

UNIVERSIDADE FEDERAL DE VIÇOSA

**Chemical castration: the state-of-the-art and novel strategies involving eugenol
and magnetite nanoparticles**

Iara Magalhães Ribeiro
Doctor Scientiae

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Thesis submitted to the Veterinary
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degree of *Doctor Scientiae*.

Adviser: Mariana Machado Neves

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Assent:

Iara Magalhães Ribeiro
Author

Mariana Machado Neves
Adviser

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ABSTRACT

RIBEIRO, Iara Magalhães, D.Sc., Universidade Federal de Viçosa, August, 2026.
Chemical castration: the state-of-the-art and novel strategies involving eugenol and magnetite nanoparticles. Adviser: Mariana Machado Neves. Co-adviser: Renner Philipe Rodrigues Carvalho.

The development of safe, effective, and low-cost non-surgical contraceptive methods remains a major challenge for large-scale population control of free-roaming animals. Beyond veterinary applications, non-hormonal, localized approaches to male contraception may also offer insights relevant to human reproductive health, where non-surgical alternatives to vasectomy remain limited. To better understand this field and propose new strategies for male contraception, the present study was divided into two parts. First, a systematic review was conducted to compile chemical castration methodologies documented in the literature for mammals. The focus was on evaluating methods involving the local administration of sclerosing substances, considering the most explored mechanisms of action and the effectiveness of these products in reducing male fertility. The systematic review concluded that methods causing osmotic stress, such as calcium chloride and hypertonic saline, applied via the intratesticular route, were able to decrease serum testosterone concentration and cause seminiferous tubule atrophy and azoospermia, without triggering systemic effects. The experimental study with adult Wistar rats evaluated the effects of local application (intratesticular and intraepididymal) of eugenol on histological parameters of reproductive organs (experiment 1), as well as the intraepididymal application of eugenol (Eug), magnetite nanoparticles (FeO), and eugenol-coated magnetite nanoparticles (Eug-FeO; experiment 2). In experiment 1, the main findings were that intratesticular administration of eugenol triggered degeneration of the seminiferous epithelium, tubular atrophy, interstitial fibrosis, and reduced morphological parameters of Leydig cells, indicating impaired spermatogenesis and testicular function. Intraepididymal administration triggered epithelial necrosis, and fibrotic remodeling, culminating in a complete loss of sperm motility without significant morphological changes in the testis, showing that infertility can be induced by localized changes in the epididymis. In experiment 2, histopathological analysis revealed a temporal progression from inflammatory infiltrates at 24 h to nodular epididymal remodeling at 14 days. These lesions persisted at 80 days and were accompanied by luminal narrowing and absence of luminal spermatozoa, whereas testicular architecture remained preserved. Oxidative and nitrosative stress markers

increased transiently at 14 days, returning to control levels by 80 days. Proteomic analysis of epididymal fluid revealed distinct group-specific protein profiles. All treatments reduced sperm motility, whereas normal sperm morphology was reduced in the Eug and Eug·FeO groups, and sperm plasma membrane integrity only in the Eug group. Mating behavior was preserved, but the male fertility index dropped to 0% after Eug treatment and to 10% after FeO or Eug·FeO treatment, relative to the control group. Intratesticular and serum testosterone, serum FSH, LH, and physiological stress indicators were not affected by treatment. These findings demonstrate that intraepididymal administration induced persistent epididymal remodeling, associated with impaired sperm quality and reduced fertility, through a localized, non-endocrine mechanism. Functionalization of FeO with eugenol attenuated the biological effects observed with free eugenol, while still maintaining a reduction in male fertility, which supports the need for further investigation of this localized, non-hormonal contraceptive approach.

Keywords: chemical sterilization; male contraception; epididymis; eugenol; magnetite nanoparticles; nanotechnology

RESUMO

RIBEIRO, Iara Magalhães, D.Sc., Universidade Federal de Viçosa, agosto de 2026. **Castração química: estado da arte e novas estratégias envolvendo o eugenol e nanopartículas de magnetita.** Orientadora: Mariana Machado Neves. Coorientador: Renner Philipe Rodrigues Carvalho.

O desenvolvimento de métodos contraceptivos não cirúrgicos seguros, eficazes e de baixo custo permanece um grande desafio para o controle populacional em larga escala de animais de rua. Além das aplicações veterinárias, abordagens não hormonais e localizadas para a contracepção masculina também podem oferecer perspectivas relevantes para a saúde reprodutiva humana, área em que alternativas não cirúrgicas à vasectomia ainda são limitadas. Para melhor entender esse universo e propor novas estratégias para a contracepção masculina, o presente estudo foi dividido em duas partes. Primeiro, realizou-se uma revisão sistemática para compilar metodologias de castração química documentadas na literatura em mamíferos. O foco foi avaliar métodos que envolvessem a administração local de substâncias esclerosantes, considerando os mecanismos de ação mais explorados e a eficácia dos produtos na redução da fertilidade masculina. A revisão sistemática concluiu que os métodos que causam estresse osmótico, como cloreto de cálcio e salina hipertônica, aplicados por via intratesticular, foram capazes de diminuir a concentração de testosterona sérica e causar atrofia de túbulos seminíferos e azoospermia, sem desencadear efeitos sistêmicos. O estudo experimental com ratos Wistar adultos avaliou os efeitos da aplicação local (intratesticular e intraepididimária) do eugenol sobre parâmetros histológicos de órgãos reprodutores (experimento 1), bem como a aplicação intraepididimária de eugenol (Eug), de nanopartículas de magnetita (FeO) e de nanopartículas de magnetita revestidas com eugenol (Eug·FeO; experimento 2). No experimento 1, os principais achados foram que a administração intratesticular de eugenol desencadeou degeneração do epitélio seminífero, atrofia tubular, fibrose intersticial, e redução dos parâmetros morfológicos das células de Leydig, indicando comprometimento da espermatogênese e da função testicular. A administração intraepididimária desencadeou necrose epitelial e remodelamento fibrótico, culminando na perda completa da motilidade espermática sem alterações morfológicas significativas no testículo, evidenciando que a infertilidade pode ser induzida por alterações localizadas no epidídimo. No experimento 2, a análise histopatológica revelou uma progressão temporal, de infiltrados inflamatórios em 24 h a remodelamento epididimário nodular em 14 dias. Essas lesões persistiram

aos 80 dias e foram acompanhadas por estreitamento luminal e ausência de espermatozoides no lúmen, ao passo que a arquitetura testicular permaneceu preservada. Os marcadores de estresse oxidativo e nitrosativo aumentaram transitoriamente em 14 dias, retornando aos níveis do controle aos 80 dias. A análise proteômica do fluido epididimário revelou perfis proteicos distintos e específicos para cada grupo. Todos os tratamentos reduziram a motilidade espermática, enquanto a morfologia espermática normal foi reduzida nos grupos Eug e Eug·FeO, e a integridade da membrana plasmática espermática apenas no grupo Eug. O comportamento de acasalamento foi preservado, mas o índice de fertilidade masculina caiu para 0% após o tratamento com Eug e para 10% após o tratamento com FeO ou Eug·FeO, em relação ao grupo controle. A testosterona intratesticular e sérica, o FSH, o LH, e os indicadores de estresse fisiológico séricos não foram afetados pelo tratamento. Esses achados demonstram que a administração intraepididimária induziu um remodelamento epididimário persistente, associado a comprometimento da qualidade espermática e redução da fertilidade, por meio de um mecanismo localizado e não endócrino. A funcionalização do FeO com eugenol atenuou os efeitos biológicos observados com o eugenol livre, mantendo, contudo, a redução da fertilidade masculina, o que sustenta a necessidade de investigações adicionais sobre essa abordagem contraceptiva localizada e não hormonal.

