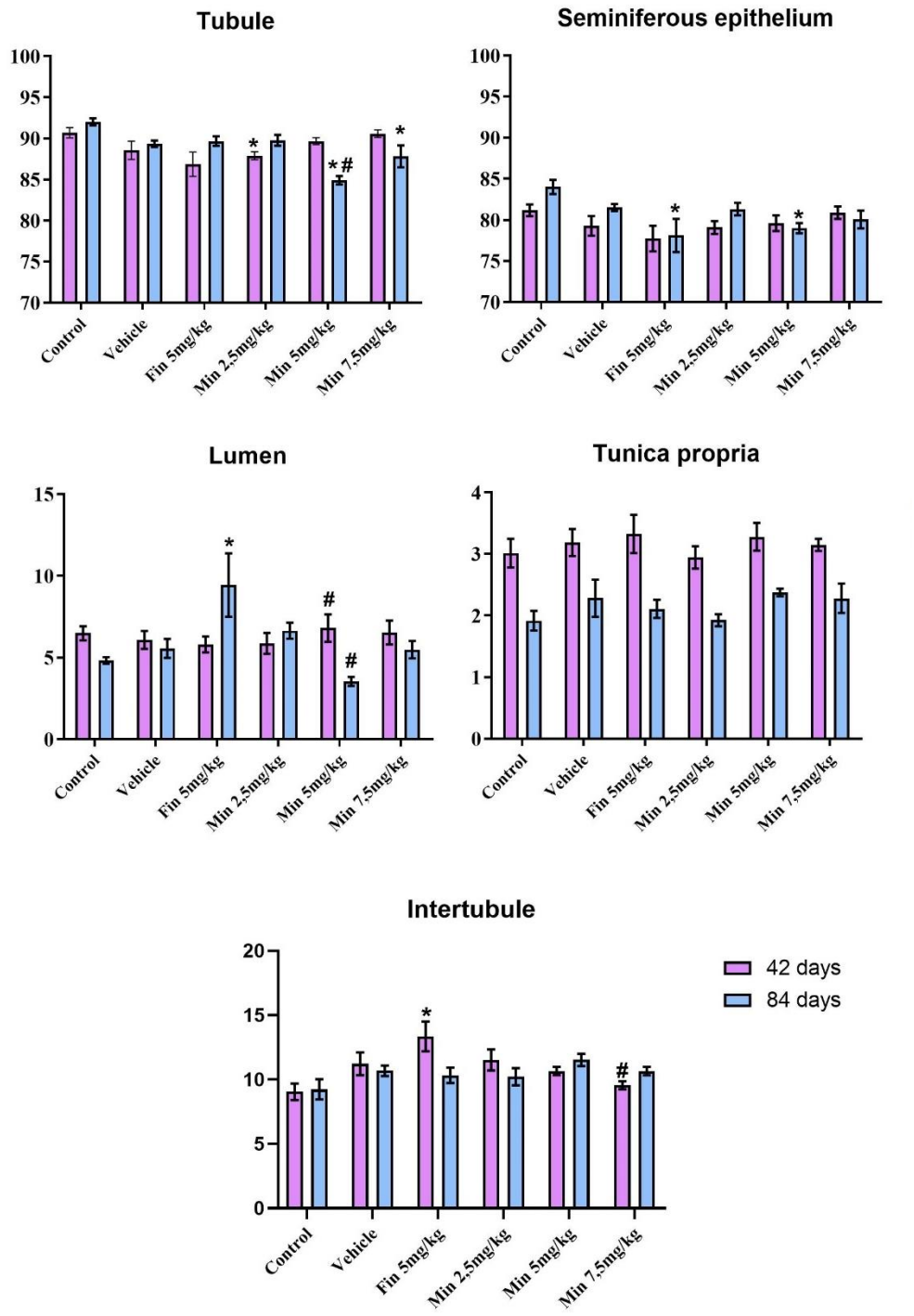

Figure 4. Proportion (%) of seminiferous tubules, seminiferous epithelium, lumen, tunica propria and intertubular tissue of Balb/c mice after long-term exposure to finasteride and minoxidil. Data expressed as mean ± SEM. (*) shows significantly different values compared to control ($p \leq 0.05$) and (#) shows significantly different values compared to finasteride, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. Fin – finasteride, Min – minoxidil. N=6/group.

3.4.2 Histomorphometry of the seminiferous tubules

Next, to investigate the alterations observed in the seminiferous tubules, we analyzed additional morphometric parameters to access whether they could be affected by the exposure to finasteride and minoxidil, as shown in **Table 4**. After 42 days, finasteride and minoxidil 7.5 mg/kg reduced the height of the seminiferous epithelium (by 6.98% and 9.57%, respectively) compared to the water control. However, no significant changes were observed in the other parameters evaluated.

The additional morphometric parameters of the seminiferous tubules were also analyzed after 84 days of exposure and the results are presented in **Table 5**. A reduction in lumen diameter was observed in the minoxidil 5 mg/kg and 7.5 mg/kg groups (44.94% and 43.69%, respectively) compared to the water control. Similarly, lumen area decreased by 70% and 68% in the 5 mg/kg and 7.5 mg/kg groups, respectively. The tubule/ epithelium ratio also declined in both groups, with a 9.48% reduction relative to the water control.

Table 4. Morphometric results of seminiferous tubules of Balb/c mice after long-term exposure to finasteride and minoxidil for 42 days

Parameters	Control	Vehicle	Fin 5 mg/kg	Min 2.5 mg/kg	Min 5 mg/kg	Min 7.5 mg/kg
TSI (%)	0.44±0.09	0.49±0.04	0.31±0.05	0.36±0.04	0.41±0.04	0.43±0.02
EPSI (%)	0.39±0.08	0.44±0.03	0.28±0.05	0.33±0.04	0.36±0.04	0.38±0.02
EH (μm)	84.90±0.48	81.38±1.53	78.97±1.32*	82.93±1.30	83.36±2.27	76.77±2.66*
TD (μm)	212.39±7.72	205.29±3.90	198.14±3.82	206.30±4.20	215.24±9.00	201.04±6.05
LD (μm)	42.59±6.94	42.53±2.80	40.21±2.65	40.42±1.82	48.52±7.07	47.51±5.99
TL (m)	4.18±0.77	4.62±0.42	3.68±0.70	3.81±0.39	4.09±0.28	5.06±0.48
TL/gT (m)	19.30±2.36	23.13±1.00	21.48±1.36	19.70±0.61	18.86±0.60	24.22±2.00
TAr (10^{-2} mm²)	3.56±0.25	3.31±0.12	3.09±0.11	3.34±0.13	3.67±0.30	3.19±0.19
LAr (10^{-2} mm²)	0.16±0.04	0.15±0.02	0.13±0.02	0.13±0.01	0.20±0.06	0.19±0.05
EAr (10^{-2} mm²)	3.40±0.21	3.17±0.11	2.96±0.11	3.21±0.12	3.46±0.25	3.00±0.16
TER	1.04±0.01	1.05±0.01	1.04±0.01	1.04±0.00	1.06±0.03	1.06±0.04

Fin – finasteride/ Min – minoxidil/ TSI - Tubulosomatic index / EPSI - Epitheliosomatic index / EH - Epithelial height / TD - Tubule diameter / LD - Lumen diameter / TL - Total length of seminiferous tubule in the testis / LT/gT - Total length of seminiferous tubule per gram of testis / TAr - Tubule area / LAr - Lumen area / EAr - Epithelial area and TER - Tubule/Epithelium ratio. Data expressed as mean ± SEM. (*) shows significantly different values compared to control ($p \leq 0.05$, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. No differences were found ($p \leq 0.05$) between minoxidil-treated groups and the finasteride group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group.

Table 5. Morphometric results of seminiferous tubules of Balb/c mice after long-term exposure to finasteride and minoxidil for 84 days

Parameters	Control	Vehicle	Fin 5 mg/kg	Min 2.5 mg/kg	Min 5 mg/kg	Min 7.5 mg/kg
TSI (%)	0.49±0.03	0.54±0.04	0.39±0.06	0.40±0.02	0.47±0.04	0.47±0.02
EPSI (%)	0.45±0.03	0.49±0.03	0.34±0.05	0.36±0.02	0.44±0.04	0.43±0.02
EH (μm)	76.58±2.99	81.55±2.35	75.49±2.58	77.80±2.29	80.39±1.80	82.90±1.49
TD (μm)	216.41±4.28	217.34±3.97	203.36±9.66	204.80±5.32	204.42±3.57	210.42±2.45
LD (μm)	79.26±5.35	54.23±3.70	52.38±8.97	49.19±3.22	43.64±3.06*	44.63±2.80*
TL (m)	4.69±0.25	4.87±0.40	4.01±0.67	4.30±0.14	4.80±0.34	5.07±0.30
TL/gT (m)	21.42±1.26	20.10±0.93	22.66±3.04	21.43±0.77	22.61±0.65	21.95±0.89
TAr (10-² mm²)	3.68±0.14	3.71±0.14	3.28±0.30	3.30±0.17	3.29±0.11	3.48±0.08
LAr (10-² mm²)	0.50±0.06	0.24±0.03	0.25±0.07	0.19±0.02	0.15±0.02*	0.16±0.02*
EAr (10-² mm²)	3.18±0.12	3.48±0.13	3.04±0.24	3.10±0.16	3.13±0.10	3.32±0.07
TER	1.16±0.02	1.07±0.01	1.08±0.02	1.07±0.01	1.05±0.01*	1.05±0.01*

Fin – finasteride/ Min – minoxidil/ TSI - Tubulosomatic index / EPSI - Epitheliosomatic index / EH - Epithelial height / TD - Tubule diameter / LD - Lumen diameter / TL - Total length of seminiferous tubule in the testis / LT/gT - Total length of seminiferous tubule per gram of testis / TAr - Tubule area / LAr - Lumen area / EAr - Epithelial area and TER - Tubule/Epithelium ratio. Data expressed as mean ± SEM. (*) shows significantly different values compared to control ($p \leq 0.05$) and (#) shows significantly different values compared to finasteride, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group.

3.4.3 Histomorphometry of the intertubular tissue

To investigate the changes observed in the intertubular testicular compartment, we analyzed the proportion and volume of its components in the testis, including blood vessels, lymphatic space, connective tissue, and Leydig cells (cytoplasm and nucleus). After 42 days of exposure, the percentage of Leydig cell cytoplasm increased in the finasteride (66.89%) and minoxidil 2.5 mg/kg (58.28%) compared to the water control. In addition, the percentage of Leydig cells increased after finasteride (65.17%), minoxidil 2.5 mg/kg (58.10%), and minoxidil 5 mg/kg (38.55%) treatments compared to the water control. The proportion of connective tissue also increased significantly after 42 days of exposure to finasteride (180%), minoxidil at 2.5 mg/kg (153.3%), and 5mg/kg (133.3%) compared to the water control **(Figure 5)**. The proportions and volumes of blood vessels, lymphatic space, and Leydig nuclei did not change significantly after exposure to either drug **(Supplementary Figure 2)**.

After 84 days of exposure, the proportions and volumes in the testis of blood vessels, lymphatic space, and Leydig cells (nucleus and cytoplasm) remained unchanged after exposure to either finasteride or minoxidil **(Supplementary Figure 3).** However, both the percentage and volume of connective tissue increased following exposure to minoxidil at 7.5 mg/kg, by 170% and 83.3%, respectively, and the percentage also increased by 170% after finasteride treatment, compared to the water control **(Figure 5).**

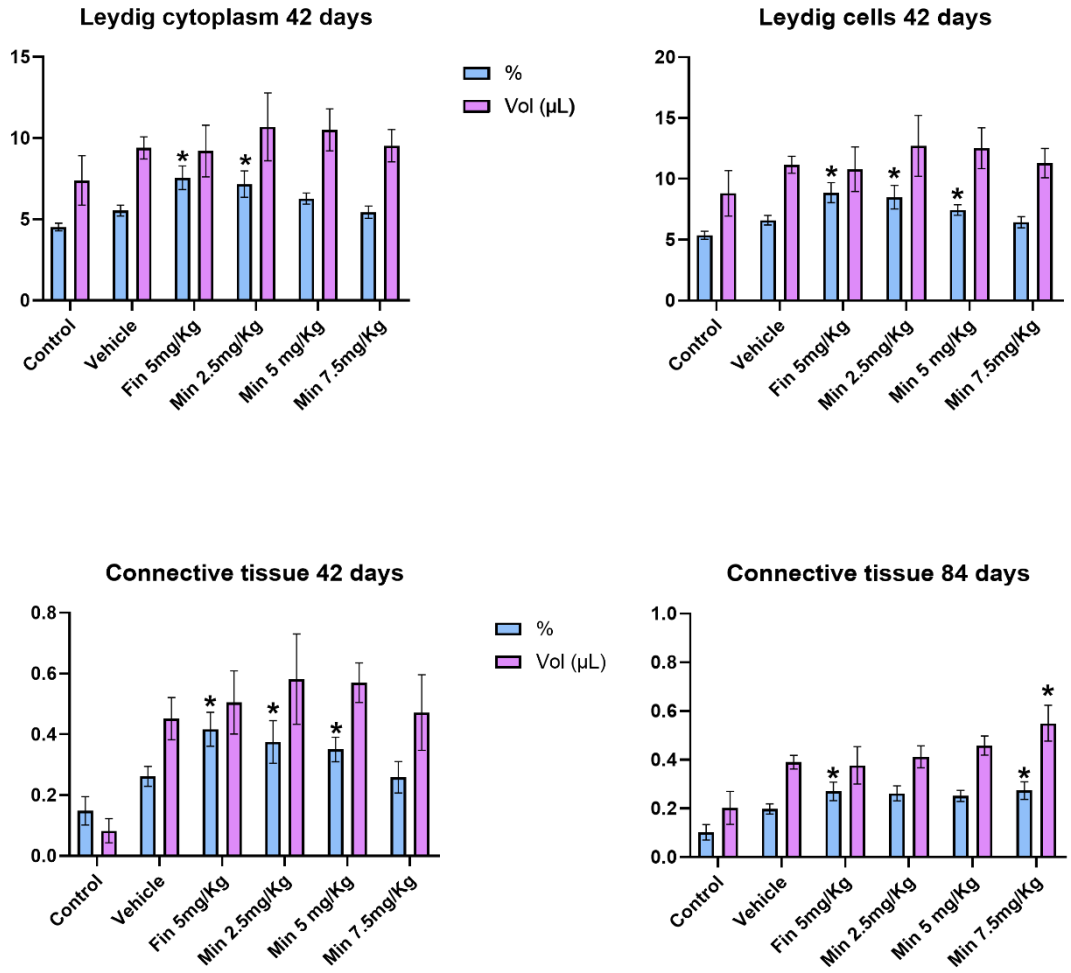

Figure 5. Proportion and volume of Leydig cytoplasm, Leydig cells, and connective tissue in the testis of Balb/c mice after long-term exposure to finasteride and minoxidil. Data expressed as mean ± SEM. (*) shows significantly different values compared to control ($p \leq 0.05$), using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. No differences were found ($p \leq 0.05$) between minoxidil-treated groups and the finasteride group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. Fin – finasteride; Min – minoxidil. N=6/group.

3.5 *Long-term exposure to finasteride and minoxidil induces subtle changes in Leydig cell morphology and density*

Using morphometric analysis, we assessed specific Leydig cell parameters, including nuclear diameter, the volume of individual Leydig cells (nucleus and cytoplasm), total Leydig cell volume, cell count within the testis, and the Leydigosomatic index (relative Leydig cell weight). After 42 days of treatment, finasteride exposure resulted in a 65.29% increase in Leydig cell volume per gram of testis. No significant

changes in these parameters were observed in the groups treated with minoxidil **(Table 6)**.

After 84 days of exposure, most Leydig cell-specific parameters remained unchanged across treatment groups. Finasteride treatment resulted in a 39.44% increase in Leydig cell volume per gram of testis compared to the water control. However, minoxidil at 5 mg/kg significantly elevated the Leydigosomatic index by 53.48% relative to the finasteride group. Furthermore, treatment with minoxidil at 7.5 mg/kg led to a 4.63% increase in total Leydig cell volume in the testis compared to finasteride **(Table 7).**

Table 6. Morphometric and stereological results of Leydig cells from Balb/c mice after exposure to finasteride and minoxidil for 42 days.

Parameters	Control	Vehicle	Fin 5mg/Kg	Min 2.5mg/Kg	Min 5mg/Kg	Min 7.5mg/Kg
LND (μm)	7.08 ± 0.18	6.93 ± 0.10	7.12 ± 0.12	6.86 ± 0.09	6.79 ± 0.16	6.97 ± 0.15
LNV (μm³)	187.59 ± 13.91	175.06 ± 7.97	190.01 ± 9.81	169.13 ± 6.80	165.15 ± 12.21	178.35 ± 12.97
LCV (μm³)	1054.42 ± 104.00	965.89 ± 108.20	1138.87 ± 141.10	941.63 ± 56.34	925.79 ± 90.46	996.84 ± 106.00
LV (μm³)	1242.01 ±113.00	1140.95 ± 107.80	1328.87 ± 149.50	1110.76 ± 55.01	1090.94 ± 97.86	1175.19 ± 118.60
LV/ts (μL)	8.80 ± 1.8	11.15 ± 0.70	10.79 ± 1.82	12.71 ± 2.50	12.52 ± 1.67	11.29 ± 1.20
LV/gt (μL)	39.86 ± 5.14	56.43 ± 2.44	65.82 ± 2.73*	63.85 ±8.13	56.25 ± 3.70	53.91 ± 4.93
LN/ts (10^{6})	7.44 ± 1.76	10.09 ± 0.87	8.50 ± 1.58	11.56 ± 2.25	12.82 ± 3.40	10.35 ± 1.57
LN/gt (10^{6})	33.65 ± 5.56	51.95 ± 5.49	52.36 ± 5.38	58.32 ±7.88	56.33 ± 11.25	48.72 ± 6.59
LSI (%)	26.93± 5.92	36.05 ± 1.90	29.78 ± 4.46	37.05 ±7.40	33.09 ±3.43	30.75 ± 3.38

Fin – Finasteride, Min – Minoxidil, LND - Leydig nuclear diameter, LNV - Leydig nuclear volume, LCV - Leydig cytoplasmic volume, LV - Leydig cell volume, LV/ts - Leydig volume in testis, LV/gt - Leydig volume per gram of testis, LN/ts - Leydig cell number in testis, LN/gt - Leydig cell number per gram of testis, LSI – Leydigosomatic index. Data expressed as mean ± SEM. (*) shows significantly different values compared to control ($p \leq 0.05$), using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. No differences were found ($p \leq 0.05$) between minoxidil-treated groups and the finasteride group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group.

Table 7. Morphometric and stereological results of Leydig cells from Balb/c mice after exposure to finasteride and minoxidil for 84 days.