Palavras-chave: esterilização química; contracepção; testículo; epidídimo; subfertilidade; nanotecnologia

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I. Introdução geral

O controle populacional de animais representa um desafio tanto para espécies domésticas quanto para espécies silvestres. Entre as domésticas, cães e gatos de rua impõem riscos à saúde pública (Ellwanger & Chies, 2019), ao bem-estar animal (Robertson, 2008) e à conservação da fauna nativa (Lessa et al., 2016; Doherty et al., 2017). Entre as silvestres, espécies invasoras — como os saguis do gênero *Callithrix*, amplamente disseminados em áreas urbanas e periurbanas do Brasil — competem com espécies nativas, promovem hibridização e alteram a dinâmica de ecossistemas onde foram introduzidas (Silva et al., 2017; Moraes et al., 2019). Em ambos os contextos, a esterilização cirúrgica permanece como a principal estratégia de controle reprodutivo. Entretanto, a aplicação da orquiectomia em programas populacionais de larga escala pode ser limitada pelos custos, pela necessidade de infraestrutura adequada, de profissionais capacitados e pelos cuidados pós-operatórios (Robertson, 2008; Jivago et al., 2021; Hess et al., 2024). Essas limitações têm estimulado a busca por métodos contraceptivos não cirúrgicos que sejam permanentes, seguros e de fácil aplicação. Para além do contexto de manejo populacional animal, o desenvolvimento de estratégias contraceptivas masculinas não cirúrgicas e não hormonais também dialoga com uma demanda ainda pouco atendida na saúde reprodutiva humana, na qual a vasectomia permanece a única opção não hormonal amplamente disponível.

Diversos agentes têm sido avaliados para induzir infertilidade por administração local, incluindo compostos esclerosantes, soluções hiperosmóticas e agentes citotóxicos. Embora muitos desses protocolos reduzam a fertilidade, os resultados permanecem heterogêneos quanto à eficácia, à duração da infertilidade e à ocorrência de efeitos adversos. Para reunir as evidências disponíveis e identificar as principais lacunas do conhecimento, foi realizada uma revisão sistemática da literatura, apresentada no Capítulo 2 (*Non-surgical sterilization of male animals using sclerosing agents: A systematic review of intratesticular and intraepididymal injection protocols*).

A revisão mostrou que a administração intratesticular foi utilizada na maior parte dos estudos disponíveis, enquanto a administração intraepididimária foi avaliada em um número reduzido de trabalhos. Também foram observadas diferenças importantes entre os protocolos experimentais, principalmente em relação aos agentes utilizados, às doses e ao período de acompanhamento. Esses resultados evidenciaram que ainda existem limitações para o desenvolvimento de métodos

quimioesterilizantes permanentes e reforçaram a necessidade de investigar novos agentes e novos locais de administração. Entre as lacunas identificadas, destacou-se o reduzido número de estudos direcionados ao epidídimo, apesar da importância desse órgão para a maturação, o armazenamento e o transporte dos espermatozoides (Robaire & Hinton, 2006; Dacheux & Dacheux, 2014).

Paralelamente, compostos de origem natural passaram a despertar interesse como potenciais agentes quimioesterilizantes. O óleo essencial de cravo-da-índia (*Syzygium aromaticum*), cujo principal constituinte é o eugenol, foi avaliado em diferentes modelos experimentais e demonstrou potencial para induzir alterações no sistema reprodutor masculino (Abshenas et al., 2013; Abu-Ahmed, 2015; Trindade et al., 2023). O eugenol apresenta propriedades antioxidantes, anti-inflamatórias e antimicrobianas, além de efeitos citotóxicos dependentes da concentração e da via de administração (Aburel et al., 2021; Carvalho et al., 2022). Resultados obtidos pelo nosso grupo demonstraram que a administração oral de eugenol reduziu as concentrações séricas de testosterona e comprometeu a viabilidade espermática em ratos Wistar (Carvalho et al., 2022), indicando que esse composto interfere na função reprodutiva masculina.

Considerando as lacunas identificadas na revisão sistemática e os resultados obtidos com o eugenol, foi conduzido um estudo preliminar, apresentado no Capítulo 3 (*Preliminary insights into intratesticular and intraepididymal administration of eugenol: histological alterations and impact on sperm motility in Wistar rats*). Nesse estudo foram comparadas as administrações intratesticular e intraepididimária de diferentes concentrações de eugenol em ratos Wistar, com o objetivo de caracterizar as alterações histológicas induzidas pelo composto e selecionar a estratégia experimental para o estudo subsequente.

As informações obtidas no estudo prévio permitiram o desenvolvimento do estudo apresentado no Capítulo 4 (*Intraepididymal injection of eugenol and magnetite nanoparticles reduces male fertility through localized, non-endocrine epididymal remodeling*), no qual foram avaliados os efeitos da administração intraepididimária de eugenol, de nanopartículas de magnetita e de nanopartículas revestidas com eugenol sobre parâmetros reprodutivos em ratos Wistar. A associação entre o eugenol e um sistema nanoestruturado foi proposta como estratégia para ampliar o potencial da

administração local e investigar uma abordagem ainda pouco explorada para contracepção masculina não cirúrgica.

Dessa forma, esta tese foi desenvolvida em etapas sequenciais, resultando em três estudos complementares, nos quais as evidências produzidas em cada etapa orientaram o desenvolvimento da etapa subsequente. Em conjunto, esses estudos ampliam o conhecimento sobre a quimioesterilização masculina, exploram o potencial do epidídimo como alvo para administração local e fornecem bases experimentais para o desenvolvimento de métodos contraceptivos não cirúrgicos, além de indicar novas perspectivas para futuras investigações.

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II. Capítulo 1 – Artigo 1: Non-surgical sterilization of male animals using sclerosing agents: A systematic review of intratesticular and intraepididymal injection protocols

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Non-surgical sterilization of male animals using sclerosing agents: A systematic review of intratesticular and intraepididymal injection protocols

Abstract

The overpopulation of free-roaming animals, particularly dogs and cats, poses a significant global public health concern, contributing to socioeconomic, ecological, political, and ethical issues. These animals reproduce freely, increasing the risk of zoonotic disease transmission, wildlife predation, and traffic accidents. Although surgical castration is the most widely used method for controlling the male animal population, it is costly, time-consuming, and requires specialized professionals and post-operative care. Therefore, there is an urgent need for practical, low-cost, large-scale, and welfare-friendly non-surgical sterilization methods. This systematic review aimed to evaluate the efficacy of intratesticular or intraepididymal injections of sclerosing agents for inducing permanent chemical sterilization. A total of 38 peer-reviewed articles were retrieved from Medline/PubMed, Web of Science, Embase, and Scopus. Among sixteen substances, calcium chloride was the most frequently studied, followed by zinc gluconate, hypertonic saline, chlorhexidine gluconate, mannitol, and glycerol. A single bilateral intratesticular and intraepididymal injection damaged testicular and epididymal tissues, thereby disrupting spermatogenesis, steroidogenesis, and sperm transport. Despite differences in the methodology and outcomes among studies, most tested agents showed promising results in disrupting reproductive function. None of the compounds fully met the two key criteria for their practical use, including the absence of side effects and consistent induction of irreversible azoospermia or infertility. Nevertheless, calcium chloride and hypertonic saline emerged as the most promising candidates, due to their robust mechanistic profiles, broad evidence base, and favorable cost-effectiveness. Therefore, researchers may prioritize these two agents in future studies to develop standardized, safe, and field-applicable protocols for large-scale population control.

Keywords: male sterilization; male contraception; chemical castration; testis; sperm.

2.1. Introduction

The overpopulation of free-roaming animals remains a global issue with significant implications for public health, environmental balance, and animal welfare. These animals may contribute to ecological disturbances, threatening wild animals through predation and competition for territory. Additionally, they may cause traffic accidents and increase the risk of zoonotic disease outbreaks, such as rabies, leishmaniasis, leptospirosis, toxoplasmosis, neosporosis, dirofilariasis, and brucellosis, affecting both humans and wildlife [1-4]. For decades, surgical castration has been considered the gold standard for male sterilization. Despite its recognized effectiveness, this method is limited to small-scale applications due to its cost, time-consuming nature, and specialized infrastructure requirements, including surgical equipment, trained professionals, a sterile environment, anesthesia, and post-operative care. Moreover, the procedure carries risks of hemorrhage, evisceration, mutilation, herniation, sepsis, and even death [5-8].

In response to these limitations, researchers have explored several non-surgical approaches since the mid-1970s, aiming to develop simpler, safer, and more cost-effective alternatives for male animal sterilization. Hormonal treatments are the most extensively studied strategies, with androgens [9], progestagens [10], and anabolic steroids [11] being investigated primarily in men, with no evidence of their use in other mammalian species. In contrast, antiandrogens were studied in rats and ferrets [12,13], androgen–progestagen combinations were tested in men and dogs [14,15], while gonadotropin-releasing hormone (GnRH) agonists and GnRH antagonists were evaluated in dogs [16] and goats [17], respectively. These compounds suppress reproductive function through receptor blockade or negative feedback mechanisms, leading to the downregulation of the hypothalamic-pituitary-gonadal (HPG) axis. Endocrine disruption affects the synthesis and secretion of GnRH, leading to the suppression of gonadotropin release and testosterone production. Consequently, spermatogenesis and secondary sexual characteristics and behavior are disrupted [18,19].

Although initially promising, hormonal treatments have failed to provide consistent and permanent sterilization. For instance, GnRH agonists have demonstrated better outcomes [15,16], but their temporary effects and the need

for repeated administration limit their use in large-scale applications. Dogs treated with deslorelin exhibited a transient increase in plasma luteinizing hormone (LH) and testosterone levels shortly after implantation. Later on, these hormone levels declined remarkably, resulting in undetectable levels in approximately 12 days and azoospermia due to suppressed spermatogenesis [20,21]. Tomcats' treatment with 4.7 mg deslorelin similarly caused low plasma testosterone, testicular volume, and disappearance of penile spines, with infertility expected upon complete testosterone suppression. However, azoospermia requires an additional period after hormone down-regulation [22]. Multiple studies have evaluated different deslorelin concentrations (2.1, 4.7, 6, 9.4, and 12 mg/implant) [20,23,24-29]. Collectively, these studies demonstrated that deslorelin implants represent a safe and well-tolerated approach for male sterilization. However, its treatment-induced effects are reversible, requiring a repeat treatment every 6 months for implants containing 4.7 mg and every 12 months for 9.4 mg deslorelin [30]. Similarly, immunocontraceptive strategies (e.g., targeting gonadotropins, GnRH [31,32], or zona pellucida proteins) have yielded variable results, with limited duration of azoospermia and the occurrence of adverse reactions [18,33,34].

Given the limitations of previously tested approaches, the direct injection of sclerosing agents into the testes, epididymides, or vas deferens has emerged as a practical and promising strategy for achieving permanent chemical sterilization. This method aims to induce irreversible degeneration of reproductive tissues, particularly the germinal epithelium, thereby impairing spermatogenesis and sperm maturation. Unlike surgical castration, direct injection offers advantages such as minimal invasiveness, reduced pain and stress, and feasibility for large-scale application [5,8]. Experimental trials have been conducted across diverse mammalian species using several types and formulations of chemical agents [5,6,35-39]. Despite their long-standing use in experimental settings, these compounds still require refinement and critical evaluation [7,40]. An ideal chemical sterilant for companion or stray animals should be safe, practical, cost-effective, and capable of inducing permanent infertility without systemic toxicity or adverse local effects [5,41,42].

Considering the diversity of tested substances, variations in methodologies, and the lack of consensus regarding their long-term efficacy and safety, a

comprehensive synthesis of the current literature is necessary to inform evidence-based decision-making. Therefore, this study aimed to systematically review the available scientific evidence on the use of intratesticular or intraepididymal injections of sclerosing agents for male chemical sterilization. By consolidating the findings of experimental studies, this review aims to support the development of standardized, field-ready protocols for application in both clinical practice and large-scale population control programs.

2.2. Methods

2.2.1. Search strategy

The research protocol for this systematic review was developed based on the PICOS strategy (Population, Intervention, Comparison, Outcome, Study design) [43], aiming to answer the following guiding question: *What is the efficacy of chemical sterilization in inducing permanent sterility?* The review is in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [44].

A comprehensive literature search was carried out using the electronic databases Medline/PubMed, Web of Science, Embase, and Scopus, including original articles published up to May, 2025. The search was performed electronically, using combinations of the following terms in titles, abstracts, and keywords: “chemical sterilization” OR “chemical castration” OR “chemical contraception” OR “medical castration”; AND testis OR testes OR testic* OR epididym* OR “efferent duct*”. The wildcard symbol “*” was used to capture variations of the search terms.