Parameters	Control	Vehicle	Fin 5mg/Kg	Min 2.5mg/Kg	Min 5mg/Kg	Min 7.5mg/Kg
LND (μm)	7.08 ± 0.15	6.93 ± 0.20	7.12 ± 0.10	6.86 ± 0.13	6.79 ± 0.09	6.97 ± 0.07
LNV (μm^3)	187.59 ± 9.22	175.06 ± 13.55	190.01 ± 7.40	169.13 ± 8.61	165.15 ± 6.04	178.35 ± 4.80
LCV (μm^3)	1054.42 ± 172.50	965.89 ± 304.80	1138.87 ± 99.02	941.63 ± 183.80	925.79 ± 108.80	996.84 ± 170.90
LV (μm^3)	1242.01 ±177.40	1140.95 ± 312.70	1328.87 ± 91.89	1110.76 ± 184.60	1090.94 ± 109.20	1175.19 ± 173.90
LV/ts (μL)	8.80 ± 1.39	11.15 ± 0.80	10.79 ± 1.47	12.71 ± 1.01	12.52 ± 1.06	11.29 ± 1.35$^{\#}$
LV/gt (μL)	39.86 ± 4.82	56.43 ± 4.54	65.82 ± 4.71*	63.85 ±6.73	56.25 ± 5.94	53.91 ± 2.41
LN/ts ($10^{6)}$	7.44 ± 1.08	10.09 ± 0.83	8.50 ± 0.95	11.56 ± 0.37	12.82 ± 0.70	10.35 ± 0.70
LN/gt ($10^{6)}$	33.65 ± 4.04	51.95 ± 4.14	52.36 ± 3.30	58.32 ±1.97	56.33 ± 3.41	48.72 ± 3.35
LSI (%)	34.60± 3.97	40.75 ± 3.18	28.27 ± 4.35	32.79 ±3.46	43.39 ±3.79$^{\#}$	41.94 ± 2.80

Fin – Finasteride, Min – Minoxidil, LND - Leydig nuclear diameter, LNV - Leydig nuclear volume, LCV - Leydig cytoplasmic volume, LV - Leydig cell volume, LV/ts - Leydig volume in testis, LV/gt - Leydig volume per gram of testis, LN/ts - Leydig cell number in testis, LN/gt - Leydig cell number per gram of testis, LSI – Leydigosomatic index. Data expressed as mean ± SEM. (*) shows significantly different values compared to control ($p \leq 0.05$) and (#) shows significantly different values compared to finasteride, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group.

3.6 Effects of long-term finasteride and minoxidil exposure on antioxidant enzyme activity and markers of oxidative stress in the testis and epididymis

We then analyzed the activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST), and the levels of oxidative stress markers, including malondialdehyde (MDA), nitric oxide (NO), and protein carbonyl (CP), in the testes and epididymis of animals exposed to finasteride and minoxidil for 42 or 84 days.

After 42 days of exposure, the highest dose of minoxidil decreased the CAT activity (89.31%) in the testis compared to the water control. The activities of SOD and GST were not significantly affected by the drugs, nor were the levels of MDA and NO. In contrast, CP levels were significantly increased after administration of minoxidil at 2.5 mg.kg and 5 mg/kg (246.8% and 163.3%, respectively) compared to finasteride **(Figure 6)**.

In the epididymis, the 42-day exposure did not significantly change the activity of antioxidant enzymes or the levels of NO and CP compared to the water control. However, minoxidil at 5 mg/kg decreased MDA levels (69.65%) compared to the water control. Compared to finasteride, minoxidil 5 mg/kg and 7.5 mg/kg decreased CP levels (58.91% and 58.61%) **(Figure 7)**.

After 84 days of exposure to either finasteride or minoxidil, neither the activity of antioxidant enzymes nor the levels of oxidative stress markers were affected in the testis **(Supplementary Figure 4)** and epididymis **(Supplementary Figure 5).**

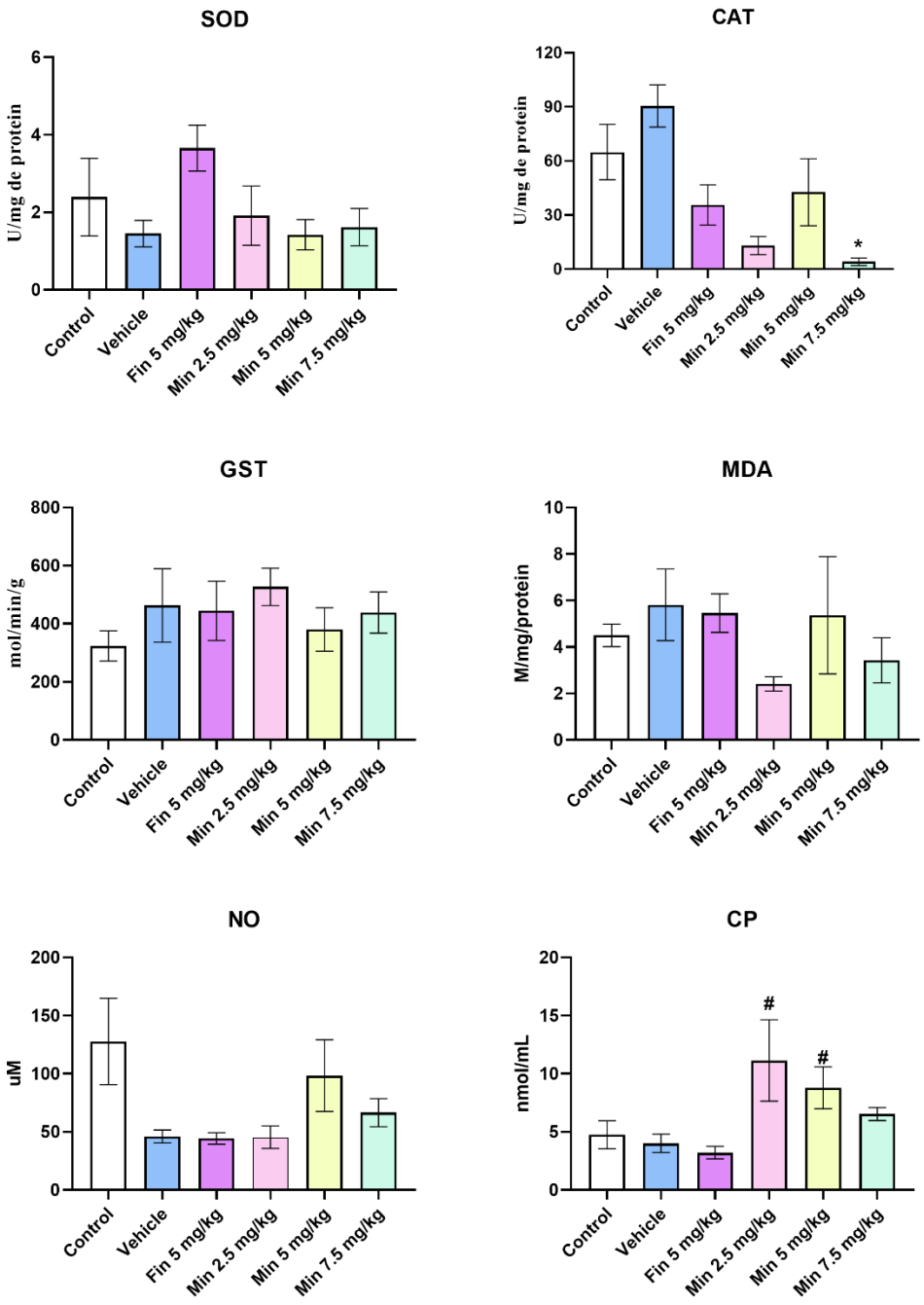

Figure 6. Activity of antioxidant enzymes and oxidative stress markers in the testes of Balb/c mice exposed to finasteride and minoxidil for 42 days. Fin – Finasteride, Min – Minoxidil, SOD – superoxide dismutase, CAT – catalase, GST – glutathione S-transferase, MDA – malondialdehyde, NO – nitric oxide, CP – carbonyl protein. Data expressed as mean ± SEM. (*) shows significantly different values compared to control ($p \leq 0.05$) and (#) shows significantly different values compared to finasteride, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group.

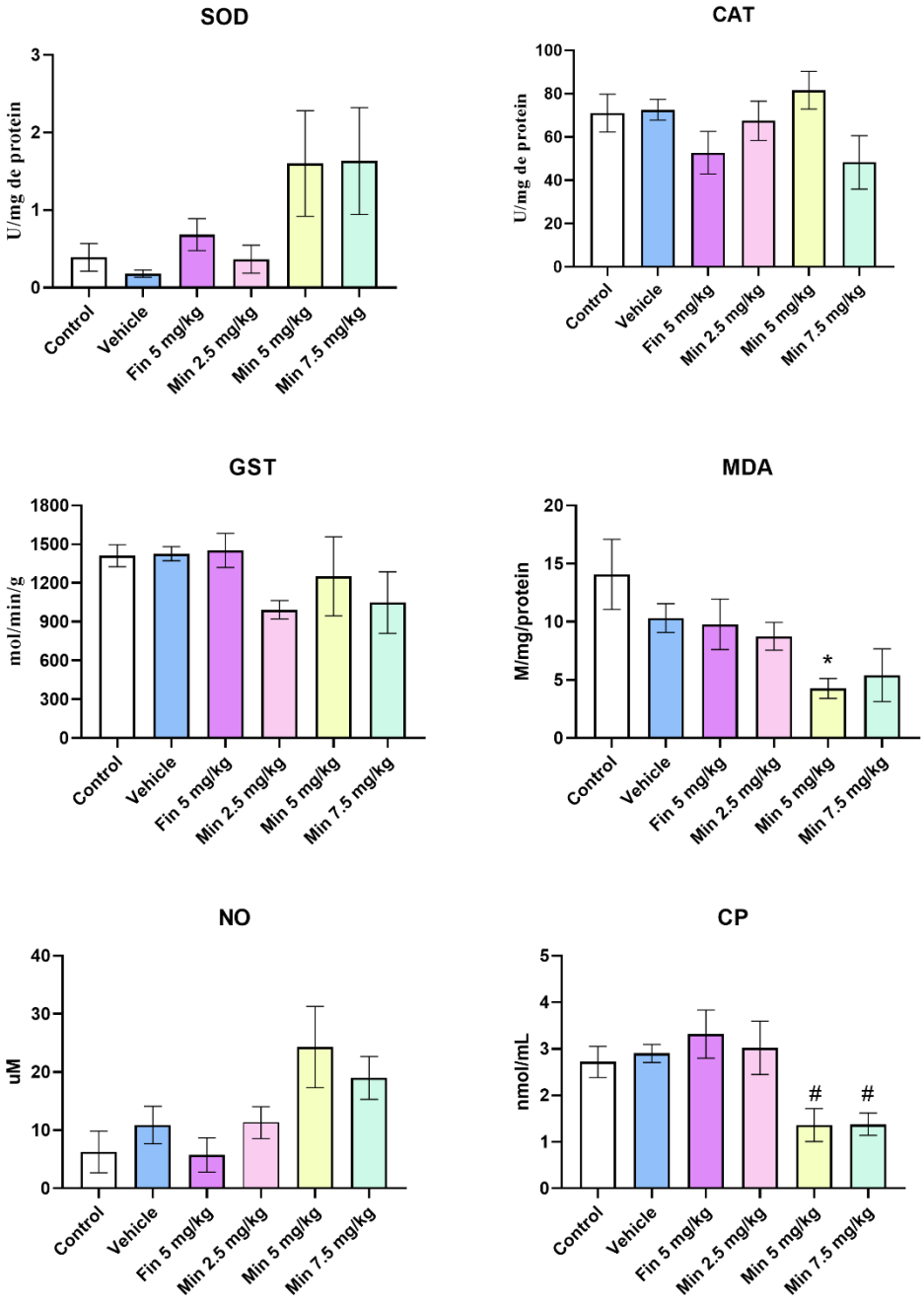

Figure 7. Activity of antioxidant enzymes and oxidative stress markers in the epididymis of Balb/c mice exposed to finasteride and minoxidil for 42 days. Fin – Finasteride, Min – Minoxidil, SOD – superoxide dismutase, CAT – catalase, GST – glutathione S-transferase, MDA – malondialdehyde, NO – nitric oxide, CP – carbonyl protein. Data expressed as mean ± SEM. (*) shows significantly different values compared to control ($p \leq 0.05$) and (#) shows significantly different values compared to finasteride, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group.

3.7 *Sperm count, motility, and transit time in the epididymis after long-term exposure to finasteride and minoxidil*

To investigate whether the changes found in the testis and epididymis could affect the sperm production and quality, we next determined sperm count and transit time in the epididymal regions (caput/corpus and cauda). We also evaluated total sperm transit time in the epididymis and assessed sperm motility.

As a result, finasteride increased the total sperm transit time in the epididymis after 42 and 84 days of exposure (21.37% and 13.98%, respectively) compared to the water control **(Table 8)**. In addition, finasteride specifically decreased the number of sperm in the cauda (per gram of the organ) (27.94%) after 84 days of exposure. The other sperm parameters, such as sperm number in the caput/corpus and sperm motility, were not significantly affected **(Supplementary Table 5)**.

The 42-day and 84-day exposures to minoxidil did not significantly affect any of the sperm parameters examined (**Table 8** and **Suppl Table 5**). However, compared to finasteride, minoxidil at 2.5 mg/kg significantly reduced the total sperm transit time by 13.46% after 84 days of exposure. Additionally, minoxidil at doses of 5 mg/kg and 7.5 mg/kg decreased the number of spermatozoa in the caput/corpus regions by 20.38% and 19.48%, respectively, while increasing the sperm count in the cauda region by 37.85% and 37.50%, relative to finasteride.

Table 8. Sperm analyses in the epididymis of Balb/c mice after long-term exposure to finasteride and minoxidil

	Control	Vehicle	Fin 5 mg/kg	Min 2.5 mg/kg	Min 5 mg/kg	Min 7.5 mg/kg
42 days						
Caput/corpus epididymis sperm number ($\times 10^6$/g organ)	181.00±19.46	196.56±30.61	199.38±5.21	205.79±15.66	218.80±11.50	170.75±15.20
Cauda epididymis sperm number ($\times 10^6$/g organ)	490±44.57	435.63±26.80	535.31±50.30	499.0±22.85	487.5±34.04	395.0±19.74
Total transit time (days)	7.72±0.51	8.28±0.27	9.37±0.19*	8.26±0.56	8.30±0.41	8.55±0.08
84 days						
Caput/corpus epididymis sperm number ($\times 10^6$/g organ)	198.25±14.01	220.9±17.02	192.81±7.02	161.00±5.48	153.50±5.22#	155.25±5.59#
Cauda epididymis sperm number ($\times 10^6$/g organ)	490.50±28.70	526.56±25.67	353.44±5.62*	411.75±31.07	487.25±24.07#	486.0±24.01#
Total transit time (days)	8.08±0.19	7.99±0.10	9.21±0.04*	7.97±0.20#	8.35±0.26	8.53±0.19

Fin – Finasteride, Min – Minoxidil. Data expressed as mean ± SEM. (*) shows significantly different values in relation to the control ($p \leq 0.05$) using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. No differences were found ($p \leq 0.05$) between minoxidil-treated groups and the finasteride group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group.

4 DISCUSSION

Despite the increasing *off-label* use of oral minoxidil to treat AGA, its potential adverse male reproductive effects remain under-investigated. Thus, this study represents the first comprehensive investigation comparing the testicular and epididymal effects of oral minoxidil and finasteride, specifically focusing on hormonal, histopathological, and oxidative stress parameters. Here, we provide evidence that long-term exposure to minoxidil affects testicular architecture by inducing epithelial detachment and vacuolization, among other morphometrical changes such as decreased seminiferous epithelium height and seminiferous tubule percentage, possibly due to oxidative stress in the sub-chronic exposure. Although minoxidil exposure induced subtle changes in Leydig cell morphology and density, we did not find significant changes in testosterone levels. In contrast, estradiol levels were reduced after 42 days of minoxidil exposure. The epididymis was also a target of minoxidil effects as evidenced by histological changes such as epithelial vacuolization and the presence of detached cells in the lumen. Alongside oxidative stress, the observed decrease in estradiol levels may contribute to the testicular and epididymal morphological alterations, as estradiol plays a crucial role in maintaining male reproductive homeostasis (SCHULSTER et al., 2016).

Although testosterone levels were not altered after finasteride and minoxidil exposure, our results indicate a trend toward decreased 17β-estradiol levels with finasteride (5 mg/kg), whereas minoxidil (2.5 mg/kg) caused a significant reduction after 42 days of exposure. In the testes, the enzyme responsible for the conversion of testosterone to estrogen is P450arom, encoded by the CYP19 gene. In adults, this enzyme is predominantly found in Leydig cells, making them a major source of estrogen synthesis (PAYNE & HARDY, 2007). Powlin et al. (1998), in an *in vitro* study using rat testicular parenchyma cells, found a decrease in estradiol levels after exposure to finasteride. The authors suggested that this decrease may be due to cytotoxicity. This effect could also occur after minoxidil exposure, or minoxidil could potentially directly interfere with aromatase activity. In either case, minoxidil treatment may be associated with endocrine dysregulation, and a decrease in estrogen could negatively impact fertility, as normal estrogen levels are essential for spermatogenesis and maintaining a good sperm concentration (LUCAS et al. 2011). While the hormonal alterations observed at 42 days were not sustained at 84 days, indicating a potential lack of long-term hormonal effects, this does not preclude sustained structural and functional damage to the testes and epididymis that has already occurred.

Although no changes in testicular weight were observed, histological analyses revealed that the seminiferous tubules were affected by the administration of finasteride and minoxidil, as decreased in its proportion (minoxidil doses of 2.5 mg/kg after 42 days, and 5 mg/kg and 7.5 mg/kg after 84 days). Our morphometrical findings revealed that specifically the epithelium seminiferous were the target in the seminiferous tubules (not the lumen), in which a decrease in seminiferous epithelial height was observed after 42 days of exposure to finasteride and the highest doses of minoxidil, compared to the negative water control. Studies suggest that a decrease in epithelial height may indicate tubular cell degeneration, with a loss of epithelial cells, and a decrease in the number of haploid cells (secondary spermatocytes and spermatids) (WRIGHT et al., 1996). Indeed, our histopathological analysis showed the onset of vacuolization (after 42 days of exposure to the 5 mg/kg and 7.5 mg/kg doses of minoxidil and after 84 days of exposure to all doses of minoxidil) as well as cell detachment (after 42 days of exposure to 2.5 mg/kg and 7.5 mg/kg and after 84 days of exposure to 5 mg/kg and 7.5 mg/kg) in minoxidil-treated animals, supporting this hypothesis.