2.2.2. Studies reviewed

For a study to be included in this review, the following eligibility criteria had to be met: (1) use of chemical sterilization via intratesticular or intraepididymal injection; and (2) assessment of at least one sperm quality parameter, including structural (e.g., morphology, concentration, membrane or chromatin integrity) or functional (e.g., motility, viability, acrosomal reaction) analyses, or in vivo fertility testing. The inclusion of studies evaluating sperm parameters is justified by their recognized use as indirect indicators of male fertility potential, especially when fertility trials are not feasible [45].

Studies were excluded if they met any of the following criteria: (1) not related explicitly to chemical castration; (2) focused on cancer treatment; (3) involved human participants; (4) conducted on non-mammalian species; (5) employed chemical castration through alternative routes of administration (e.g., oral, subcutaneous, intraperitoneal); (6) lacked sperm quality/function assessments or in vivo fertility evaluation; (7) were review articles; or (8) had inaccessible full texts.

2.2.3. Data extraction and synthesis

Two independent reviewers screened the search results to determine whether the studies met the eligibility criteria. Any disagreements were resolved through consultation with a third reviewer. Data were extracted for each included study, encompassing the following elements: chemical compound, animal species/breed/lineage, administration method (route, single or multiple doses, unilateral or bilateral injection, and administered dose), post-intervention evaluation period, and sample size per group. Main findings related to the effects in hormones (testosterone, cortisol, LH, and follicle stimulating hormone [FSH]), testis (histology, biometry, antioxidant enzyme activity and oxidative stress markers), epididymis (histopathology, histomorphometry, and sperm content), semen (semen volume), sperm (motility, viability, concentration, and membrane and DNA integrity) and fertility performance (implantation sites and number of fetuses) and side effects were also extracted. Moreover, the reporting quality of all eligible studies was assessed using the SYRCLE Risk of Bias tool [46], developed by the Systematic Review Centre for Laboratory Animal Experimentation.

2.3. Results

2.3.1. Search results and study characteristics

The initial search retrieved 1,173 records from PubMed, Web of Science, Embase, and Scopus. An additional five records were identified through other sources. After removing duplicates and screening for eligibility, a total of 38 articles published between 1976 and 2024 were included in this systematic review (Fig. 1).

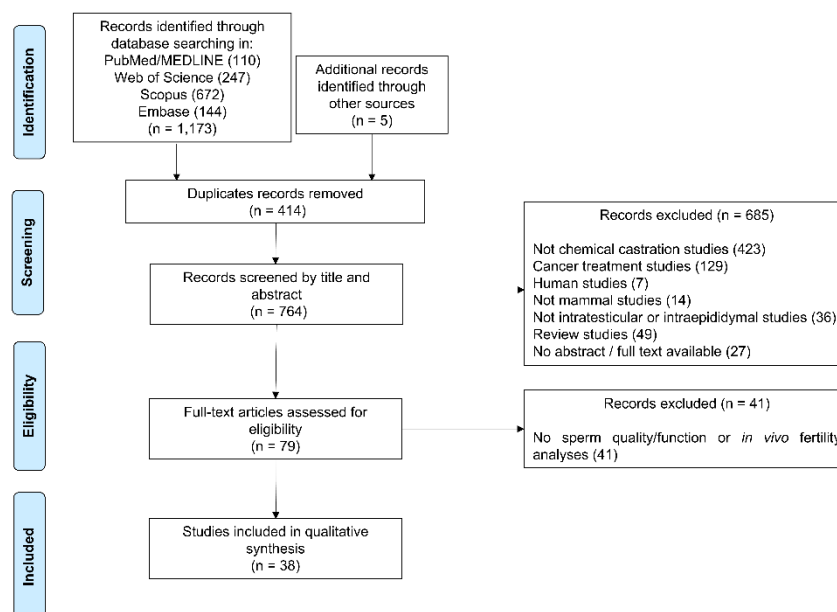


Fig. 1. Flow diagram of search results to define the articles to be included in the systematic review, according to PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses). (n) = number of studies

The most frequently studied chemical agent was calcium chloride (CaCl_2 ; $n = 14$), followed by zinc gluconate and hypertonic saline ($n = 6$ each), chlorhexidine gluconate ($n = 3$), mannitol ($n = 2$), and glycerol ($n = 2$; Table 1). Other agents evaluated included chlorhexidine gluconate + cetrimide, zinc acetate, zinc tannate, zinc arginine, benzalkonium chloride + cetrimide, 4-vinylcyclohexene 1,2-monoepoxide (VCM), ethanol, urea-ethanol, metallic copper particles, methoxy poly(ethylene glycol)-modified gold nanorods (GNR), and formalin ($n = 1$ each; Table 1).

In addition to chemical diversity, the studies employed a wide range of animal models, with dogs being the most frequently used ($n = 13$), followed by rats ($n = 9$), cats ($n = 5$), calves ($n = 2$; sexually immature males [up to 6–9 months of age]), and bulls (sexually mature males [from 12 months of age onward]), horses, donkeys, mice, goats, bucks, bears, guinea pigs, and monkeys ($n = 1$ each; Table 1). The predominant route of administration was a single bilateral intratesticular injection ($n = 32$), followed by a single bilateral intraepididymal injection ($n = 5$), two bilateral intratesticular injections ($n = 2$), daily bilateral intratesticular injections, and a single bilateral vas deferens injection ($n = 1$ each; Table 1). Three studies employed more than one administration route.

The volumes administered varied proportionally to animal size and administration route. In small animals, injection volumes ranged from 0.05 to 0.75 mL in rats, 0.2 to 0.3 mL in squirrel monkeys, and 0.25 mL in guinea pigs. Cats, dogs, and goats received volumes between 0.25 and 0.5 mL, 0.1 and 4.6 mL, and 1.0 and 2.5 mL, respectively. Larger animals, which required substantially greater volumes, received values between 1.0 and 4.0 mL in calves, 1.5 and 2.5 mL in bears, 10 mL in stallions, 15 mL in bulls, and up to 20 mL in donkeys. Notably, doses were sometimes expressed in concentration (e.g., 20% CaCl_2), mass (e.g., mg Kg^{-1}), or volume (e.g., mL/testis), reflecting methodological heterogeneity across studies.

Post-intervention evaluation periods also exhibited considerable variability. Broadly, assessment windows ranged from a few hours to several years, depending on the chemical agent (Table 1). Calcium chloride and zinc gluconate were evaluated over extended periods (7–365 days and 7–1,450 days, respectively), whereas mannitol (60 days), VCM (90 days), and formalin (60 days) had more limited assessment intervals. Some substances were monitored at multiple fixed points, including chlorhexidine and cetrimide (3 to 30 days), ethanol and urea-ethanol (1 to 6 months), and copper-oil mixtures (40 to 173 days). Ultimately, the number of animals per treated groups ranged from as few as 3 to as many as 55 animals, with most studies including 5 to 10 animals per group. Larger samples were occasionally observed in long-term evaluations, such as with zinc tannate in rats ($n = 55$) and calcium chloride in dogs ($n = 37$), reflecting variability in study design and scope. This temporal and quantitative heterogeneity highlights the variability in study design and follow-up rigor across the included literature.