Based on our previous systematic review (SANTANA et al., 2023a) regarding the effects of minoxidil on male reproductive organs, the drug may impair testicular function through several mechanisms, particularly by inducing oxidative stress. Our findings demonstrated a decrease in catalase (CAT) activity in animals exposed to 7.5 mg/kg of minoxidil for 42 days. Furthermore, elevated levels of carbonylated proteins (CPs) were observed in the groups treated with 2.5 and 5 mg/kg, exceeding those of the finasteride and water control groups, suggesting that CP accumulation may serve as a more sensitive marker of minoxidil-induced oxidative damage. Catalase plays a key role in the antioxidant defense system by decomposing hydrogen peroxide (H_2O_2) into water and oxygen (PAITHANKAR et al., 2021), thereby reducing the availability of H_2O_2 for conversion into highly reactive hydroxyl radicals via the Fenton reaction. These radicals promote lipid peroxidation, leading to membrane damage and cellular dysfunction (HALLIWELL & CHIRICO, 1993; IGHODARO & AKINLOYE, 2017). Protein carbonylation indicates irreversible oxidative modifications through the introduction of aldehyde and ketone groups into protein side chains (LEVINE et al., 1994). Aldehydes can arise as secondary products of lipid peroxidation, these alterations impair protein structure and function, leading to enzyme inactivation, aggregation, and impact the proteolytic degradation (AKAGAWA, 2020; SANTOS et al., 2023). Although Nguyen and Donaldson (2005) reported that CAT retained considerable enzymatic activity despite high

levels of carbonylation, in biological systems oxidized proteins are usually targeted for degradation (GRUNE et al., 1997). Thus, under chronic or severe oxidative stress, carbonylation may contribute to the functional depletion of CAT, compromising the redox balance. Together, lipid peroxidation and protein carbonylation make mitochondrial membranes and cytoplasmic proteins highly vulnerable to damage. In metabolically active and proliferative tissues such as the testis, oxidative stress can be particularly harmful (TURNER & LYSIAK, 2008), impairing spermatogenesis through Leydig cell dysfunction—which affects testosterone production—and disruption of the blood–testis barrier (WALKE et al., 2023). These mechanisms likely explain the morphological changes observed in this study.

Finasteride has previously been reported to induce testicular damage through oxidative stress (KOLASA et al., 2008; OLAYINKA et al., 2020). Similarly, a study by Ozturk et al. (2020) demonstrated that minoxidil administration to the testes of Wistar rats led to oxidative stress and histomorphological alterations in the seminiferous tubules. In the present study, oxidative stress appears to occur earlier, as redox imbalances observed at 42 days of exposure were no longer present at 84 days, despite the persistence of morphological consequences. Moreover, estrogen has been recognized as an antioxidant, acting as a scavenger of reactive oxygen species (ROS) and modulating the expression of antioxidant enzymes (CHAINY & SAHOO et al., 2019). In this study, a decline in 17β-estradiol levels was confirmed following exposure to 2.5 mg/kg of minoxidil, which may be associated with the observed redox imbalance.

Our morphometric results showed that finasteride and minoxidil at 2.5 mg/kg increased the proportion of Leydig cells in the testis after 42 days of exposure, and specifically the proportion of Leydig cell cytoplasm after treatment with finasteride and minoxidil at 2,5 and 5 mg/kg. In the finasteride group, this change was also reflected in an increase in the proportion of the intertubular compartment in the testis and in the volume of Leydig cell per gram of testis. Although the mean volumes of individual Leydig cells—including the nucleus, cytoplasm, and total cell volume—did not change and showed no trend toward alteration, there was a non-significant trend toward an increased number of Leydig cells per gram of testis in all treated groups after 42 days of exposure. Tash et al. (2002) suggested that an apparent increase in Leydig cells (hyperplasia) can sometimes be attributed to seminiferous tubule atrophy, which results in Leydig cells appearing more densely packed rather than truly proliferating. However, Prahalada et al. (1994) demonstrated that mice treated with 250 mg/kg/day of finasteride

exhibited a higher incidence of diffuse Leydig cell hyperplasia. The main cause of Leydig cell hyperplasia is primary hypogonadism, which results from disorders in which the testes no longer respond to LH and FSH, leading to a cessation of testosterone production. However, since no decrease in testosterone levels was observed in this study, only a decrease in estrogen, an alternative hypothesis could be the relationship between TGF-β1 and estrogen. Liu et al. (2013) showed that TGF-β1 has a significant inhibitory effect on estrogen production by rat Leydig cells, possibly through the negative regulation of aromatase gene expression and activity. Tomsig and Turner (2006) and Rocio et al. (2013) suggest that TGF-β1 is involved in morphogenetic processes such as growth and is known to affect both Leydig and Sertoli cells in the testis by activating the JNK, p38 MAPK and ERK pathways, which are also involved in the processes of hyperplasia. However, a study demonstrated that minoxidil inhibits the TGF-β1/Smad3 signaling pathway in the lung tissue of mice (SHAO et al., 2018). Whether a similar regulatory effect occurs in testicular tissue remains to be elucidated.

Similarly, our findings indicate that minoxidil treatment increased the percentage of connective tissue in the testis at doses of 2.5 and 5 mg/kg after 42 days, while a significant increase in connective tissue volume was also observed at the 7.5 mg/kg dose after 84 days of exposure. The connective tissue in this region contains immune cells and fibroblasts, which are responsible for producing the extracellular matrix—predominantly composed of collagen. However, this increase in connective tissue may not be primarily attributed to a fibrotic process, as previous studies have shown that minoxidil can suppress collagen synthesis (MURAD & PINNELL, 1987; LACHGAR et al., 1996; SHAO et al., 2018). Although an elevation in the cellular component of the connective tissue could suggest an inflammatory response, our histomorphological analyses did not reveal any clear signs of inflammation. Notably, the observed reduction in seminiferous epithelium height after 42 days of exposure suggests degenerative changes in either germ cells or Sertoli cells. While the tubular diameter remained unaltered, epithelial thinning may have contributed to a relative expansion of the interstitial space. This interstitial enlargement may, in turn, account for the observed trend toward Leydig cell hyperplasia and connective tissue accumulation, as interstitial expansion is commonly associated with tubular regression in certain experimental models (JEMERIN, 1937). Adamczewska et al. (2022) emphasized that, under physiological conditions, the testicular interstitial compartment is primarily composed of Leydig cells and blood vessels. However, in individuals with impaired spermatogenesis, this compartment becomes markedly expanded due

to excessive deposition of fibrotic connective tissue. The same review referenced histological findings from testicular biopsies of men with AZFa deletions—a genetic mutation strongly associated with non-obstructive azoospermia (NOA) and severe spermatogenic failure. These samples exhibited a combination of features, including normal to thickened tunica propria, reduced seminiferous tubular diameter, enlarged intratubular spaces, Leydig cell hyperplasia, and histological patterns consistent with Sertoli cell-only syndrome. Therefore, the coexistence of these alterations in our model may reflect testicular remodeling induced by minoxidil exposure.

The epididymis was also a target of histomorphological alteration such as epithelial detachment, vacuolization, and clear cell hypertrophy induced by 42 and 84-days exposure to finasteride and/or minoxidil. In addition, finasteride exposure increased total sperm transit time and decreased the number of sperm in the cauda. Despite these changes, sperm motility remained unaffected across all treatments. Histological evidence suggests that damage to the epididymal epithelium—particularly the basal cell layer—is closely linked to abnormal sperm morphology (CUPPS & BRIGGS, 1957). Zhou et al. (2010) reported that chemical exposure in rats induces dose-dependent epididymal damage by disrupting antioxidant defenses, thereby compromising epithelial integrity and the environment required for sperm maturation. In the present study, although epithelial detachment and vacuolization were observed after 42 days of minoxidil treatment, MDA levels were reduced in the group treated with 5 mg/kg/day compared to controls. This apparent discrepancy may be explained by a compensatory upregulation of antioxidant systems, which could have mitigated oxidative stress and consequently lowered lipid peroxidation. Supporting this hypothesis, SOD activity—though not statistically significant—was higher in groups with reduced MDA levels.

Clear cells, which are characterized by their apical region containing vesicles, lysosomes, and lipid droplets, play an important role in absorbing proteins secreted by the epithelium and cytoplasmic droplet contents, particularly in the cauda of the epididymis (JOSEPH et al., 2011). These cells are known to become abnormally large and filled with lysosomes when the normal function of the testis and epididymis is disrupted (ROBAIRE & HINTON, 2002). A potential contributing factor to this phenomenon may be estrogen deficiency. In our study, we observed a decrease in serum estradiol levels after 42 days of minoxidil treatment. Joseph et al. (2011) and Hess et al. (2001) explain that estrogen regulates the function of the epididymal epithelium, specifically through estrogen receptors (ESR1 and

ESR2) and genes such as aquaporin 9 (AQP9), which are expressed in specific epithelial cells, including the clear cells. ESR1 deficiency or antiestrogen treatment significantly reduces AQP9 expression, which may affect cell morphology.

In terms of daily sperm production, sperm count, epididymal sperm transit time and sperm motility, finasteride appeared to have a more aggressive effect than minoxidil. A review by Robaire and Henderson (2006) had already provided evidence that inhibition of 5α-reductase, as promoted by finasteride, reduces the conversion of testosterone to dihydrotestosterone (DHT), a hormone that plays a key role in sperm maturation, storage, and transit. Garcia et al. (2012) demonstrated showed that after treating rats with 5 mg/kg finasteride for 56 days, a significant reduction in DHT levels was associated with decreased sperm motility and a lower percentage of live sperm. The increase in sperm transit time observed in our study following finasteride administration could also increase the risk of negative effects of ROS as well as insufficient maturation time, leading to lower sperm quality (FERNANDEZ et al., 2008).

Furthermore, Meistrich et al. (1975) studied the effects of estradiol on sperm transit time in the epididymis of mice and found that estradiol alone was effective in reducing sperm transit time. Since we observed a reduction in estradiol levels in the minoxidil-treated animals, and non-significant increase in transit time in days, it is possible that this reduction contributed to the delayed sperm transit between the testis and the epididymis. It is also important to note that some studies, such as Guimaraes-Ervilha (2024), have shown that hypertension can reduce sperm transit time. Given that minoxidil is primarily a vasodilator, it could alter blood flow to the epididymis and affect the local environment necessary for sperm distribution and maturation. However, there is insufficient evidence in this study to conclude that minoxidil has a direct effect on blood flow to the epididymis. Despite the increased in transit time, sperm motility remained unaffected by either finasteride or minoxidil at the doses tested.

5 CONCLUSIONS

This study offers a comprehensive evaluation of the long-term effects of minoxidil on testicular and, for the first time, epididymal morphology and oxidative status in mice, shedding light on its potential to compromise male reproductive health. After 42 and 84 days of exposure, minoxidil led to a reduction in the proportion of seminiferous tubules, along with epithelial detachment and vacuolization. Although no significant alterations were observed in

testosterone levels, estradiol concentrations were notably decreased, particularly at 42 days. Additionally, minoxidil exposure was associated with a trend toward Leydig cell hyperplasia and increased interstitial connective tissue, suggesting ongoing structural remodeling. These alterations coincided with markers of oxidative stress, including reduced catalase activity and elevated levels of carbonylated proteins (CP), indicating that redox imbalance may be a key driver of histopathological changes.

Compared to finasteride—which predominantly impaired sperm count and transit time—minoxidil induced a broader and more extensive spectrum of tissue damage, particularly in the testis and epididymis. Notably, the most severe effects were observed at 84 days with 5 mg/kg minoxidil, marked by persistent degeneration and interstitial expansion. However, early oxidative damage was also evident at 42 days, especially at higher doses, suggesting an acute onset of toxicity. It is important to note, as demonstrated by studies such as Mouro et al. (2019), that chronic low-dose exposure to toxicants can sometimes result in more pronounced testicular damage than single high-dose exposures. This paradox may stem from the inability of lower doses to activate adaptive cellular responses, such as antioxidant defenses or protective autophagy, thereby allowing the gradual accumulation of reactive intermediates and cellular injury over time. This concept may help explain why the highest dose of minoxidil did not always correlate with the greatest damage at later time points.

In conclusion, while finasteride primarily disrupts functional parameters of sperm maturation, minoxidil exerts more profound structural and oxidative damage within male reproductive tissues. These findings provide new insights into the reproductive toxicity of minoxidil, particularly its understudied impact on the epididymis, and underscore the role of estradiol depletion and oxidative imbalance in its mechanism of action. The results hold important implications for the safe clinical use of minoxidil and emphasize the need for further investigation into potential protective strategies.

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7 SUPPLEMENTAL MATERIAL

Supplementary table 1. Body and reproductive organs parameters of male Balb/c mice after chronic exposure to finasteride and minoxidil for 42 days.

	Control	Vehicle	Fin 5 mg/kg	Min 2.5 mg/kg	Min 5 mg/kg	Min 7.5 mg/kg
BW (g)	33.14±1.83	30.99±1.46	35.71±1.71	34.32±0.74	37.31±1.32	36.88±1.08
TW (g)	0.20±0.03	0.20±0.01	0.17±0.02	0.20±0.02	0.22±0.02	0.21±0.01
AW (g)	0.05±0.01	0.03±0.01	0.04±0.01	0.05±0.01	0.05±0.01	0.04±0.01
PW (g)	0.16±0.02	0.17±0.01	0.13±0.02	0.15±0.01	0.17±0.02	0.17±0.01
EW (g)	0.07±0.02	0.08±0.02	0.07±0.02	0.07±0.01	0.08±0.01	0.06±0.01
GSI (%)	0.62±0.10	0.64±0.04	0.45±0.06	0.56±0.05	0.58±0.04	0.56 ±0.02
PSI (%)	0.48±0.09	0.55±0.03	0.35±0.05	0.42±0.04	0.45±0.05	0.47±0.02
ESI (%)	0.23±0.03	0.27±0.03	0.19±0.01	0.20±0.01	0.22±0.01	0.17±0.01

Fin – Finasteride/ Min- Minoxidil/ BW - Body weight / TW - Testicular weight / AW - Albuginea weight / PW - Parenchyma weight / EW - Epididymis weight/ GSI - Gonadosomatic Index / PSI - Parenchymosomatic Index / ESI - Epididymosomatic Index. Data expressed as mean ± SEM. No differences were found between treated groups and the water control group, nor between minoxidil-treated groups and the finasteride group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group.

Supplementary table 2. Body and reproductive organs parameters of male Balb/c mice after chronic exposure to finasteride and minoxidil for 84 days.

	Control	Vehicle	Fin 5 mg/kg	Min 2.5 mg/kg	Min 5 mg/kg	Min 7.5 mg/kg
BW (g)	36.15±1.12	33.58±0.84	33.61±1.38	36.24±0.99	33.49±1.15	37.23±1.08
TW (g)	0.22±0.01	0.24±0,01	0.18±0,02	0.20±0.01	0.21±0.01	0.23±0.01[#]
AW (g)	0.04±0.02	0.04±0.02	0.03±0.01	0.04±0.01	0.03±0.01	0.03±0.01
PW (g)	0.19±0.01	0.20±0.01	0.14±0.01	0.17±0.01	0.18±0.01	0.20±0.01[#]
EW (g)	0.08±0.01	0.08±0.01	0.07±0.01	0.07±0.01	0.06±0.01	0.08±0.01
GSI (%)	0.54±0.06	0.71±0.02	0.53±0.06	0.57±0.03	0.64±0.05	0.62±0.03
PSI (%)	0.52±0.02	0.60±0.04	0.43±0.06	0.45±0.02	0.55±0.04	0.54±0.02
ESI (%)	0.22±0.01	0.24±0.01	0.21±0.01	0,20±0.01	0.19±0.01	0.21±0.01

Fin – Finasteride/ Min- Minoxidil/ BW - Body weight / TW - Testicular weight / AW - Albuginea weight / PW - Parenchyma weight / EW - Epididymis weight/ GSI - Gonadosomatic Index / PSI - Parenchymosomatic Index / ESI - Epididymosomatic Index. Data expressed as mean ± SEM. No differences were found between treated groups and the water control group, nor between minoxidil-treated groups and the finasteride group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. N=6/group.

Supplementary table 3. Semi-quantitative data from the histopathological analyses of Balb/c mice's epididymis after chronic exposure to Finasteride and Minoxidil for 42 days

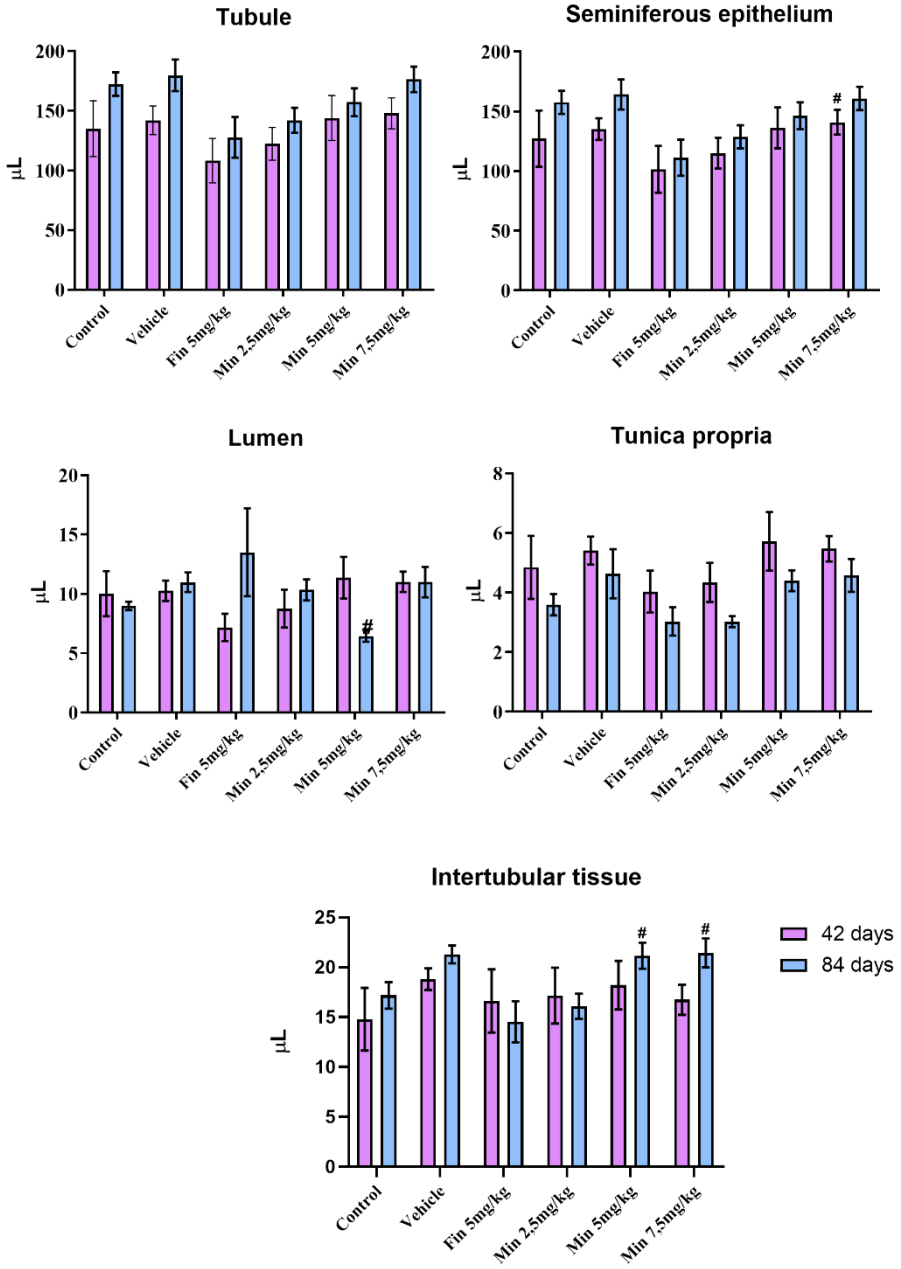
	Epithelial vacuolization				Cryptic epithelium				Detached cells in the lumen			
	Initial segment	Caput	Corpus	Cauda	Initial segment	Caput	Corpus	Cauda	Initial segment	Caput	Corpus	Cauda
Control	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5(0%)	3/5(60%)	3/5(60%)
Vehicle	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	3/5(60%)	3/5(60%)	4/5(80%)
Fin 5 mg/kg	0/5 (0%)	0/5 (0%)	3/5 (60%)	1/5 (20%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	3/5(60%)	4/5(80%)	3/5(60%)
Min 2.5 mg/kg	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5(0%)	4/5(80%)	3/5(60%)
Min 5 mg/kg	0/5 (0%)	0/5 (0%)	0/5(0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5(0%)	3/5(60%)	1/5 (0%)
Min 7.5 mg/kg	0/5 (0%)	0/5 (0%)	2/5(40%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	4/5(80%)	3/5(60%)	3/5(60%)
	Sperm in the lumen				Hypertrophy of clear cells				Inflammatory infiltrates			
	Initial segment	Caput	Corpus	Cauda	Initial segment	Caput	Corpus	Cauda	Initial segment	Caput	Corpus	Cauda
Control	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Vehicle	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Fin 5 mg/kg	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	4/5 (80%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Min 2.5 mg/kg	5/5(100%)	4/5(80%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5(0%)	0/5(0%)	3/5 (60%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Min 5 mg/kg	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	4/5 (60%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Min 7.5 mg/kg	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	3/5 (60%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	1/5 (20%)

Fin – Finasteride/ Min- Minoxidil. Numbers indicate the ratio of animals exhibiting pathology to the total number of animals examined except for the sperm in the lumen parameter, as animals that do not have a decrease in this parameter are considered normal.