Table 1. Characterization of studies included in the systematic review. Details include the chemical compound used, animal model, administration method, volume/dose administered, post-intervention assessment time, number of animals in treated groups, and study reference.

Chemical compound	Animal species/breed/lineage	Administration method	Volume/dose	Post-intervention assessment time	Sample size per group	References
Calcium chloride (CaCl ₂ .2H ₂ O)	Wistar rats	Single bilateral intratesticular injection	0.1 mL/100 g/body weight (BW) (2.5, 5, 10, 20 mg CaCl ₂ /100 g/body wt.)	30 days	12	[47]
Calcium chloride (CaCl ₂ .2H ₂ O)	Wistar rats	Single bilateral intratesticular injection	0.1 mL/100 g/BW (5, 10, 15, 20 mg CaCl ₂)	21 days	12	[48]
Calcium chloride (CaCl ₂ .2H ₂ O)	Stray dogs	Single bilateral intratesticular injection	1 mL in 1% lignocaine hydrochloride (5, 10, 15, 20 mg/Kg BW)	45 days	6	[49]
Calcium chloride dihydrate in saline solution	Cats	Single bilateral intratesticular injection	0.25 mL in 1% lignocaine hydrochloride (5, 10, 20% CaCl ₂)	60 days	6	[50]

Calcium chloride (20% CaCl ₂) in 1% lidocaine chlorhydrate	Mixed-breed dogs	Single bilateral intratesticular injection	0.8 – 1 mL (20g/100mL)	2, 6, 12 months	21	[51]
Calcium chloride (20% CaCl ₂) in 95% ethanol						
Calcium chloride (CaCl ₂ 10, 20, 30, 60%)	Mixed-breed dogs	Single bilateral intratesticular injection	0.8 – 1 mL	2, 6, 12 months	10	[52]
Calcium chloride dihydrate in saline solution (CaCl ₂ .2H ₂ O)	Guinea pigs	Single bilateral intratesticular injection	0.25 mL (15 mg/100 g)	90 days	10	[53]
		Daily bilateral intratesticular injection for 3 consecutive days				
Calcium chloride	Wistar rats	Single bilateral intratesticular injection	10 mg/100 g BW in 0.5mL distilled water 20 mg/100 g BW in 0.5mL distilled water	30 days	4	[54]

Calcium chloride (7.5% CaCl ₂) in 0.5% dimethyl sulfoxide (DMSO)	Crossbred dogs	Single bilateral intratesticular injection	1 – 4.6 mL (15 mg/Kg/testis)	15, 30, 60 days	6	[55]
Calcium chloride (20% CaCl ₂) in 0.5% DMSO	Stray cats	Single bilateral intratesticular injection	0.25 mL (20g/100mL)	30 days	6	[56]
Calcium chloride (20% CaCl ₂) in 95% ethanol	Crossbred dogs	Single bilateral intratesticular injection	0.1 – 1mL (20g/100mL)	3-9 months	37	[57]
		Single bilateral intraepididymal injection (caput)				
Calcium chloride (20% CaCl ₂) in 1% lidocaine	Mixed-breed stray tomcats	Single bilateral intratesticular injection	0.25 mL	60 days	6	[58]
Calcium chloride (40% CaCl ₂) in 0.5% DMSO	Nelore bulls	Single bilateral intratesticular injection	15 mL	15, 60, 150 days	20	[59]
Calcium chloride dihydrate in saline solution (20% CaCl ₂)	Wistar rats	Single bilateral intratesticular injection	0.3 mL	7, 14, 21 days	5	[60]

Zinc gluconate in 0.5% DMSO	Mixed breed dogs	Single bilateral intratesticular injection	26.2 mg/mL (0.5 – 2 mL)	Every 15 days totaling 12 evaluations (spermogram)	11	[61]
		Two bilateral intratesticular injections		120, 135, 150 days (libido)		
				5 and 6 months (ultrasonographic analysis)		
				7 months (histopathological analysis)		
0.2M of zinc gluconate (Testoblock®)	Mongrel dogs	Single bilateral intratesticular injection	13.1 mg/mL (0.2 – 1 mL)	Every 2 months until 180 days after treatment	5	[62]
0.2M of zinc gluconate (Testoblock®)	Mongrel dogs	Single bilateral intratesticular injection	13.1 mg/mL (0.2 – 1 mL)	Once weekly for the first 5 weeks after treatment, and then every 2 weeks until orchiectomy (semen evaluation and testosterone levels)	10	[63]

				Every 2 weeks for 90 days (libido)		
Zinc gluconate in arginine (Neutersol®)	Black bears (<i>Ursus americanus</i>)	Single bilateral intratesticular injection	1.5 – 2.5 mL (13.1 mg/mL)	57 – 1.450 days	8	[64]
Zinc gluconate in DMSO	Mongrel dogs	Two bilateral intratesticular injections in a 1- month interval	1 mL	15 days – 5.5 months after the 1 st injection	15	[65]
Zinc gluconate in arginine	Stray mongrel dogs	Single bilateral intratesticular injection	2 mL (10 mg/mL)	7, 15, 30 days	6	[66]
Hypertonic saline solution (20% NaCl)	Sprague-Dawley rats	Single bilateral intratesticular injection	750 µl (0.75 mL)	4 weeks	-	[67]
Hypertonic saline solution (20% NaCl)	Crossbred beef calves	Single bilateral intratesticular injection	Up to 4 mL	60 days	8	[68]
Hypertonic saline solution (15%, 20%, 25% NaCl) in 2% lidocaine	Feral cats	Single bilateral intratesticular injection	0.25 mL (NaCl 15%, 20%, 25%)	30 days	4	[69]

Hypertonic saline solution (20% NaCl)	Wistar rats	Single bilateral intratesticular injection	0.5 mL	60 days	10	[70]
Hypertonic saline solution (20% NaCl)	Donkeys	Single bilateral intratesticular injection	20 mL	60 days	3	[71]
Hypertonic saline solution (20% NaCl)	Goat kids	Single bilateral intratesticular injection	1 mL	2, 4, 6, 8, 9 months	8	[72]
Hypertonic saline solution (40% NaCl)						
Chlorhexidine (1%) in 90% DMSO	Stallions	Single bilateral intraepididymal injection (cauda)	10 mL	2, 3 years	16	[73]
Chlorhexidine gluconate (4%, 5% w/v)	Stray mongrel dogs	Single bilateral intratesticular injection	2 mL	7, 15, 30 days	6	[66]
Chlorhexidine gluconate (4%)	Stray dogs	Single bilateral intraepididymal injection (cauda)	0.5 – 1 mL	2-56 days	10	[74]
Mannitol (20%)	Wistar rats	Single bilateral intratesticular injection	0.5 mL	60 days	10	[70]