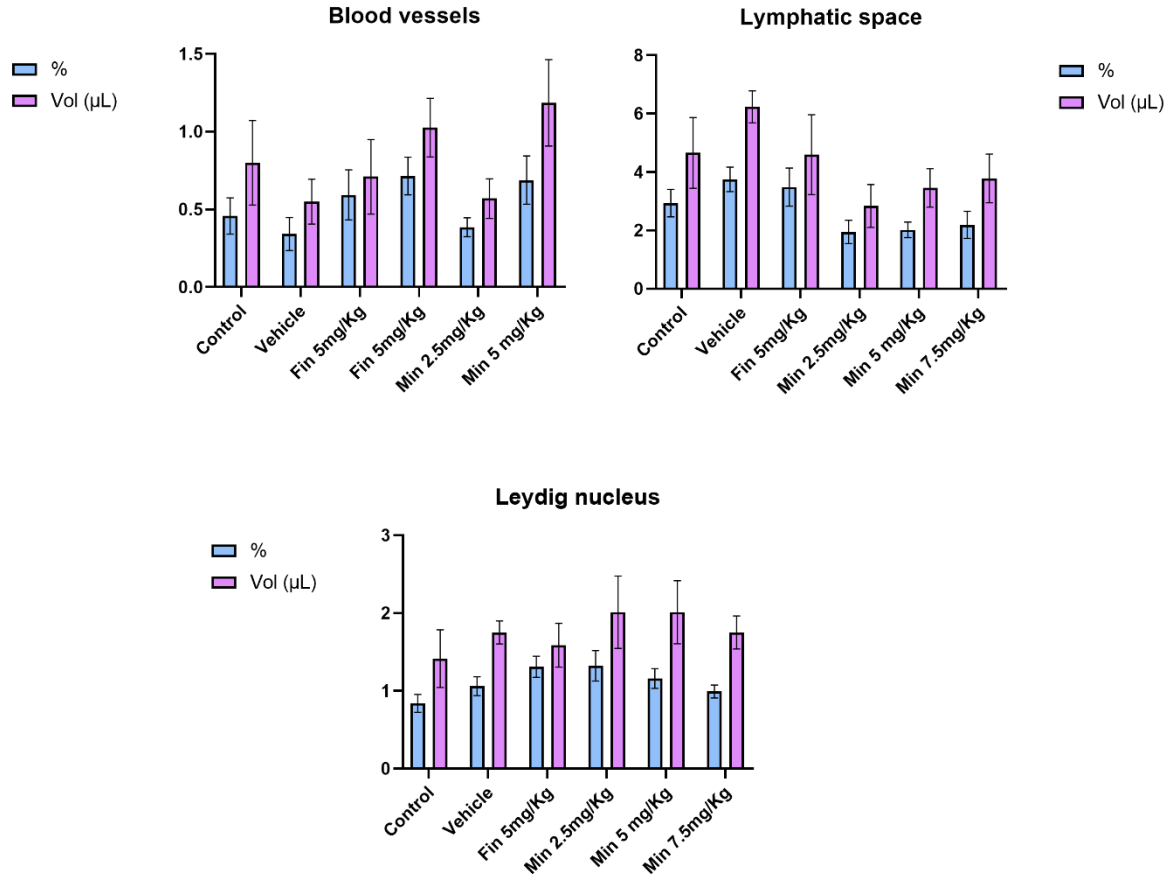
Supplementary table 4. The semi-quantitative data from the histopathological analyses of Balb/c mice's epididymis after chronic exposure to Finasteride and Minoxidil for 84 days

	Epithelial vacuolization				Cryptic epithelium				Detached cells in the lumen			
	Initial segment	Caput	Corpus	Cauda	Initial segment	Caput	Corpus	Cauda	Initial segment	Caput	Corpus	Cauda
Control	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	2/5(40%)	3/5(60%)	3/5(60%)
Vehicle	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	1/5 (20%)	0/5 (0%)	5/5(100%)	0/5(0%)	0/5(0%)
Fin 5 mg/kg	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	4/5(80%)	1/5(20%)	3/5(60%)
Min 2.5 mg/kg	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	1/5 (20%)	0/5 (0%)	3/5(60%)	0/5(0%)	0/5(0%)
Min 5 mg/kg	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	4/5(80%)	3/5(60%)	2/5 (40%)
Min 7.5 mg/kg	0/5 (0%)	0/5 (0%)	2/5 (40%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	1/5 (20%)	0/5 (0%)	4/5(80%)	3/5(60%)	2/5(40%)
	Sperm in the lumen				Hypertrophy of clear cells				Inflammatory infiltrates			
	Initial segment	Caput	Corpus	Cauda	Initial segment	Caput	Corpus	Cauda	Initial segment	Caput	Corpus	Cauda
Control	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Vehicle	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	2/5 (40%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Fin 5 mg/kg	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Min 2.5 mg/kg	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	2/5(40%)	2/5(40%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Min 5 mg/kg	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	2/5 (40%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)
Min 7.5 mg/kg	5/5(100%)	5/5(100%)	5/5(100%)	5/5(100%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)	0/5 (0%)

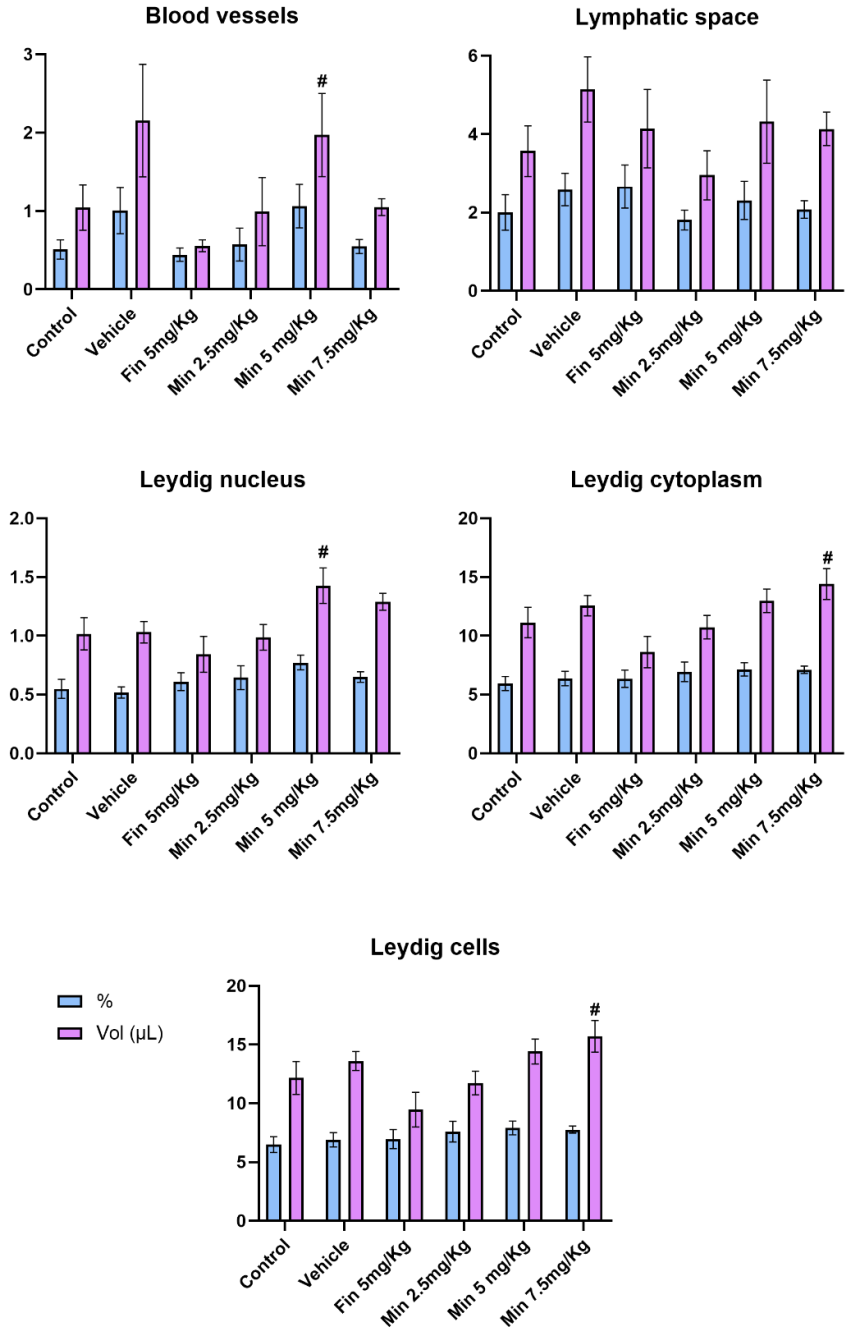
Fin – Finasteride/ Min- Minoxidil. Numbers indicate the ratio of animals exhibiting pathology to the total number of animals examined except for the sperm in the lumen parameter, as animals that do not have a decrease in this parameter are considered normal.



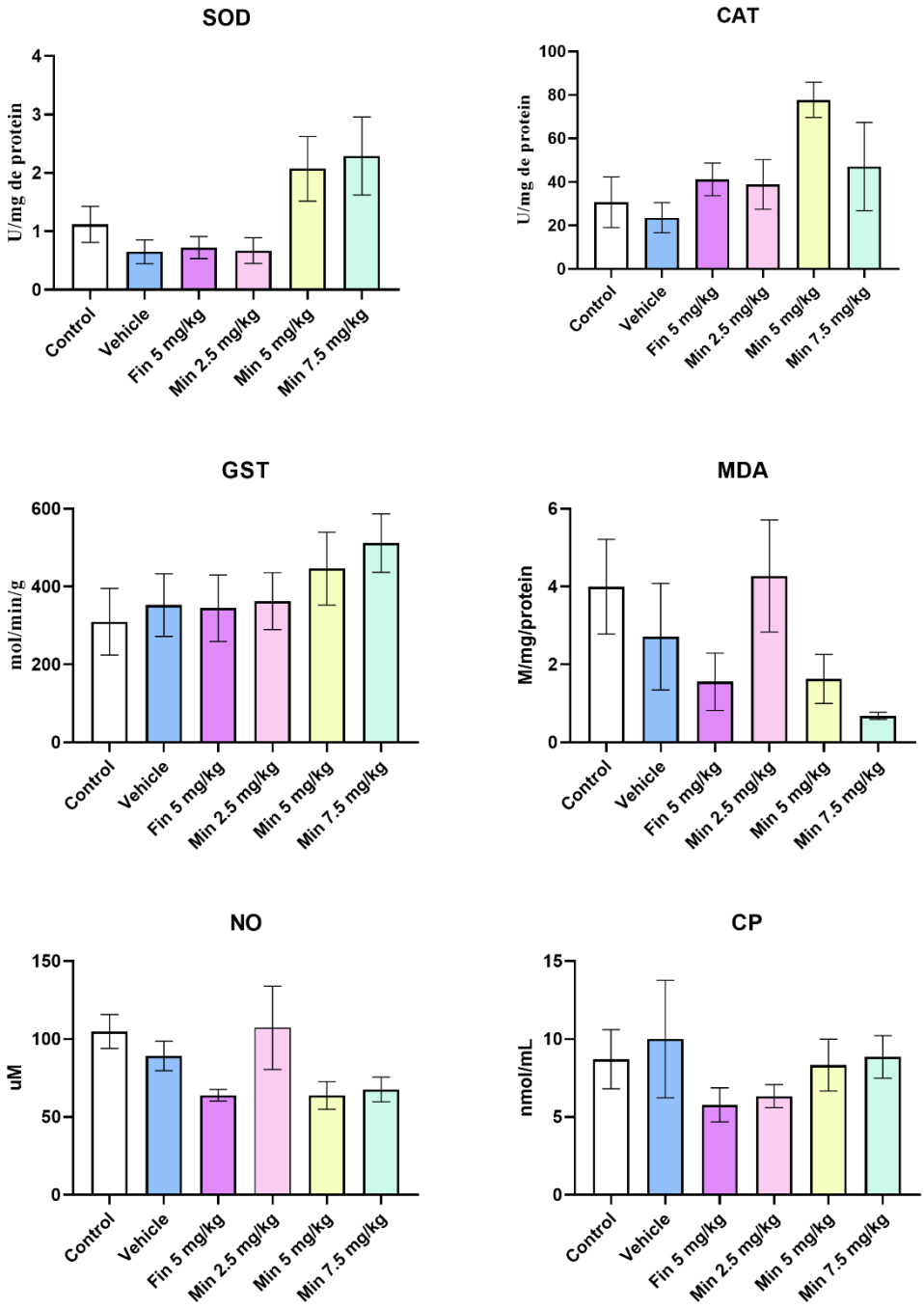
Supplementary figure 1. Volume (µL) of seminiferous tubules, seminiferous epithelium, lumen, tunica propria and intertubular tissue of Balb/c mice after long-term exposure to finasteride and minoxidil. Data expressed as mean ± SEM. (#) shows values significantly different from finasteride ($p \leq 0.05$) using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. No differences were found between treated groups and the water control group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. Fin – Finasteride, Min- Minoxidil. N=6/group.



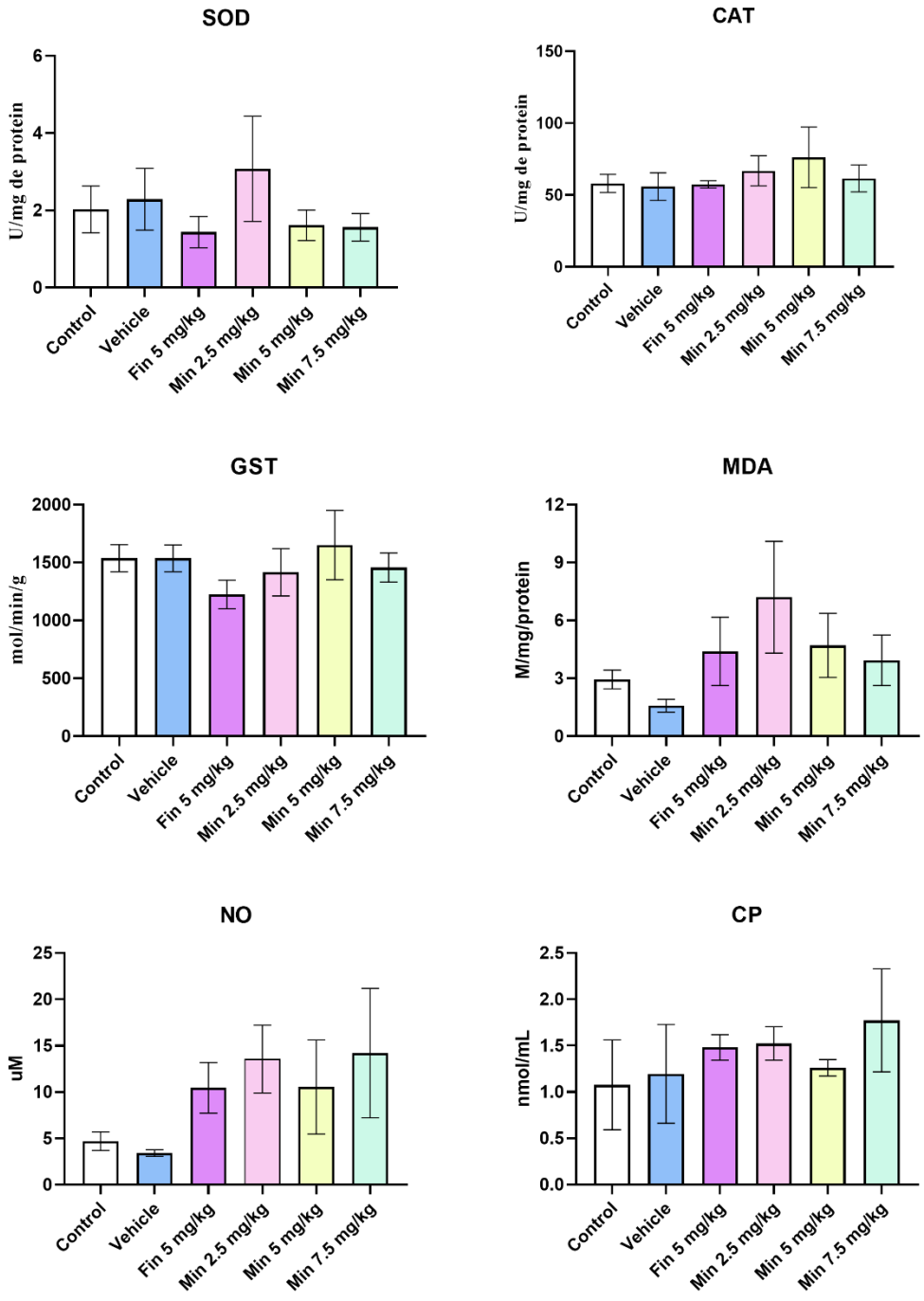
Supplementary figure 2. Proportion and volume of blood vessels, lymphatic space and Leydig nucleus of Balb/c mice after 42 days exposure to finasteride and minoxidil. Data expressed as mean ± SEM. No differences were found between treated groups and the water control group, nor between minoxidil-treated groups and the finasteride group using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. Fin – Finasteride, Min- Minoxidil. N=6/group.