Mannitol (20%)	Donkeys	Single bilateral intratesticular injection	20 mL	60 days	3	[71]
Glycerol	Bolivian and Guyanan squirrel monkeys ((<i>Saimiri sciureus</i>))	Single bilateral intratesticular injection	0.2 – 0.3 mL	22 months	10	[75]
Glycerol (70%)	Egyptian Mau and short-haired cats	Single bilateral intratesticular injection	0.5 mL	3 weeks – 2 months	7	[76]
Chlorhexidine gluconate (0.3%) + cetrimide (3%)	Borno bucks	Single bilateral intratesticular injection	2.5 mL	3, 10, 16, 20. 30 days	6	[77]
Zinc acetate	<i>Bos indicus</i> bull calves	Single bilateral intratesticular injection	1 mL of 57.75 mg/mL (17.75 mg Zn) 1 mL of 71.75 mg/mL (21.4 mg Zn)	35 – 860 days	10	[78]

Zinc tannate	Holtzman rats	Single bilateral intratesticular injection	0.1 mL (3 mg zinc tannate)	4 hours – 1 year	55	[79]
Zinc arginine	Mixed breed dogs	Single bilateral intraepididymal injection (cauda)	0.5 mL (50 mg)	1 month – 1 year	10	[80]
Benzalkonium chloride (4%) + Cetrimide (BC) (0.4%)	Stray dogs	Single bilateral intraepididymal injection (cauda)	0.5 – 1 mL	2 – 56 days	10	[74]
4-vinylcyclohexene 1,2-monoepoxide (VCM) in 1% lidocaine hydrochloride	Guinea pigs	Single bilateral intratesticular injection Daily bilateral intratesticular injections for 3 consecutive days	240 mg/Kg	90 days	10	[53]
95% ethanol	Holtzman rats	Single bilateral vas deferens injection	50 µl (0.05 mL)	1, 2, 3, 6 months	6	[81]
0.006% urea in 95% ethanol						

Metallic copper particles (copper-oil mixture)	Wistar rats	Single bilateral intraepididymal injection (caput)	0.05 mL (200 mg/mL)	40, 50, 90, 165, 173 days	9	[82]
Methoxy poly (ethylene glycol)-modified gold nanorods (GNR) + near-infrared (NIR) irradiation	ICR mice	Single intratesticular injection	105 μ M	7, 30, 60 days	-	[83]
			145 μ M			
Formalin solution (10%)	Dogs	Single bilateral intratesticular injection	2 mL	60 days	6	[84]

2.3.2. Main findings

Herein, we describe the main findings for the sclerosing agents individually, along with their side effects, as reported by the authors. A summary of the main histopathological and functional alterations in the testis, epididymis, spermatozoa, and fertility outcomes associated with each sclerosing agent is in Figure 2.

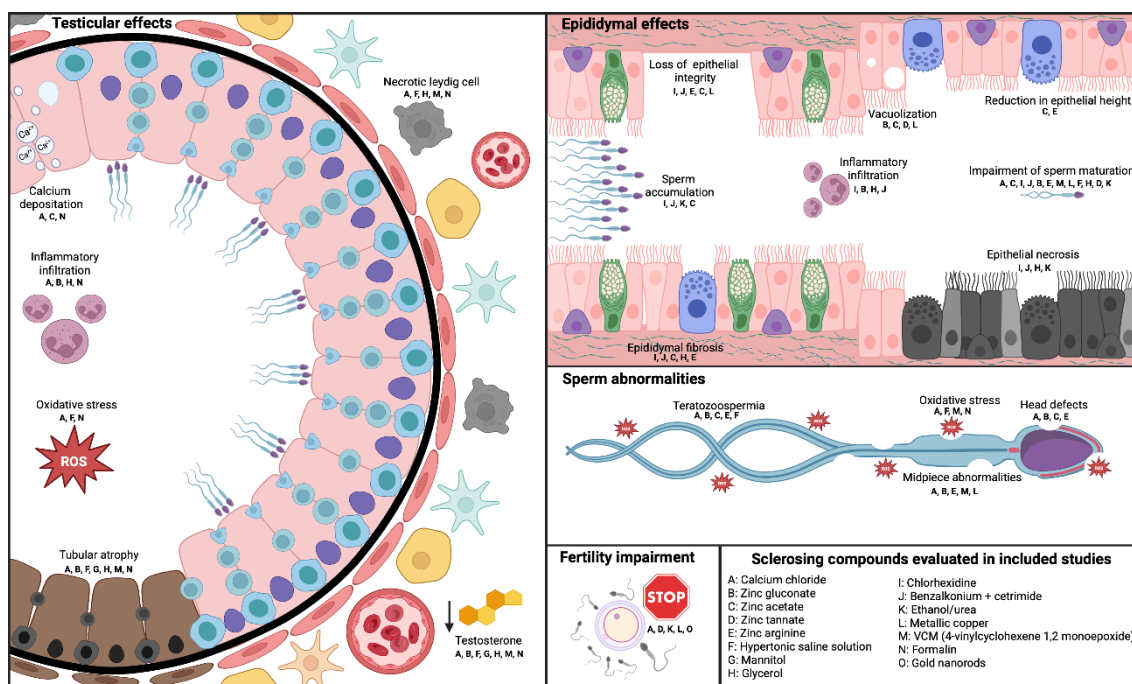


Figure 2. Schematic representation of the main testicular, epididymal, and sperm alterations, as well as fertility impairment, induced by intratesticular and intraepididymal administration of sclerosing agents in animal models. The compounds are coded from A to O at the bottom right corner. Each lesion or alteration is associated with the corresponding compounds based on the findings extracted from the included studies.

Calcium chloride was the sclerosing agent mostly studied (n =14)

The efficacy of calcium chloride was evaluated after administering via intratesticular (n = 13) and intraepididymal (n = 1) injections in rats, dogs, cats, guinea pigs, and bulls. Most studies (n = 11) documented alterations in hormonal balance, particularly reductions in serum [47,51-54,56,57,59] and intratesticular [48-50] testosterone levels (Table 2). Two studies reported only a non-significant downward trend [55,60], and one did not analyze hormonal parameters [58].

Serum testosterone levels reached values compatible with orchiectomy, indicating a suppression comparable to surgical castration [47,48,50,53,59]. Additionally, four studies reported increased circulating levels of LH and FSH following treatment [47-49,54]. While 12 studies reported no adverse effects [47-51,53-58,60], two documented significant complications, including testicular swelling, local inflammation progressing to scrotal ulcers and fistulas, and necrosis of the scrotal skin [52,59].