Supplementary figure 3. Proportion and volume of blood vessels, lymphatic space and Leydig nucleus of Balb/c mice after 84 days exposure to finasteride and minoxidil. Data expressed as mean ± SEM. (#) shows values significantly different from finasteride ($p \leq 0.05$) using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. No differences were found between treated groups and the water control group. Fin – Finasteride, Min- Minoxidil. N=6/group.



Supplementary figure 4. Activity of antioxidant enzymes and oxidative stress markers in the testes of Balb/c mice- exposed to finasteride and minoxidil for 84 days. Data expressed as mean ± SEM. No differences were found between treated groups and the water control group, nor between minoxidil-treated groups and the finasteride group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. Finasteride, Min- Minoxidil. N=6/group.



Supplementary figure 5. Activity of antioxidant enzymes and oxidative stress markers in the epididymis of Balb/c mice- exposed to finasteride and minoxidil for 84 days. Data expressed as mean ± SEM. No differences were found between treated groups and the water control group, nor between minoxidil-treated groups and the finasteride group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. Fin – Finasteride, Min- Minoxidil. N=6/group.

Supplementary table 5. Sperm analyses in the epididymis of Balb/c mice after long-term exposure to finasteride and minoxidil

	Control	Vehicle	Fin 5 mg/kg	Min 2.5 mg/kg	Min 5 mg/kg	Min 7.5 mg/kg
42 days						
Caput/corpus epididymis sperm number ($\times 10^6$/organ)	4.32±0.44	4.27±0.23	4.66±0.20	4.38±0.65	4.44±0.44	4.36±0.36
Sperm transit time in the caput/corpus epididymis (days)	3.31±0.34	3.25±0.18	4.51±0.71	3.31±0.36	3.44±0.30	3.50±0.19
Cauda epididymis sperm number ($\times 10^6$/organ)	5.78±0.58	6.61±0.09	6.94±0.27	6.42±0.54	6.22±0.58	6.37±0.33
Sperm transit time in the cauda epididymis (days)	4.41±0.35	5.03±0.14	5.61±0.17	4.95±0.45	4.86±0.48	5.09±0.27
Sperm motility (%)	92.92±1.16	92.40±0.91	92.45±0.64	92.61±0.70	92.48±0.88	92.61±0.70
84 days						
Caput/corpus epididymis sperm number ($\times 10^6$/organ)	4.50±0.47	4.61±0.31	4.39±0.11	4.21±0.12	4.57±0.11	4.60±0.12
Sperm transit time in the caput/corpus epididymis (days)	3.49±0.33	3.49±0.13	3.83±0.04	3.24±0.07$^{\#}$	3.57±0.11	3.77±0.16
Cauda epididymis sperm number ($\times 10^6$/organ)	5.91±0.34	5.97±0.18	6.16±0.12	6.18±0.36	6.11±0.31	5.85±0.23
Sperm transit time in the cauda epididymis (days)	4.59±0.25	4.50±0.13	5.38±0.03	4.73±0.19	4.78±0.28	4.76±0.12
Sperm motility (%)	93.00±0.90	92.86±0.63	92.98±0.45	92.65±0.66	92.65±0.66	92.65±0.66

Data expressed as mean ± SEM. No differences were found between treated groups and the water control group, nor between minoxidil-treated groups and the finasteride group, using Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test. Fin – Finasteride, Min- Minoxidil. N=6/group.

CHAPTER 3

Minoxidil's histological, hormonal, and gene expression effects on human testis: an *ex vivo* culture study across fetal and adult stages

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ABSTRACT: Minoxidil, used in the treatment of androgenetic alopecia, has been associated with potential adverse effects on reproductive function, but its specific impacts on the human testis remain poorly understood. This study aimed to evaluate the effects of minoxidil on human testicular function using ex vivo cultures of fetal and adult testes, focusing on cellular function, hormone production, and gene expression. Fetal testes were collected during the first trimester, while adult testes were obtained from organ donors. Testicular cultures were exposed to three concentrations of minoxidil (10^{-5}, 10^{-6}, and 10^{-7} M), and histological, hormonal, and gene expression analyses were performed to assess its effects on Leydig, Sertoli, and germ cells, as well as testosterone production and apoptosis. In fetal testes, minoxidil did not induce significant cellular or endocrine changes, with stable enzyme expression and unchanged testosterone levels. In adult testicular fragments, two culture periods (2 and 9 days) were examined. After two days of exposure, a reduction in the expression of certain germ cell markers was observed, including CHEK1 (30% reduction at 10^{-7} M and 40% at 10^{-6} M) and SYCP3 (25% reduction at 10^{-6} M). Additionally, a decrease in steroidogenic gene expression was noted, with reductions of 35% in CYP17A1 (10^{-7} M and 10^{-6} M) and 20% in CYP11A1 (10^{-5} M). A 30% decrease in S5RD2 was observed at 10^{-6} M, suggesting potential impairment

of Leydig cell steroidogenic function. In long-term cultures, a trend toward reduced seminiferous tubule cellularity was observed, along with a transient 50% increase in testosterone levels after six days of exposure, which returned to baseline by day nine. These results suggest that minoxidil may influence spermatogenesis and steroidogenic function in adult testes. However, the lack of significant effects in fetal testes indicates that the impacts of minoxidil may be developmentally stage-dependent. Future studies should focus on human clinical trials to confirm these findings and explore strategies to minimize adverse events.

1 INTRODUCTION

Minoxidil was originally developed to treat high blood pressure for its vasodilating properties thanks to its ability to open potassium channels (PATEL et al., 2025). However, it is better recognized for the treatment of hair loss or alopecia topically or orally (*off-label*) at low doses (GUPTA et al., 2021), thanks to other possible mechanisms such as induction of the Wnt/β-catenin pathway, anti-inflammatory and possibly anti-androgenic properties (GUPTA et al., 2021). Since alopecia reaches half of white men by the age of 50 years old (HAMILTON, 1951; LOLLI et al., 2017) and 50% of women by the age of 60 (SINCLAIR, 2017), adherence to the treatment has become frequent, with a slight prevalence in the use of oral minoxidil (compared to other treatment options) starting in 2020 in both males and females, relative to the proportion of diagnosed patients (GUPTA et al., 2025).

Reported side effects of minoxidil use include loss of libido, reduced semen volume and ejaculatory dysfunction in men, and menorrhagia or irregular menstrual bleeding in women (WU et al., 2016), suggesting a possible iatrogenic hormonal imbalance. Pharmaceuticals that have antiandrogenic properties can either reduce the amount of available testosterone or interfere with the androgen receptor (AR) signaling (EZECHIÁŠ et al., 2016). These so-called endocrine disrupting chemicals (EDC's) ultimately affect the spermatogenic process which is highly coordinated by steroid hormones (RUSSEL et al., 1990), leading to altered fertility. Our previous results in male mice of reproductive age show some effects of 42 and 84 days of exposure to minoxidil in the testes and epididymis, with hormonal and histopathological changes, and oxidative stress (Chapter #2). Fine anti-androgenic mechanisms of action were identified for hair follicles and include inhibition of the expression of 5 alpha-reductase type II gene (SRD5A2) and interference with AR activity (GUPTA et al., 2024). However, despite the increasing use of oral minoxidil off-label, its direct impacts on human testicular physiology

have not yet been systematically investigated, representing an important gap in the field of reproductive toxicology. In addition, minoxidil's antihypertensive and follicular effects are dependent on the tissue's ability to convert it to its active metabolite, minoxidil sulfate, via the sulphotransferase system (ROSSI et al., 2012), which is poorly characterized in the human testis throughout life.

During organogenesis, such gonad-derived hormone imbalance can trigger congenital malformations such as cryptorchidism (abnormal testis descent), hypospadias (abnormal opening of the urethra), increased urogenital distance, and potential infertility in adulthood (SKAKKEBAEK et al., 2001; SVINGEN et al., 2018). EDCs can alter testicular Leydig cell testosterone production, but also Sertoli cell-triggered Leydig cell differentiation (O'SHAUGHNESSY et al., 2000). Minoxidil exposure during fetal life can trigger several altered angiogenesis-related congenital malformations in humans (SMORLESI et al., 2003), and macrosomia in rats (TURKINA et al, 2021), which is often accompanied by increased risk of abnormal development of organs and systems. There is no precise data on minoxidil use during pregnancy, but as minoxidil use is sometimes necessary and alopecia prevalence rise 8% in women aged 20–29 (child-bearing age) (MÜLLER RAMOS et al., 2023), there are a potential risk for the fetuses. If few cases of such major congenital malformations have been reported in women, this raises concerns about the use of minoxidil by pregnant women and the risk of EDC-induced malformations in baby boys, which to our knowledge have not been studied.

Finally, according to the Organisation for Economic Co-operation and Development (OECD) (2023), regulatory assays to identify steroidogenesis disorders include the use of a steroidogenic cell line (OECD TG456). Furthermore, earlier studies by the same organization highlight the importance of *in vivo* exposure in rodent models for toxicity evaluations (OECD, 2016; OECD, 2018). These assays provide the first evidence to answer this black box, but human and rodent fetal Leydig cells differ in their differentiation and endocrine properties (SHARPE, 2020), as well as in their sensitivity to EDCs (HABERT et al., 2014; BEN MAAMAR et al., 2015), highlighting the need to challenge sensitivity to EDCs directly in human organs. Therefore, our study is the first to evaluate the direct effects of minoxidil on the human testis at different stages of development, using ex vivo models that allow direct analysis of the cellular and hormonal effects. The results may provide insight into the risks of minoxidil use on male reproductive function, helping to develop safer clinical guidelines.

2 MATERIAL AND METHODS

2.1 Ethics statement

First-trimester human fetuses (8-12 DW) were obtained from legally induced terminations of pregnancy performed in Rennes University Hospital. Tissues were collected following women's written consent, following the legal procedure agreed upon by the National Agency for Biomedical Research (authorization #PFS09-011; Agence de la Biomédecine), and the ethical approval of the whole procedure by the Local ethics committee of Rennes Hospital (advice # 11-48). None of the terminations were for pathological reasons of the pregnancy or fetal abnormality. Tissues were kept at 4° from aspiration and processed within 2 hours. The adult testes were obtained from multiorgan donors (n=8; mean age:57.9±8.9y). The local ethics committee approved the protocol (Agence de la Biomédine; authorization no. PFS09-015), and informed consent was obtained from all donors or their next of kin.

2.2 The human fetal gonad assay

When collected, the testes were immediately processed and cultured according to our validated method (MAZAUD-GUITTOT, et al. 2013). Briefly, the retrieved testes were cut into approximately 1 mm^3 pieces. Four wells were prepared for 4 different culture conditions (1 control and 3 minoxidil concentrations). These wells contained 4 to 5 pieces of a single testis. The explants were cultured in inserts (0.4µm pores; Falcon, Becton-Dickinson, Le Pont de Claix, France) placed in 24-well culture plates (Becton-Dickinson). Each well was filled with phenol red-free medium M199 (Invitrogen, Life Technologies) supplemented with 50 mg/ml gentamicin and 2.5 mg/ml amphotericin B (Sigma-Aldrich Chemicals). Human chorionic gonadotrophin (hCG, Sigma-Aldrich Chemicals) was added at a final concentration of 0.1 IU/ml (HALLMARK et al., 2007) to sustain steroidogenesis, and the cultures were incubated at 37 °C for 96h. There were three consecutive days of chemical exposure, with the medium changed daily. The medium was removed every 24h and divided into at least 2 aliquots immediately snap-frozen on dry ice and stored at −80 °C. To assess concentration response effects, after the first 24 hours of culture (D0), explants were exposed to either the control, DMSO at a final concentration of 0.1%, or minoxidil (Sigma Aldrich M4145, CAS nb # 38304-91-5) at concentrations of 10^{-7}, 10^{-6}, and 10^{-5}M added to the medium. Concentrations were chosen based on the study carried out by Hsu et al., (2014) which describes that the most frequently reported values in the literature for the use of minoxidil are 0.1 to 10 µM for oral

administration and approximately 1 mM for topical use, assuming a 1.7% absorption rate. The authors also described that in *in vitro* organ culture or animal studies, higher concentrations, ranging from 1 to 100 mM, have been detected in skin tissue. At the end of exposure, explants from each well and condition were collected, fixed in Bouin's solution, and embedded in paraffin. The fixed explants were cut into 5.0µm thick sections and stored at 4ºC until staining.

2.3 The human adult testis explant assay (TEXAS)

We used a validated organ model assay (Testis Explant Assay) for a short-term culture (2 days) (DESDOITS-LETHIMONIER et al, 2012) and long-term culture (9 days) (MATUSALI et al., 2018). Freshly collected testes were placed at 4°C and rapidly processed for experimentation. Only testes showing alternating dark and light, reflecting dynamic spermatogenesis, were selected by transillumination. Four 3-mm^3 testis explants were placed onto a polyethylene terephthalate insert (Falcon Labware, USA) at the interface of air in 1 mL of medium. In short-term culture assay, the Dulbecco's Modified Eagle's Medium (Thermo Fisher Scientific, 31053028), was supplemented with 1X of antibiotics (Gibco), 4 mM glutamine (Gibco), 1 mM sodium pyruvate, 10 µg/ml insulin, 5 µg/ml transferrin, 200 ng/ml vitamin E, 100 ng/ml vitamin A, 50 ng/ml vitamin C, and 1 IU/ml hCG (all from Sigma) for culture, in a well of a 12-well plate. In the long-term culture assay, the DMEM F12 medium was supplemented with 10% FCS (eurobio), 1% antibiotics, 1% non-essential amino acids (Sigma, M7145) and 1% ITS (Gibco). The medium contained 0.1% DMSO or minoxidil diluted in three concentrations ranging from 10^{-5}M to 10^{-7}M. For each condition, randomly selected testis explants were cultured in three different wells, corresponding to technical replicates, for 48 h with medium change every 24 h for short-term culture and for 9 days with medium change every 72 h for long-term culture. Media from the replicates were stored at -80ºC for testosterone measurements. At the end of exposure, six explants, one from each well of each condition, were randomly collected and stored at -80ºC for reverse transcription quantitative real-time PCR (RT-qPCR) and six were fixed in Bouin's or 4% paraformaldehyde (PAF) solution and embedded in paraffin. The fixed explants were cut into 5.0µm thick sections and stored at 4ºC until staining.

2.4 Testosterone measurements

Testosterone levels were assayed in duplicate in culture media with specific radioimmunoassays (RIA): a direct kit was used for the fetal testis explants culture media and

an indirect kit - with ether-based steroid extraction - for TEXAS culture media (both from Beckman Coulter, Villepinte, France). The intra- and inter-assay coefficients of variation were ≤ 10.6% and ≤ 19%, respectively for the direct kit, and ≤ 11.6% and ≤ 13.5% respectively for the indirect kit.

2.5 Immunohistochemistry

Histological sections of the fetus and adult human testis explants from each treatment condition were submitted to analysis of immunohistochemistry (IHC) to observe Leydig cells and estimate the number of testicular cells undergoing apoptosis, and proliferation as already described (MAZAUD-GUITTOT et al., 2013; DESDOITS-LETHIMONIER et al., 2012; DESDOITS-LETHIMONIER et al., 2017). Briefly, after dewaxing and rehydration, sections followed a step of heat-mediated antigen retrieval, either basic Tris 10 mM, EDTA 1mM, pH 9 for cleaved caspase 3 or acidic citrate buffer, 10 mM, pH6 for the others, and a subsequent step of antigen blocking in BSA 10% in PBS.

Leydig cells were labeled with a rabbit primary antibody directed against the cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1, 1:250; Sigma-Aldrich, Germany), apoptotic cells with a polyclonal rabbit primary antibody directed against cleaved caspase-3 (1:250; Asp175, Cell Signaling Technology, Ozyme, France), and germ cells with a polyclonal rabbit antibody directed against LIN28 (1:2000; Abcam ab46020). Primary antibody was reacted with a biotinylated polyclonal goat anti-rabbit secondary antibody (1:500; Dako, Denmark). For proliferation assessment of fetal testis, histological sections were also labeled with a monoclonal mouse primary antibody directed against Ki-67 (1:150; Dako, Denmark) and then a biotinylated polyclonal goat anti-mouse secondary antibody (1:500, Dako, Denmark). Sertoli cells were labelled with a goat AMH primary antibody (1:400; Santa Cruz Biotechnology, CA, USA) followed by a biotinylated polyclonal rabbit anti-goat secondary antibody (1:500, Dako, Denmark). Sections were developed with streptavidin-horseradish peroxidase (Vectastain ABC kit; Vector Laboratories) and 3,3-diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and counterstained with Masson's hemalun.

Caspase-3, KI-67-, and AMH-stained sections from ten fetal testes and Caspase-3 and CYP11A1 from six adult testes were examined (qualitative histopathological assessment) and photographed under a light microscope (Olympus Provis AX60). Histological morphometric analyses in fetal testis explants were performed by using ImageJ software. The relative cord area and the relative interstitial tissue area were determined thanks to positively- or negatively-

AMH stained tissue, respectively, within the total tissue surface of the explants, on 8-10 randomly selected histological explant sections. Relative Leydig cell area was estimated by determining the ratio of the area occupied by CYP11A1-positive Leydig cells to the AMH-negative interstitial tissue area on a full section of 8-10 randomly selected histological explant sections.

2.6 Data acquisition

The profile of expression of six genes (SRD5A1, SRD5A2, SRD53, AKR1C2, AKR1C4, and SULTA1) were retrieved from Bulk RNA sequencing data of human fetal gonads aging 7-17 PCW (LECLUZE et al, 2020). The expression profiles of the same genes in addition to SOX9, CYP17A1, CYP11A1, POU5F1, and DDX4 were investigated at the single cell level by using single cell RNA seq data retrieved from Lardenois et al., (2023) and Di Persio et al., (2021) requested from the reprogenomics viewer (RGV https://rgv.genouest.org/) database. Different single-cell RNAseq datasets were used to confirm these patterns retrieved from Guo et al., (2018); Sohni et al., (2019); Nie et al., (2022); Guo et al., (2021) sourced at Human Testis Atlas (https://humantestisatlas.shinyapps.io/humantestisatlas1/). The protein expression for these genes was also retrieved from the Human protein atlas (https://www.proteinatlas.org/) to confirm by immunohistochemistry the presence of these enzymes in human adult testes.

2.7 RNA extraction and quantitative PCR

RNA was extracted from frozen adult human testis explants by using RNeasy® Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity of RNAs was estimated by nanodrop D-1000 spectrophotometer (Thermo Scientific). Reverse transcription was performed on 100 ng of RNA using the iScript cDNA Synthesis Kit (Biorad) according to the manufacturer's protocol. RT-qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad) according to the manufacturer's instructions. The amplification program was as follows: an initial denaturation of 3 min at 95 °C; 40 cycles of 10-s denaturation at 95 °C; and 30 s at 62 °C for annealing and extension. Dissociation curves were produced with the thermal melting profile performed after the last PCR cycle. To avoid amplification of contaminating genomic DNA, primer pairs were selected on two different exons (For genes studied, see Table 1). BZW1, GAPDH, and GUSB mRNA were used as internal controls for normalization. Results

were calculated by the ΔΔCT method as n-fold differences in target gene expression concerning the reference gene and the calibration sample.

Table 1. Primers used in RT-qPCR in adult human testis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
BZW1	TCAGAGTCGGAGAGGAACAAG	CATGCTGACTTTCCGAAGACT	207
GAPDH	CATCAATGGAAATCCCATCA	GACTCCACGACGTACTCAGC	90
GUSB	CGTCCCACCTAGAATCTGCT	TTGCTCACAAAGGTCACAGG	94
TSPO	GGCTTCACAGAGAAGGCTGT	ACTGACCAGCAGGAGATCCA	132
STAR	GGCTGGCATGGCCACAGACT	TTGGGCAGCCACCCCTTGA	162
CYP11A1	AGACCTGGAAGGACCATGTG	TCCTCGAAGGACATCTTGCT	163
HSD3B2	GCCTGTTGGTGGAAGAGAAG	GCAGGCTCTTTTCAGGAATG	158
HSD17B3	TCCTTGGCCTCTCTACTCCA	AGACAGCATATGGGGTCAGC	125
CYP17A1	GTGGAGACCACCACCTCTGT	GCTGAAACCCACATTCTGGT	108
CYP19A1	CCAGGTCCTGGCTACTGCAT	GATCCCCATCCACAGGAATCT	69
SRD5A1	CTTGAGCCATTGTGCAGTGT	CATGCCCGTTAACCACAAGC	85
SRD5A2	ATATTGCGCCAGCTCAGGAA	ACCGAGGAAATTGGCTCCAG	93
SDR5A3	AGGAATGCCTACATAACAGGGAA	CTCCAAATGGGATCCTGTGGT	181
INSL3	GCGACCGTGAGTTGCTACAG	GGTCCCAGCGTGAGATTACTG	82
INHBB	AGCCTCCAGGATACCAGCAA	TCTCCGACTGACAGGCATTTG	71
AMH	CGCCTGGTGGTCCTACAC	GAACCTCAGCGAGGGTGTT	162
PGK2	ACCAGAGGATCAAGGCTTCC	TCAGGCCGACCTAGATGACT	94
CD90 (THY1)	AGCATCGCTCTCCTGCTAACA	CTCGTACTGGATGGGTGAACT	138
ACTA2	GCTGTTTTCCCATCCATTGT	TTTGCTCTGTGCTTCGTCAC	99
CCND1	CCGTCCATGCGGAAGATC	GAAGACCTCCTCCTCGCACT	75
CHEK1	GGTGCCTATGGAGAAGTTCAA	TCTACGGCACGCTTCATATC	89
TP53	CCATCCTCACCATCATCACACTG	CACAAACACGCACCTCAAAGC	78
FGFR3	CCGAGCGGATGGACAAGAAG	GACCAGGCTCCACTGCTGAT	175
PIWL4	CATCAAGTTCTCCCGTGTGC	GACACAGAAATGGCAAACCC	165
UTF1	CGGCTCCCAGCGAACCAG	GACGGGCTGAAGCGGAGC	87
SYCP3	TCAAAGGCAGAAGCTTAACCAA	CTTGCTGCTGAGTTTCCATCA	320
REC8	GTTGGGAATTCTGTGCCCTA	TGGTCTTTCACCCTCGATTC	76

ACTA2 – actin alpha 2, smooth muscle; AMH – anti-Mullerian hormone; BZW1– Basic Leucine Zipper And W2 Domains 1; CCND1– Cyclin D1; CD90 – Thy-1 cell surface antigen ; CHEK1– Checkpoint kinase 1; CYP11A1 – Cytochrome P450 family 11 subfamily a member 1; CYP17A1 – Cytochrome P450 family 17 subfamily a member 1; CYP19A1– Cytochrome P450 family 19 subfamily a member 1; GAPDH – Glyceraldehyde 3-phosphate dehydrogenase; GUSB – Glucuronidase beta; HSD3B2 – Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2; HSD17B3– 17-beta hydroxysteroid dehydrogenase 3; INHBB – Inhibin subunit beta; INSL3 – Insulin like 3; PGK2 – Phosphoglycerate kinase 2; SRD5A1 – Steroid 5 alpha-reductase 1; SRD5A2– Steroid 5 alpha-reductase 2; SRD5A3– Steroid 5 alpha-reductase 3; STAR – steroidogenic acute regulatory protein; TP53 – Tumor Protein p53; TSPO – Translocator protein; FGFR3 – Fibroblast growth factor receptor 3; PIWL4 – Piwi-like protein 4; UTF1 – Undifferentiated embryonic cell transcription factor 1; SYCP3 – synaptonemal complex protein 3, REC8 – Meiotic Recombination Protein.

2.8 Statistical analysis

Data were analyzed with GraphPad Prism8 (GraphPad Software, USA). The significance of the differences between values were evaluated by an appropriate test and the value of $p < 0.05$ was considered statistically significant. Data were expressed as mean ± standard error mean (SEM).

3 RESULTS

3.1 Five alpha reductases and key enzymes of the steroidogenic backdoor pathway along testis differentiation and adulthood

We first investigated the expression of the different possible targets of minoxidil in the fetal and adult human testis. Bulk RNA sequencing (LECLUZE et al., 2020) of human fetal gonads showed that neither SRD5A1 nor SRD5A2 were detectable, whereas SRD5A3 is expressed in both first trimester testes and ovaries, with the highest levels found at the end of the 7th PCW (Figure 1A). Of the backdoor pathway alpha-ketoreductases, only AKR1C2 showed detectable levels with similar male and female profiles (AKR1C4 was undetectable). The levels of the sulfotransferase SULT1A1 were similar in testes and ovaries. Single cell RNAseq data from Lardenois et al., (2023), requested from the Reprogenomics Viewer, confirmed the broad pattern of SRD5A3 expression and undetectable levels of SRD5A2, but showed detectable levels of SRD5A1, particularly in neuroendocrine cells (Figure 1B). According to the bulk data, AKR1C4 was poorly expressed compared to AKR1C2, but both

were found preferentially in male somatic progenitor cells. SULT1A1 was widely expressed in most cell types.

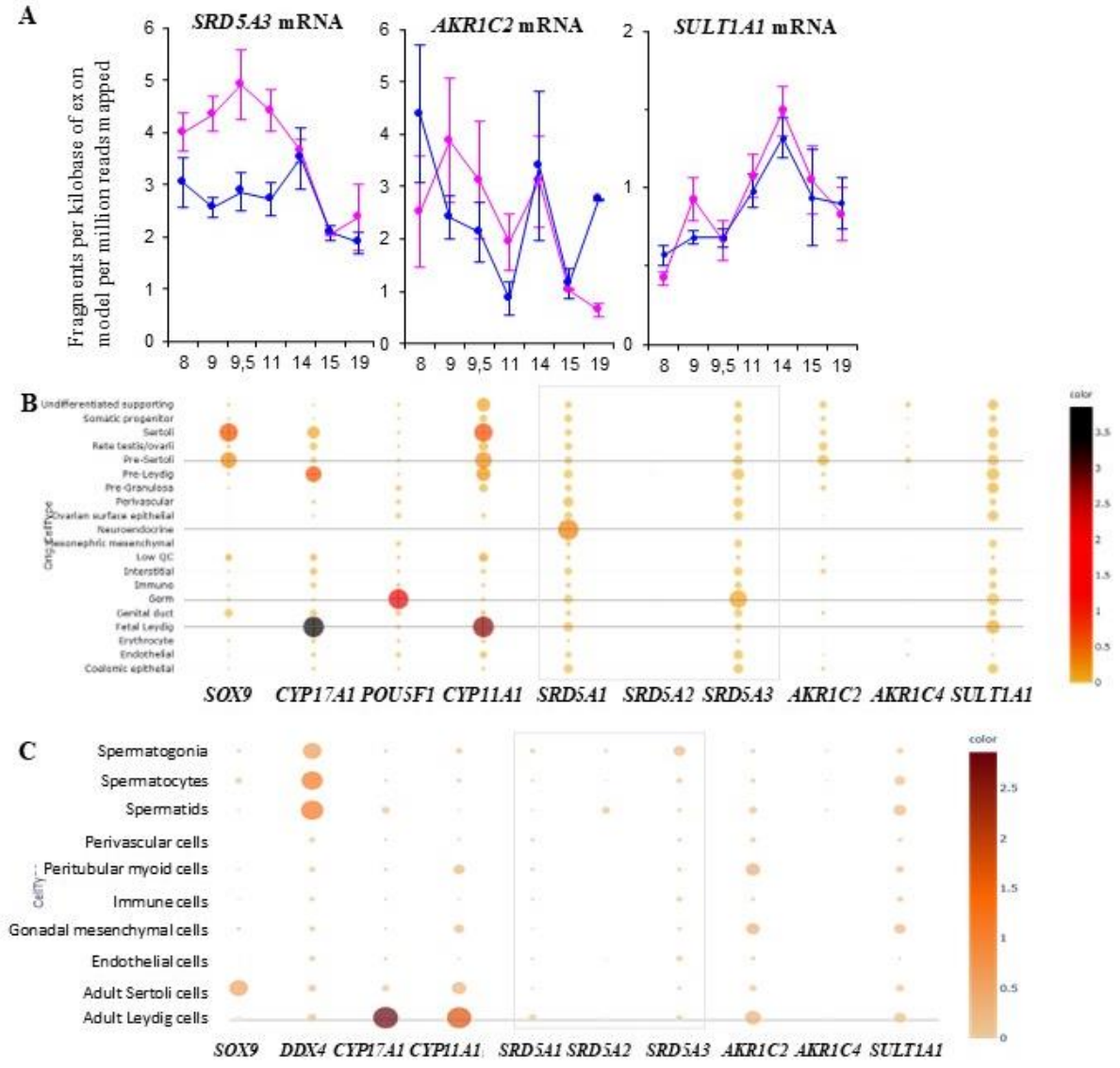

Figure 1. Expression of enzymes involved in DHT production and minoxidil metabolism in fetal and adult human testes. (A) Expression profile of the 3 isoforms of 5 alpha reductase (SRD5A1-3), aldoketoreductases of the backdoor pathway (AKR1C2/4), and of sulfotransferase (SULT1A1) as a function of age (PCW) from bulk RNA sequencing of human fetal testes (blue lines) and ovaries (pink line) (LECLUZE et al, 2020). Data are expressed as means ± SE of the mean of fragments per kilobase of exon model per million reads mapped (fpkm).; (B-C) Expression plots of typical Sertoli (SOX9), Leydig (CYP17A1 and CYP11A1) and germ (POU5F1) markers in addition to SRD5A1-3, AKR1C2/4, and SULT1A1. Data were extracted from Reprogenomics viewer from single cell RNAsequencing of human fetal gonads

(B, LARDENOIS et al, 2023) and adult human testes (C) using datasets from Lardenois et al., (2023 Biorxiv) and Di Persio et al., (2021), respectively.

Single cell RNAseq data from adult human testes (Di PERSIO et al., 2021), thanks to Reprogenomics Viewer showed that SRD5A1 mRNAs were poorly found in any cell type of young adult testis, while SRD5A2 mRNAs were mainly found in immature germ cells (spermatogonia) and SRD5A3 mRNAs in mature germ cells (from late primary spermatocytes to elongated spermatids) (Figure 1C). AKR1C2 was found in Leydig and myoid cells, whereas AKR1C4 levels were generally low. SULT1A1 was broadly expressed with the highest proportion of cells being mature germ cells. Different single-cell RNAseq datasets confirmed these patterns (Supplemental Figure 1 and 2A; GUO et al., 2018; SOHNI et al., 2019; NIE et al., 2022; GUO et al., 2021) (https://humantestisatlas.shinyapps.io/humantestisatlas1/). The protein expression profile from the Human protein atlas (https://www.proteinatlas.org/) also confirms by immunohistochemistry the presence of these enzymes in human seminiferous tubules (Supplementary Figure 2B).

3.2 Minoxidil has no major effect on cell differentiation and steroidogenic function in the human fetal testis

We used our well-characterised human fetal testis organotypic culture model to explore the possible effects of minoxidil on testicular differentiation and steroidogenic function. Histopathological analysis for apoptotic cleaved caspase-3-positive and proliferating KI67-positive cells showed that minoxidil concentrations did not alter viability (Figure 2A) or cell growth (Figure 2B) compared to the control. Immunostaining for AMH and LIN28 in control and minoxidil-exposed explants showed no major changes in Sertoli and germ cell populations (Figure 2C-D). Staining of Leydig cells for CYP11A1 showed no significant adverse effect of minoxidil compared to the control condition (Figure 3A). The relative area of interstitial tissue in the explants (Figure 3B), the relative area of CYP11A1-positive cells (Figure 3C), and the relative area of CYP11A1-positive cells in the interstitial tissue (Figure 3D) were not different in control and minoxidil-exposed explants after 72h exposure to minoxidil at three concentrations, despite a large variability among the individuals studied. Testosterone levels were not statistically altered by any of the minoxidil concentrations studied, regardless of fetal age (Figure 3E). Overall, our analysis suggests that minoxidil has no major effect on human fetal testis differentiation and testosterone synthesis but further studies are needed.

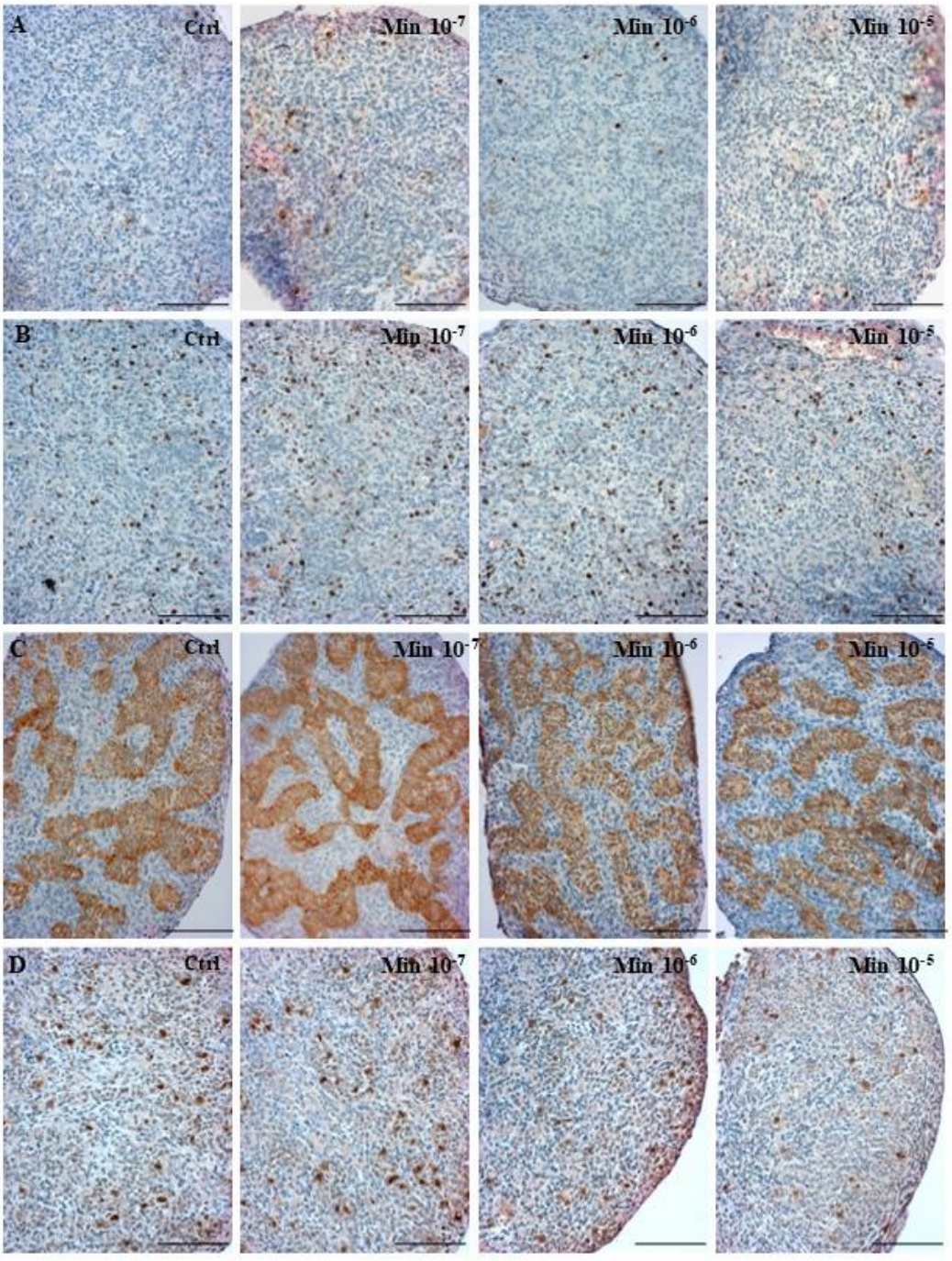

Figure 2. Minoxidil may not alter cell viability and proliferation, and testis cords differentiation in human fetal testes. Immunohistochemistry for cleaved caspase 3 (A), KI-67 (B), AMH (C), and LIN28 (D) in testicular explants after 3 days of exposure to the vehicle control DMSO (Ctrl) or MIN at 10^{-7}; 10^{-6}; and 10^{-5} M. Scale bars: 100 µm. [Magnifications: 20x for cCasp & KI67, and 10X for AMH and LIN28].