Histopathological evaluations revealed consistent findings in the testis (Table 2), such as severe disorganization of the seminiferous tubules, loss of compartmental boundaries, and intense leukocyte infiltration [47-50,52,55]. Other alterations included multinucleated giant cells [48,52], detachment and exfoliation of germ cells, germ cell depletion, and tubular atrophy. In advanced stages, the seminiferous epithelium was entirely degenerated, containing only Sertoli cells and spermatogonia [47-50,52,55-57]. Additionally, extensive necrosis of both germinal epithelium and Leydig cells was observed [47-50,52,53,55-57,60], often accompanied by calcium deposition, interstitial fibrosis, and mild hyalinization [47-50,52,53,55,57,58,60]. No signs of tissue regeneration were reported [49,50]. Two studies did not include histological analysis [51,59]. Alterations in histomorphometrical parameters were also widely reported. Ten studies documented modifications in testicular biometry, with reductions in testicular weight, width, volume, and organ indices of the epididymis and accessory glands (e.g., prostate and seminal vesicles) [47-52,54,56]. However, some findings diverged. Silva et al. [38] described a transient increase in testicular volume in the first days post-injection, while Yamada et al. [59] recorded persistent enlargement of the testes. Paranzini et al. [58] found no significant differences, and three studies did not assess these parameters [53,57,60].

Antioxidant responses in testis were evaluated in five studies, with four reporting reductions in the enzymatic activity of glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione S-transferase (GST), as well as lower levels of glutathione (GSH) and oxidized GSH (GSSG) and higher levels of malondialdehyde (MDA) [47-50]. Declines in testicular glutathione reductase (GRd), glucose-6-phosphate dehydrogenase (G-6-PDH), and γ -glutamyl transpeptidase (γ -GT) activities were detected as well [50]. In contrast, Hami et

al. [60] reported increased SOD and GPx activity, which may reflect differences in dose, species, or evaluation period.

Only one study investigated the intraepididymal route of calcium chloride administration [57], reporting azoospermia across all epididymal segments and the presence of calcified material within fibrotic capsules. Moreover, effects on semen quality and sperm function included high frequencies of teratozoospermia [55,68], necrospermia [58], oligozoospermia, or decreased sperm count and concentration [47-54,58,60], and azoospermia [51-53,55-57,59]. Sperm motility and viability were also negatively affected [52-55], along with reductions in semen volume [51,52,56]. Two studies described suppression of sexual behavior [48,57], and only one performed a direct fertility test (mating one male with three female rats for six days; n = 12 males per group), confirming infertility through the absence of implantation sites in mated Wistar rats [48].

Table 2. Summary of hormonal, testicular, epididymal, and sperm-related alterations induced by chemical sterilizing agents in animal models. Fertility performance and side effects were included when evaluated by the authors.

Chemical compound [References]	Hormonal effects	Testicular effects			Epididymal effects	Semen and sperm effects	Fertility performance	Side effects
		Histopathological damage	Testicular biometry	Antioxidant activity/oxidative stress markers				
Calcium chloride [47-60]	↑ plasma LH and FSH levels; ↓ plasma and intratesticular testosterone levels.	Disarrangement of the tubular architecture; presence of multinucleated giant and epithelioid cells; infiltration of leukocytes in tubular and interstitial tissue; degeneration and atrophy of seminiferous tubules; necrosis of interstitial cells of Leydig and whole germinal	↓ gonadosomatic index; ↓ testicular volume; ↓ testicular width, and weight.	↓ testicular GPx, SOD, GST, GRd; G-6-PDH, and γ-GT activities; ↓ testicular content of GSH/GSSG ratio; ↑ testicular contents of MDA and conjugated dienes.	Absence of sperm cells; Irregularly sparse encapsulations of calcified material in high-density areas of collagen fiber reactions.	Teratozoospermia; necrospermia; ↓ epididymal sperm count; azoospermia; ↓ sperm motility; ↓ sperm viability; ↓ semen volume.	Rare number of sperm in vaginal smears; no implantation sites in laparotomy.	Two studies described testicular swelling, local inflammation progressing to scrotal ulcers and fistulas, and necrosis of the scrotal skin.

		epithelium; calcium deposition in seminiferous tubules; presence of fibrous and hyaline tissue in tubular and interstitial spaces.						
Zinc gluconate [61-66]	↓ plasma testostero ne levels.	Degeneration of seminiferous tubules; loss of tissue architecture; intratubular calcification and fibrosis; vacuolated Sertoli cells; necrosis of seminiferous tubule; testicular atrophy; focal mononuclear inflammatory	↓ scrotal circumfere nce; ↓ paired testicular volume; ↓ testicular weight.	-	Focal mononuclear inflammatory infil-trate; reduced or no spermatozoa content.	Teratozoospe rmia; ↓ sperm motility; ↓ total sperm count; azoospermia; aspermia.	-	One study described systemic side effects (vomiting, anorexia, lethargy, and neutrophili a) and local adverse reaction (scrotal necrotizin g ulcers and

draining tracts).

No side effects.

		infiltrate, neutrophilic infiltration and calcification foci.					
Hypertonic saline solution [67-72]	↓ serum testosterone levels.	Degeneration and atrophy of seminiferous tubules; infiltration of immune cells in tubular space; degeneration of interstitial Leydig cells; dehydration of testicular parenchymal cells and extensive testicular fibrosis; dense and loose connective tissue with vascular components associated	↓ gonadosomatic index.	↓ total antioxidant capacity, GPX, SOD, glutathione; ↑ MDA, NADP+/NADPH	-	Teratozoospermia; necrospermia; ↓ sperm count; ↓ sperm total and progressive motility; ↓ sperm viability; ↑ % of sperm with DNA fragmentation (↓ sperm DNA integrity); ↑ % of damaged sperm plasma membrane functionality; aspermia; ↓ sperm concentration.	-

with
coagulative
necrosis of
Leydig cells
and
seminiferous
tubules;
vacuolation;
presence of
multinuclear
giant cells;
exfoliation of
germ cells;
degeneration
of germ cells
and spermatid
retention.

Chlorhexidine gluconate
[66,73,74]

-

↓ scrotal
circumference;
paired
testicular
volume;
testicular
weight.

-

Epididymal
tubules' wall
destruction;
infiltration of
inflammatory
cells;
formation of
sperm
granulomas;
hemorrhagic
suppurative
necrosis in
intra and

Azoospermia; -
↓ sperm
motility;
↓ sperm
viability;
↓ semen
volume.

One study
described
scrotal
edema,
hemorrhage in
cauda
epididymis,
hydrocele,
fibrosis of
the
vaginal

					extratubular fibrotic tissue.		and albuginea tunics with adhesions , and epididyma l sclerosis. Free of side effects.
Mannitol [70,71]	↓ serum testostero ne levels.	Testicular degeneration and atrophy; cracks, vacuolation, vascular occlusion, inflammatory cell infiltration, accumulation of edematous fluid and dilation of the interstitial space; degeneration of Leydig cells with pyknotic nuclei; amorphous and irregular Sertoli cells	↓ gonadoso matic index.	↓ total antioxidant capacity, GPX, SOD, glutathione; ↑ MDA, NADP+/NAD PH.	-	Necrospemia - ; ↓ sperm count; ↓ sperm motility; ↓ sperm viability; ↓ semen concentration; ↑ % of sperm with DNA fragmentation (↓ sperm DNA integrity); ↑ % of damaged sperm plasma membrane functionality.	