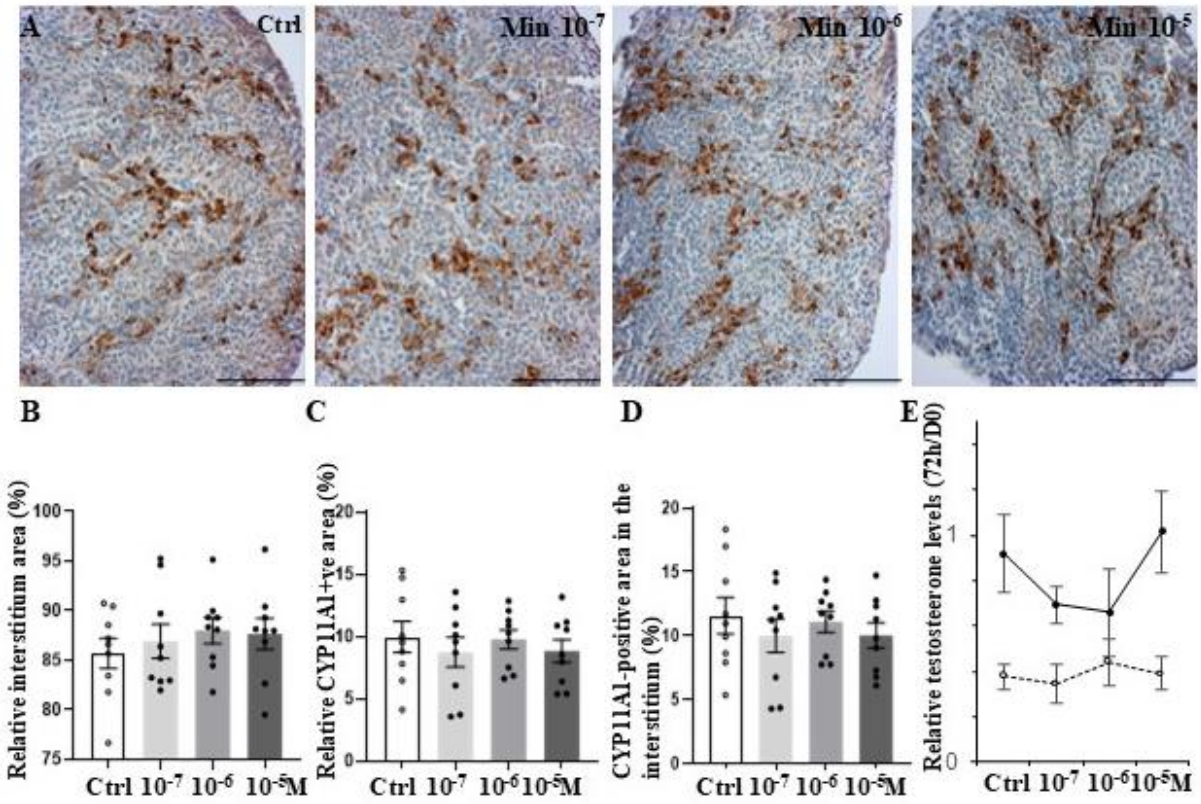

Figure 3. Leydig cells in fetal testis after exposure to three concentrations of minoxidil. (A) Immunohistochemistry for CYP11A1 in testicular explants after 2 days of exposure to the vehicle control DMSO (Ctrl) or MIN at 10^{-7}; 10^{-6}; and 10^{-5} M. (B) Relative percentage of the area occupied by interstitial tissue (n=9); (C) Relative percentage of the area occupied by CYP11A1-positive Leydig cells (n=9). (D) Relative percentage of the area occupied by Leydig cells in the interstitial tissue (n=9). (E) Testosterone production after culture of 8–10 DW (solid lines) and 10–14 DW (dotted lines) human fetal testes in the presence of the vehicle control DMSO (Ctrl) or MIN at 10^{-7}; 10^{-6}; and 10^{-5} M for 2 days of exposure. Results are expressed as fold change from the first day of culture (D2 compared with D0). A Wilcoxon test was performed for pairwise comparisons. Scale bar: 100 µm.

3.3 Minoxidil effects on human adult testis

We used our 2-day standardised organotypic culture model of the adult human testis to investigate the potential effects of minoxidil on testicular cell functions (Desdoits-Lethimonier et al 2012**;** 2017). We observed no major change in the global amount of cleaved caspase-3-positive apoptotic cells in testis fragments exposed for 2 days to the three minoxidil concentrations compared to the control DMSO ones (Figure 4A). The absence of alteration of

cell viability was confirmed by the unaltered levels of *CCND1* and *TP53* mRNAs after exposure to minoxidil (Figure 4B). The testicular morphology (Figure 4A) was not obviously altered (Figure 4A). Quantitative PCR showed that specific Sertoli (*INHBB* and *AMH*) and peritubular cell type markers (*CD90* and *ACTA2*) were not changed after exposure to any of the concentrations of minoxidil (Figure 4C). We quantified the expression of a range of mRNAs used as germ cell markers (Figure 4D). While levels of spermatogonia markers (*FGFR3*, *PIWIL4*, *UTF1*) were unchanged, levels of spermatocytes (*CHEK1*) and spermatids (*SYCP3*) markers were decreased by at least one concentration of minoxidil (Figure 4D).

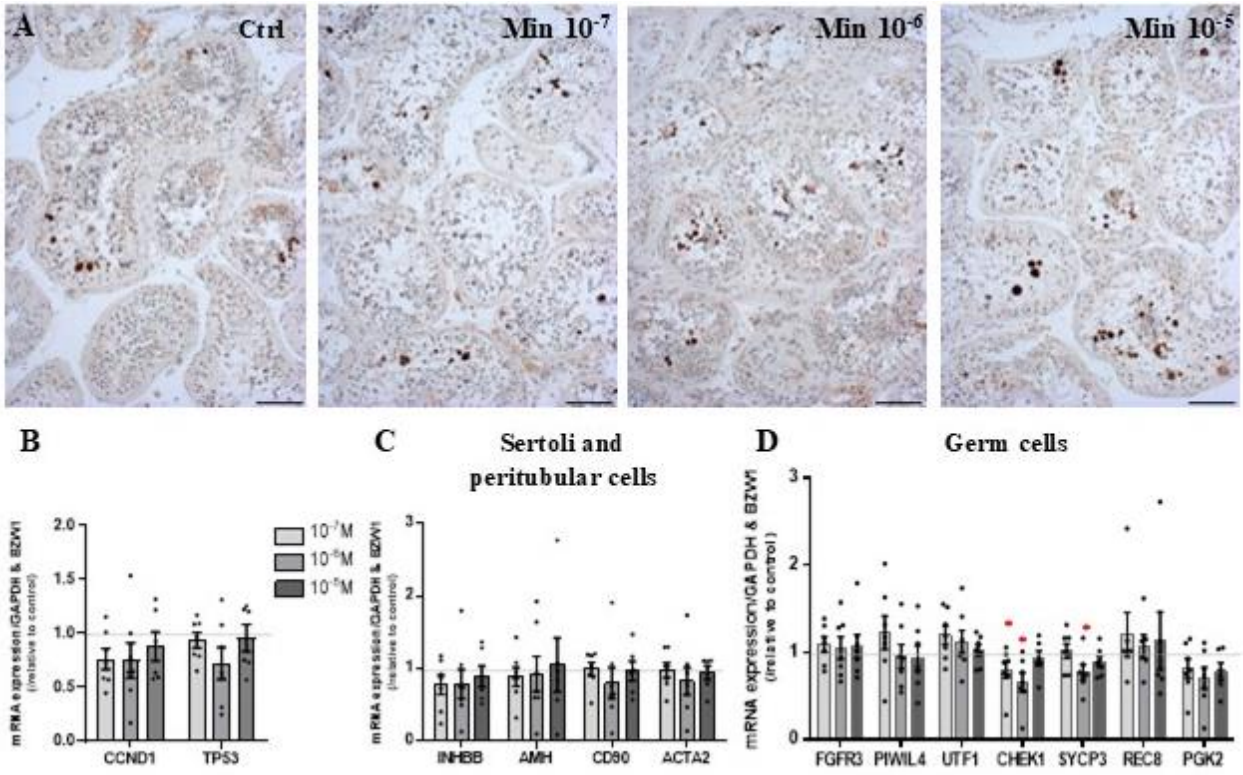

Figure 4. Cell viability in adult testes is not altered by the three concentrations of minoxidil. Immunohistochemistry for cleaved caspase 3 in testicular explants after 2 days of exposure (A) to vehicle control DMSO (Ctrl) or MIN at 10^{-7}; 10^{-6}; and 10^{-5} M. Scale bar: 100 µm. [Magnification 10x]. Levels of mRNA encoding markers of genes involved in cell cycle and apoptosis (cyclin D1, CCND1; tumor protein p53, TP53) (B), Sertoli cells (Inhibin B; INHBB, anti-üllerian hormone; AMH), and peritubular myoid cells (CD90; ACTA2) (C) and germ cells (Fibroblast growth factor receptor 3, FGFR3; Piwi-like protein 4, PIWL4; Undifferentiated embryonic cell transcription factor 1, UTF1; checkpoint kinase 1, CHEK1;

synaptonemal complex protein 3, SYCP3; Meiotic Recombination Protein, REC8; phosphoglycerate Kinase 2, PGK2 (D). A Wilcoxon test was performed for pairwise comparisons.

Staining of CYP11A1 in Leydig cells was similar in all experimental conditions (Figure 5A), and testosterone levels were not altered after exposure to either concentration of minoxidil for 1 and 2 days (Figure 5B). Quantitative analysis of mRNAs encoding proteins and enzymes of the steroidogenic pathway, in addition to the Leydig cell marker *INSL3*, showed that *CYP17A1* levels were decreased after exposure to minoxidil at 10^{-7} and 10^{-6}M, those of CYP11A1 were decreased after exposure to minoxidil at 10^{-5}M, in contrast to *TSPO*, *STAR*, *HSD17B3*, *HSD3B2*, *CYP19A1* mRNAs. Regarding the three 5 alpha-reductases, SRD5A2 mRNA levels were decreased after exposure to minoxidil at 10^{-6}M, whereas SRD5A1 and SRD5A3 mRNAs were unchanged regardless of minoxidil concentration (Figure 5C). Finally, *INSL3* mRNA levels were unchanged.

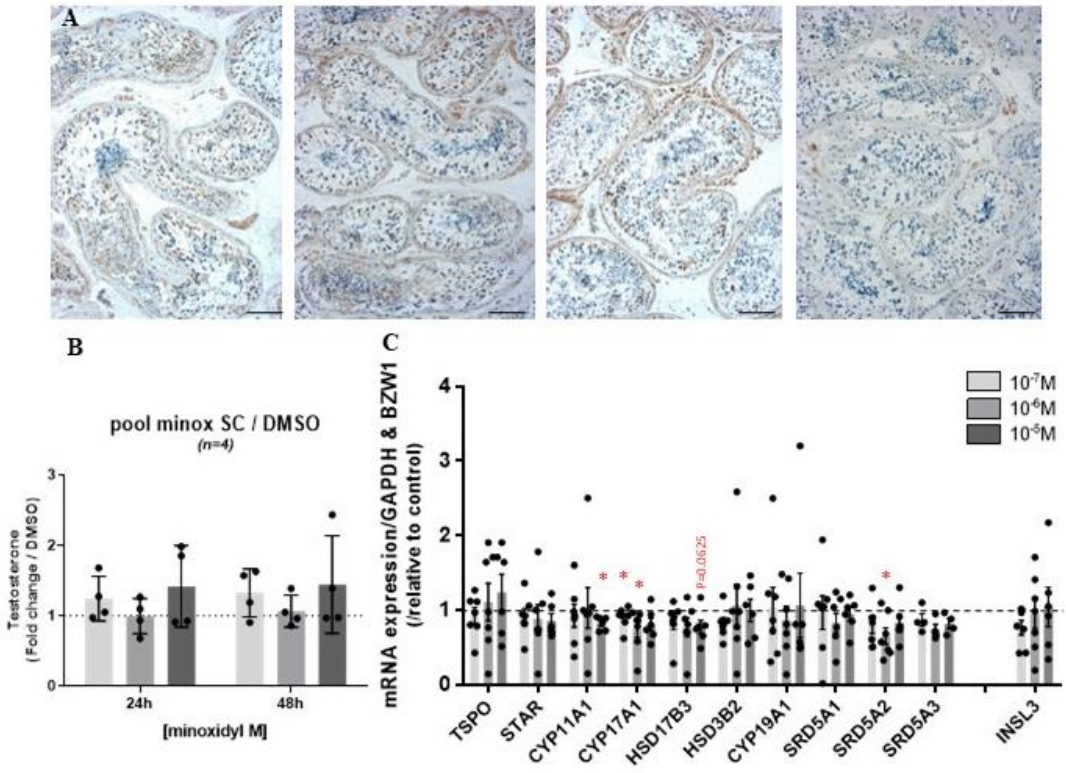

Figure 5. Leydig cells in adult testis after exposure to three concentrations of minoxidil. Immunohistochemistry for CYP11A1 in testicular explants after 2 days of exposure (A) to vehicle control DMSO (Ctrl) or MIN at 10^{-7}; 10^{-6}; and 10^{-5} M. Scale bar: 100 µm. [Magnification 10x] Testosterone levels after 1 (24h) and 2 days (48h) of exposure to vehicle

control DMSO (Ctrl) or MIN at 10^{-7}; 10^{-6}; and 10^{-5} M (B). Data are the means +/- SEM of the fold change of the Ctrl (DMSO) explants of the corresponding donor. Individual values are shown as dots. (C) Levels of mRNA encoding proteins and enzymes of the steroid biosynthesis pathway and of INSL3. Data are normalised with housekeeping genes, and are expressed as means +/– SEM with single values shown as dots. A Wilcoxon test was performed for pairwise comparisons.

We studied a longer exposure to minoxidil, increasing the culture time from 2 to 9 days (MATUSALI et al., 2020). Testicular morphology was broadly maintained, although seminiferous tubule cellularity tended to be looser, irrespective of treatment (Figure 6A). However, there was no major difference between control and minoxidil in cleaved caspase3 staining, suggesting absence of minoxidil-induced alteration in cell viability (Figure 6A). Testosterone levels tended to increase after 6 days of minoxidil exposure, but this was not seen after 9 days (Figure 6B).

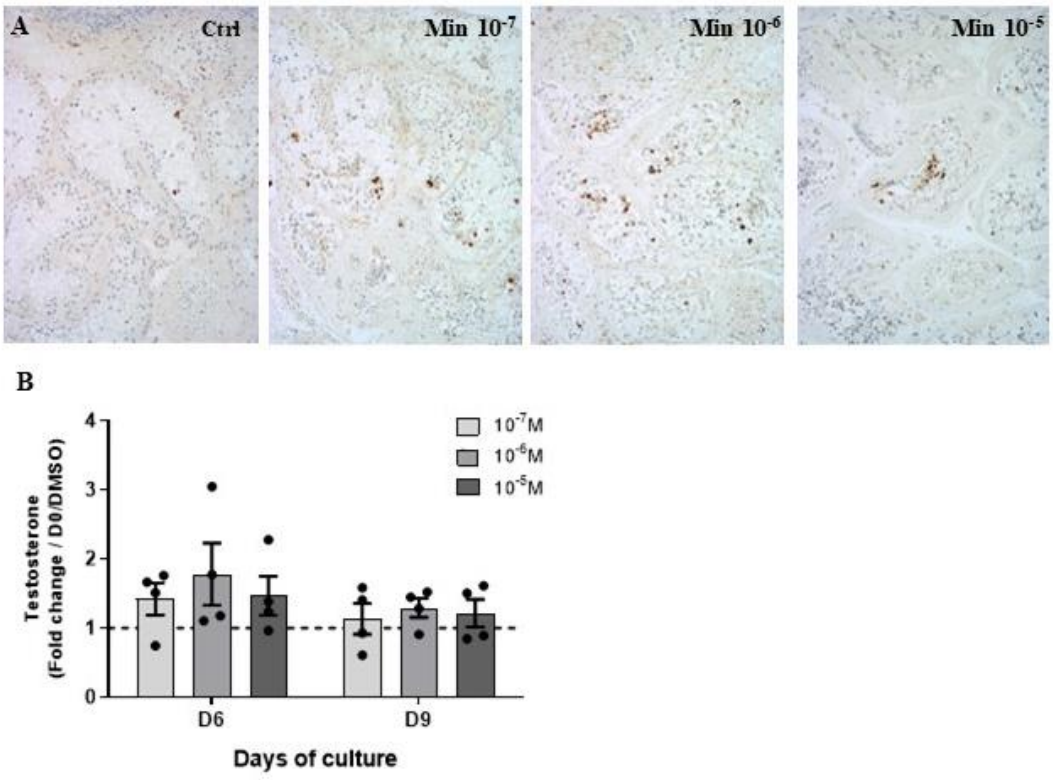

Figure 6. Nine-day long exposure of adult human testes to minoxidil. (A) Immunohistochemistry for cleaved caspase 3 in testicular explants after 9 days of exposure to vehicle control DMSO (Ctrl) or MIN at 10^{-7}; 10^{-6}; and 10^{-5} M. Scale bar: 100 µm. (B) Testosterone levels after 1 (24h) and 2 days (48h) of exposure to vehicle control DMSO (Ctrl)

or MIN at 10^{-7}, 10^{-6}, and 10^{-5}M. Data are the means +/- SEM of the fold change of the Ctrl (DMSO) explants of the corresponding donor. Individual values are shown as dots. A Wilcoxon test was performed for pairwise comparisons.

4 DISCUSSION

Minoxidil (topically or orally) is currently the most used treatment for men and women who suffer from androgenic alopecia, due to its efficacy and lack of side effects (ANASTASSAKIS, 2022). To the best of our knowledge, this is the first study to evaluate the direct impact of minoxidil on human fetal and adult testicular fragments *ex vivo*. Using an original approach of organ culture explants, our results show that minoxidil at the concentrations evaluated has no apparent effect at the cellular and endocrine levels in human fetal testis at 8-12 PCW. In adult organs, subtle changes were found in enzymes of the steroidogenesis pathway without alteration of testosterone levels after 2 days of exposure. After 6 days, testosterone levels tend to be elevated, an alteration that was attenuated at day 9. This was associated by a possible alteration of the germ cell lineage.

The approved treatment of alopecia with minoxidil is done through applications topically at the recommended doses of the drug (SUCHONWANIT et al., 2019). Its systemic bioavailability is minimal, thereby minimizing the risk of adverse effects in adults and potential complications in fetal differentiation during pregnancy (SUCHONWANIT et al., 2019). However, oral minoxidil has gained popularity due to its efficacy and convenience, particularly at low doses (ASILIAN et al., 2023). These low doses effectively stimulate hair follicles while minimizing side effects, as the effects of the drug are dose-dependent, higher doses increase the risk of side effects (MÜLLER RAMOS et al., 2023. Female pattern hair loss (FPHL), previously referred to as androgenic alopecia (AGA), is a prevalent condition in women, with two incidence peaks: one during reproductive years and another after menopause, suggesting a hormonal influence on its onset (BERTOLI et al., 2020). In a study conducted in a Brazilian population, FPHL was found in 32.3% of adult women, with prevalence increasing from 8% in those aged 20–29 to higher rates in older groups (MÜLLER RAMOS et al., 2023). Despite its benefits, caution is essential when prescribing minoxidil to women of childbearing age. Classified as risked for pregnancy category C by the FDA, minoxidil has been associated with potential adverse effects, including fetal malformations such as cryptorchidism (ROGERS et al., 2008). While category C indicates that risks cannot be ruled out, the potential benefits may

sometimes justify its use. This highlights the importance of this research for the safety and long-term effects of minoxidil, in the development of the male system. In healthy adults, the maximum plasma peak levels after oral treatment range between 16.8 ng/mL to 74.7 ng/mL (i.e. 8.03 10^{-8}M to 74.7 ng/mL: 3.5 10^{-7}M) (FLEISHAKER et al., 1989). We found no toxicity of minoxidil at a clinically relevant concentration of 10^{-7}M, but not at higher concentrations up to 10^{-5}M, either in fetal or adult tissue cultures. We therefore believe that it is unlikely that minoxidil would cause massive testicular toxicity *in vivo*.

Among the proposed mechanisms of action (MoA), the minoxidil-induced vasodilatation occurs through the opening of ATP-sensitive potassium channels, which enhance blood flow to the treated areas (GUPTA et al., 2021). It may also increase local angiogenesis, as reported in a case of a pregnant woman using topical minoxidil that experienced fetal malformations, including vascular abnormalities, such as excessive capillary formation (SMORLESI et al., 2003). In dermal papilla cells, MoAs include the dose-dependent increase of expression of vascular endothelial growth factor (VEGF) where it stimulates the formation of new capillaries around hair follicles (MESSENGER et al., 2004). In the mouse, VEGF-dependent angiogenesis and vasculogenesis from mesonephros-derived capillaries was shown to play a key morphogenetic role in the sex-specific vasculature and testis cord formation (COOL et al., 2010; SARGENT et al., 2015). Animal and *in vitro* study highlighted the crucial role of the VEGFA isoform in testis cord structure (SARGENT et al., 2015). In our experimental model, the testicular fragments used come from fetal tissues in which vascularization has already taken place, which precludes investigating a possible alteration in angiogenesis, especially as they are cultured without mesonephric tissue. In addition, minoxidil-exposed fragments had well-organized testis cords that expressed AMH and included germ cells. Although our results did not show significant changes in vascularity of the explants, it is possible that minoxidil is promoting the formation of new blood vessels. In adult testicular tissues, future studies should investigate whether minoxidil-induced angiogenesis might be related to the temporary increase in testosterone levels observed after 6 days of exposure.

Among the minoxidil MoA, interference with steroid biosynthesis and androgen action were proposed. Testosterone production relies on enzymes such as cytochrome P450 superfamily (CYP), including CYP11A1, CYP17A1 and CYP19A1, and hydroxysteroid dehydrogenases (HSD), which are essential for converting cholesterol into testosterone (CONNAN-PERROT et al., 2021). *In silico* evidence and exposure of human epidermal dermal

papilla cells to 10^{-7}M of minoxidil showed altered expression of CYP17A at the mRNA and protein levels, and altered CYP19A1 activity (SHEN et al., 2023), suggesting that CYP enzymes could be a target of minoxidil. We investigated the effects of minoxidil on human fetal testes between 8- and 12-weeks post-conception (PCW), after the differentiation of somatic Sertoli and Leydig cells (CONNAN-PERROT et al., 2021), as gonads prior to 7 weeks are undifferentiated and lack clear phenotypic sexual characteristics at this stage (MAKIYAN, 2016). In human fetal explants, the density of CYP11A1-positive Leydig cells was unchanged, we did not observe any change in staining intensity, and testosterone levels were unchanged after exposure to any concentration of minoxidil, suggesting that minoxidil may not interfere with this family of enzymes in the human fetal testis. In adult human testicular fragments, minoxidil decreased the CYP11A1 and CYP17A1 mRNA levels, while having no effect on enzymes and proteins investigated. This reduction suggests that minoxidil may interfere with the steroidogenic pathway, possibly affecting androgen production. Although testosterone levels were not altered after 2 days of exposure, the decrease in CYP17A1 expression may indicate a long-term impact on Leydig cell function. Furthermore, the reduction in SRD5A2 expression observed after minoxidil exposure may lead to a decrease in the conversion of testosterone to DHT, which could affect androgen signaling in the adult testis. The reason why CYP17A1 is insensitive to minoxidil in the human fetal testis but responsive in the adult testis requires further investigation. However, we must consider that limited data on minoxidil's placental transfer raises concerns about its fetal concentrations, and the immaturity of fetal testicular function may reduce responsiveness. Additionally, the timing of exposure may not coincide with critical developmental windows when the testis is most sensitive.

Another reported MoA of minoxidil is the alteration of testosterone to dihydrotestosterone (DHT) conversion. Indeed, minoxidil can reduce the expression levels of 5α-reductase type 2 (5α-R2, SRD5A2) in a human keratinocyte cell line (PEKMEZCI & TÜRKGLU, 2017). It was also reported to slightly increase 5a-reductase activity in cultured dermal papilla cells of bald scalp but not in dermal papilla cells from non-bald scalp and dermal fibroblasts (SATO et al., 1999), suggesting subtle local effects. In human epidermal dermal papilla cells, exposure to minoxidil decreased DHT levels (SHEN et al., 2023). The effect of minoxidil on SRD5A enzymes is questionable in the human fetal testis due to the lack of expression of the SRD5A2 isoform, at least at the mRNA level, and the specific expression of SRD5A1 in neuroendocrine cells (LECLUZE et al., 2020, LARDENOIS et al 2023). SRD5A3,

which is consistently found from fetal age to adulthood, plays a distinct role in cellular metabolism and protein glycosylation, with less clear functions about steroid metabolism (NICOLAOU et al., 2021). A single study dating back to 1982 indirectly measured 5α-reductase activity by evaluating the conversion of radiolabeled [³H]pregnenolone and [³H]progesterone steroid precursors into metabolites including DHT in human fetal testis homogenates (32 weeks old) (AZOURY & SPRINGER, 1982). It was shown that human fetal testis lacks 5a reductase activity, despite the presence of the enzyme since it can be influenced by steroids with 3-hydroxy structure (AZOURY & SPRINGER, 1982). The highly sensitive and specific mass spectrometry approach more recently showed that human fetal testis can produce DHT *ex vivo* (BEN MAAMAR et al., 2017), supporting the hypothesis of a backdoor pathway for the production of DHT that do not require SDR5A2 activity, but SRD5A1, and 3α-reductases AKR1C2 and AKR1C4 (FLÜCK et al., 2011). Nevertheless, the absence of testosterone level alteration after exposure to minoxidil suggests a minor impact of this pathway. Since expression of SRD5A2 was previously reported in differentiating human genital tract organs as the prostate, for example (ELLSWORTH & HARRIS, 1995), we cannot rule out an effect of minoxidil in the local conversion of testis-derived testosterone.

In the adult human testis, low levels of SRD5A1 and A2 isoform mRNAs were found in several single cell studies (NIE et al., 2022; Di PERSIO et al., 2021; GUO et al., 2018). SRD5A2 was consistently found in spermatids, while SRD5A1 is possibly expressed by immature germ cells and SRD5A3 in several cell types. Accordingly, DHT was measurable in culture media of adult human testes by using highly sensitive mass-spectrometry (DESDOITS-LETHIMONIER et al., 2017). Although testosterone levels were unchanged by either of the minoxidil concentrations after 2 days, a yet-to-be confirmed by additional samples increase of testosterone levels after 6 days would be consistent with a decreased SRD5A2 activity. In addition, levels of SRD2A2 were decreased after 2 days of exposure, overall suggesting that minoxidil may have an effect on human adult Leydig cell steroidogenic function. Overall, the transient increase in testosterone levels after 6 days of exposure, followed by a normalization at 9 days, may indicate an adaptive effect of Leydig cells to minoxidil, involving distinct sensitivities of several key enzymes of the steroidogenic biosynthetic pathway. Endocrine effects of minoxidil on the human adult testis warrants further investigation thanks to randomized controlled clinical trials with men exposed to minoxidil, although direct alteration

of testosterone synthesis can be compensated *in vivo* by pituitary feedback as shown for ibuprofen (KRISTENSEN et al., 2018).

Exposure of adult mice to minoxidil leads to oxidative stress, damaged testicular structures and reduced E2 levels (Chapter #2). Consistently, combined *in silico* and *in vitro* analyses revealed that minoxidil can suppress the expression of CYP17A1, boost the activity of CYP19A1, resulting in decreased formation and binding of DHT but also in increased E2 production without changes in their expression (SHEN et al., 2023). In human adult testis cultures, while CYP17A1 expression was decreased, that of CYP19A1 was unchanged after exposure to minoxidil, but this does not exclude a possible effect at the level of enzymatic activity. Further investigation of E2 levels in human adult testis fragments exposed to minoxidil is warranted.

As earlier introduced, drugs that have antiandrogenic properties can either reduce the amount of available testosterone or interfere with the AR signaling (EZECHIÁŠ et al., 2016). Minoxidil was previously shown to suppress AR functions, including transcriptional activity, protein stability, and interactions with cofactors, peptides, and DHT in several models including prostate cancer cell lines and dermal papilla cells (SHEN et al., 2023; HSU et al., 2014). Further study of the expression levels of typical genes sensitive to ARs in adult explants could provide clues to this hypothesis. Spermatogenesis is well known to be dependent on a finely tuned steroid balance, including both androgens and estrogens (O'SHAUGHNESSY et al., 2014; SMITH & WALKER, 2014; LUCAS et al., 2011). In short cultures, peritubular, Sertoli, and germ cells were screened to identify damage at the morphological and/or gene expression level, but no changes were identified for Sertoli and peritubular cells. By contrast, the reduction in CHEK1 and SYCP3 expression suggests that minoxidil may impair spermatogenesis at specific stages. Since CHEK1 is involved in cell cycle checkpoint, a decreased expression may reflect altered DNA damage management (ABE et al., 2018). In particular, whether minoxidil induces other modifications in the germ cell lineage such as epigenetic changes that could affect future generations warrants specific studies. Future studies are needed to clarify the long-term effects of minoxidil on adult testicular function, as early molecular disruptions as seen in 2 days may precede more substantial alterations over extended exposure periods.

5 CONCLUSIONS

Minoxidil is an effective and widely used treatment for androgenic alopecia, but we here addressed its possible direct effects on the human testis from fetal age to adulthood by using an original combination of organotypic cultures. We show that minoxidil does not cause significant changes in testicular differentiation or steroidogenic function in the human fetal testis. However, more studies are needed to demonstrate safety during pregnancy and breastfeeding, in particular in testicular hormone-target organs. By contrast, minoxidil may impact adult Leydig cell steroidogenic function by altering the expression of several genes of the biosynthesis pathway that may have important implications for male fertility, especially in patients using long-term minoxidil. Nevertheless, despite a trend for an increase after 6 days, testosterone levels were not massively altered by minoxidil. Although these data require further investigations of other testis-derived steroids like DHT and E2, minoxidil may induce discrete effects on adult testis steroidogenesis. In addition, alteration of expression of some markers of mature germ cells suggests a potential alteration in spermatogenesis, that may impact fertility. Studies on men populations investigating whether chronic exposure to minoxidil may lead to long-term testicular dysfunctions, such as changes in spermatogenesis or endocrine function would be of interest.

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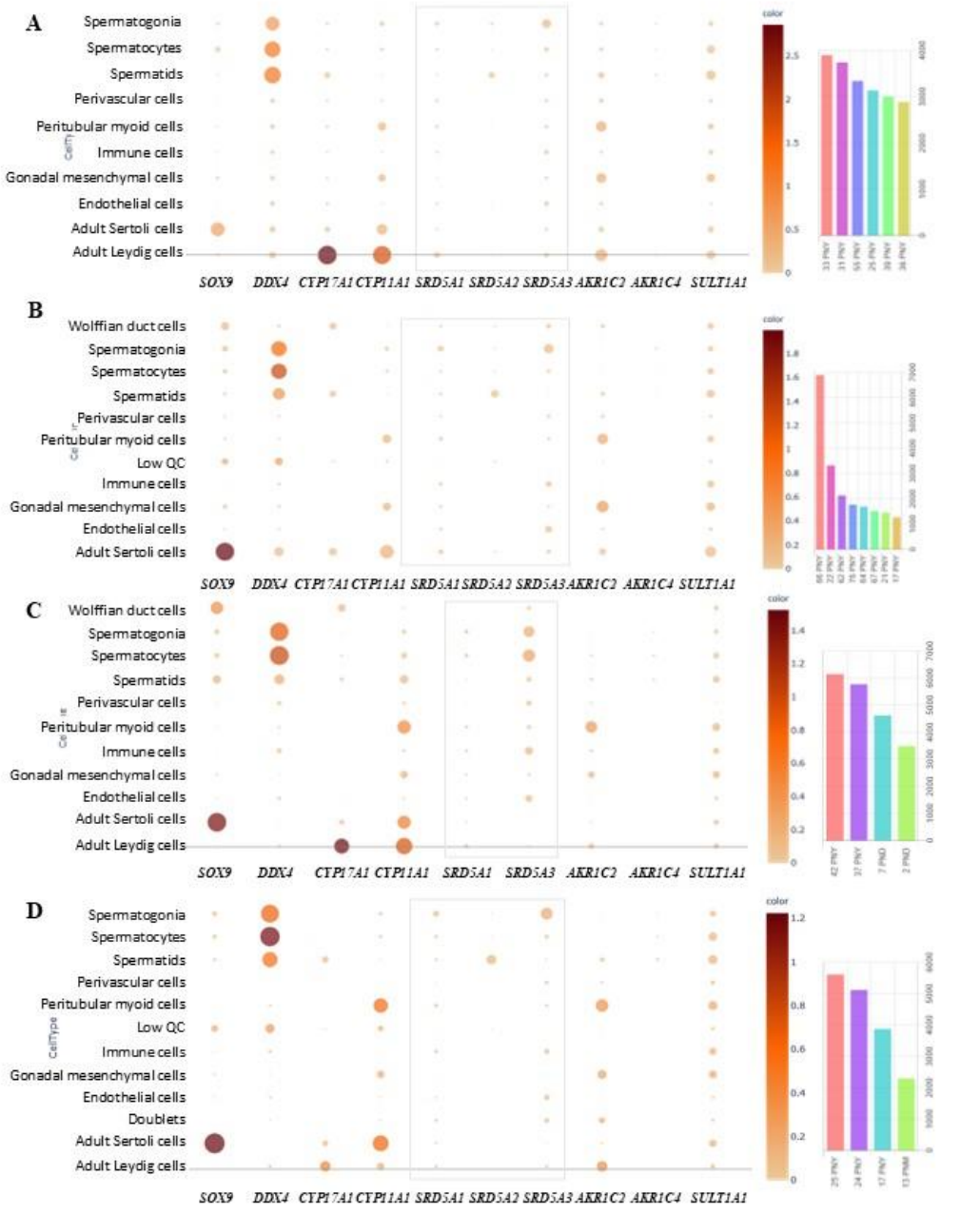
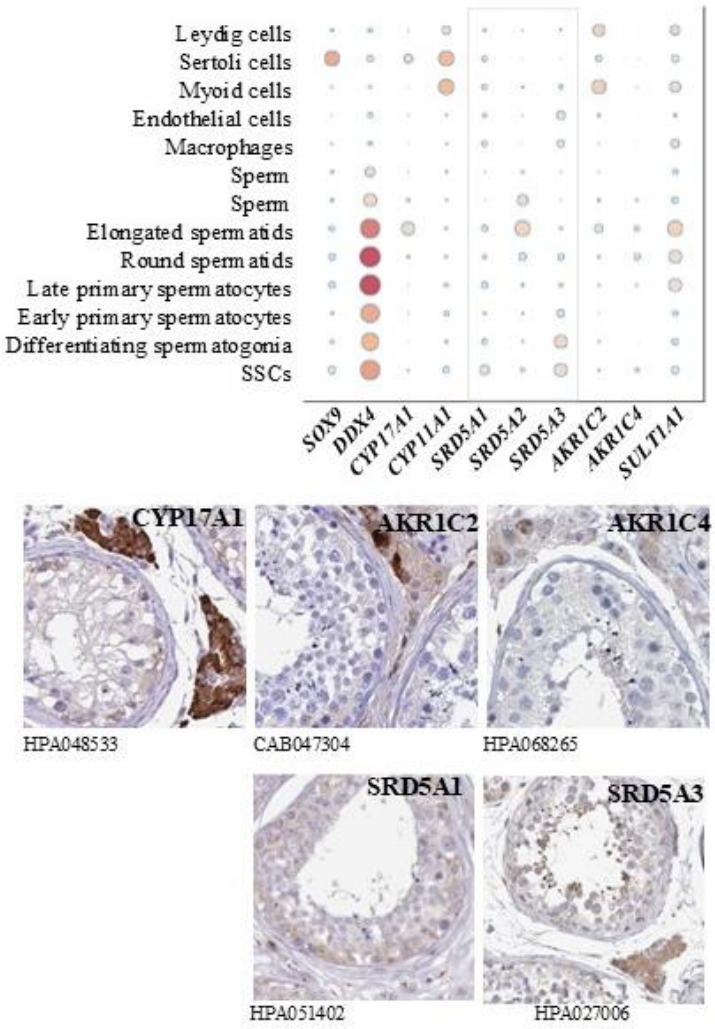
7 SUPLLEMENTAL MATERIAL

Supplemental Figure 1



Supplemental Figure 1. Comparative profiles of expression of genes in the adult human testis. Single cell RNA seq datasets were analyzed with ReproGenomics Viewer using datasets from Persio et al 2021 (A); Nie et al 2022 (B); Sohni et al 2019 (C); and Guo et al 2018 (D). Note that the population of Leydig cells is absent in the dataset from Nie et al and that SRD5A2 was not detected in the dataset from Sohni et al. Right panels indicate ages of samples (PND : post-natal day, PNY : post-natal year).



Supplemental Figure 2. Gene expression profiles in the adult human testis at the mRNA level (accessed from the human testis atlas https://humantestisatlas.shinyapps.io/humantestisatlas1/), and at the protein level (from the Human protein atlas https://www.proteinatlas.org/).

GENERAL CONCLUSIONS

The findings presented throughout this study offer a comprehensive evaluation of minoxidil's effects on male reproductive health, particularly regarding testicular and epididymal integrity. The systematic review (Chapter 1) revealed that minoxidil may exert mechanisms comparable to finasteride, a commonly co-prescribed drug, with both agents promoting oxidative stress and disrupting the blood–testis barrier. Although finasteride has well-documented detrimental effects on the epididymis, the impact of minoxidil on this organ had not been previously explored. *In vivo* experiments in mice (Chapter 2) demonstrated that minoxidil induces testicular morphological alterations, including epithelial detachment, vacuolization, and interstitial remodeling—most pronounced after 84 days of exposure to 5 mg/kg. While all tested doses induced mild epididymal changes, these did not significantly alter sperm transit time. These alterations were associated with reduced estradiol levels and oxidative stress markers. *Ex vivo* assays using human testicular tissues (Chapter 3) showed that minoxidil did not significantly impair fetal testis differentiation or steroidogenesis but may affect adult spermatogenesis by decreasing markers of mature germ cells and altering steroidogenic gene expression in Leydig cells. Collectively, the results suggest that chronic minoxidil exposure may have long-term reproductive consequences, warranting further investigation into its mechanisms of action and safety profile.

Future studies should examine DHT levels and related enzyme activity to elucidate minoxidil's pharmacodynamics. Investigations in pregnant mice are also needed to assess potential developmental impacts, given the paucity of data on placental transfer and male genital tract formation. Extended testicular organ cultures could be used to monitor testosterone production and evaluate the expression of key proteins, such as CYP17A1 and INSL3, to further characterize minoxidil's testicular effects. Finally, clinical studies in reproductive-age men exposed to oral minoxidil are essential to better understand its systemic and reproductive implications, considering species-specific hormonal regulation.