Glycerol [75,76]	↓ serum testosterone levels.	with smaller nuclei.	↓ testicular length; ↓ testicular weight.	-	Interstitial fibrosis, necrosis and hyalinization of tubular lining epithelium; cystic dilatation of tubules; absence of sperm cells.	Azoospermia. -	No side effects reported.
		Cystic dilatation; necrosis and atrophy of seminiferous tubules; infiltration of mononuclear cells; fibrous connective tissue proliferation in interstitial tissue.					
Chlorhexidine gluconate + cetrimide [77]	-	-	↓ scrotal circumference	-	-	↓ sperm motility; ↓ sperm viability; azoospermia, aspermia. -	Pyrexia (40±1.5 °C), reduced appetite, and depression.
Zinc acetate [78]	-	Degeneration and atrophy of seminiferous tubules;	↓ Testicular diameter and total	-	Reduced duct diameter and epithelial height;	Teratozoospermia; ↓ sperm motility. -	No side effects reported.

		Sertoli cells nuclear and cytoplasmic vacuolation; multifocal areas of granulomatous inflammation and mineralization; interstitial fibrosis.	testicular weight.		underdevelopment of microvilli; epididymal epithelium atrophy; epididymal ducts surrounded by embryonal appearing mesenchymal tissue; interstitial fibrosis; absence of sperm cells.			
Zinc tannate [79]	↓ plasma testosterone levels.	Decrease in cells of seminiferous tubules and Leydig cell population; caseous cell necrosis within seminiferous tubules.	↓ testicular weight	-	Attenuated cytoplasm; secretion of thick viscous material by gland cells; dark staining debris being sloughed from cells, and acinar cells not displaying cell	Denaturation of the DNA-protein complex.	None of the females that were mated with treated males became pregnant.	No side effects reported.

					boundaries; vacuoles within apical portion of the cytoplasm of epithelial cells; sloughing of cellular debris into the lumen;		
Zinc arginine [80]	-	↓ cross diameter of rete testis; ↑ connective tissue surrounding rete testis.	-	-	Absence of sperm cells; ↓ columnar epithelium height; ↑ fibrous tissue.	Teratozoospe rmia; Azoospermia; ↓ sperm motility; ↓ semen volume;	Free of adverse effects.
4% benzalkoniu m chloride + 0.4% cetrimide [74]	-	-	-	-	Epididymal tubules' wall destruction; infiltration of inflammatory cells and fibrin; formation of sperm granulomas; loss of tubular	Azoospermia; ↓ sperm motility; ↓ sperm viability; ↓ semen volume.	Severe local reactions, such as hydrocele formation, fibrosis of the vaginal tunic and tunica

					architecture; hemorrhagic suppurative necrosis in intra and extratubular fibrotic tissue; extensive and severe fibrosis.		albuginea, adhesions, hemorrhage, purulent inflammatory process in the cauda epididymis. Free of adverse effects.	
4-vinylcyclohexene 1,2-monoepoxide (VCM) [53]	↓ serum testosterone levels.	Testicular atrophy, fibrosis and necrosis; Leydig cells necrosis.	-	-	-	Azoospermia; ↓ sperm motility; ↓ sperm viability.	-	Free of adverse effects.
95% ethanol and 0.006% urea in 95% ethanol [81]	-	Occlusion of the vas deferens; testis with necrotic areas, atrophic with shrunken seminiferous tubules and extensive	-	-	Epididymis with areas of necrosis and tubules with sperm granuloma.	-	100% of the animals treated with either ethanol or with urea-ethanol mixture were infertile.	Free of adverse effects.

interstitial cell
hyperplasia.

Copper-castor oil mixture [82]	Serum testosterone levels showed no significant difference .	Pyknosis, vacuolation, karyorrhexis, and cytolysis; exfoliated cells, multinucleated giant cells, and acidophylic homogenous masses in the tubular lumen; seminiferous tubules depopulated, and only a single layer of cells consisting of Sertoli cells and spermatogoni	↓ testicular weight. -	The cytoplasm of the epithelial cells of the caput epididymis was stained paler than normal with perinuclear vacuolation and shedding of stereocilia; clumps of aggregated foreign particles found mainly in the interstitium and occasionally between the	↓ sperm count; ↓ % live sperm.	↓ fertility 40, 50, and 90 days after treatment; on day 165, the number of fetuses per rat was still significantly lower in the treated than in the control animals, but there was no significant difference	Abscess formation in the epididymides.
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		a remained; necrotic tubules.			epithelial cells. These particles were apparently copper.		in the numbers of pregnant rats between the 2 groups.	
Methoxy poly(ethylen e glycol)- modified gold nanorods (GNR) + near-infrared (NIR) irradiation [83]	No significant difference s in FSH, LH, and testostero ne plasma levels.	Degeneration of spermatogeni c (mainly spermatocyte s and spermatozoa) cells in seminiferous tubules.	↓ testis indices (weight % of testis to the mouse body weight).	-	Complete disappearanc e of sperm cells.	-	↓ fertility indices	No side effects reported.
10% formalin solution [84]	↓ testostero ne levels; ↑ cortisol levels.	Coagulative necrosis of seminiferous tubules, dystrophic calcification and hemorrhages. Interstitial	-	↑ MDA level; ↓ NO level; ↑ GSH level.	Azoospermia.	-	-	No side effects reported.

fibrosis and
heavy
infiltration of
inflammatory
cells as
lymphocytes.

Zinc gluconate (n = 6 studies)

The efficacy of intratesticular injection of zinc gluconate was evaluated in dogs and bears (Table 2). Of these, only half addressed hormonal changes. Vannucchi et al. [65] reported a reduction in serum testosterone levels, while Oliveira et al. [63] observed a gradual decrease in plasma testosterone levels over time. However, this decline was not statistically significant. Otherwise, Brito et al. [64] noted an unexpected increase in testosterone levels following treatment. These authors also observed systemic adverse reactions (e.g., vomiting, anorexia, lethargy, and neutrophilia) and local reactions observed after treatment, including scrotal necrotizing ulcers and the development of draining tracts, probably due to poor injection technique [64]. The other authors reported the absence of adverse reactions [61-66].

Histopathological effects were described in three studies, revealing significant testicular degeneration. Observed changes included vacuolation of the seminiferous epithelium, sloughing of germ cells into the lumen, reduction in epithelial height, and loss of multiple germ cell layers, culminating in complete degeneration of the seminiferous epithelium and collapse of tubule architecture [61,64]. Additional alterations involved the presence of multinucleated giant cells, vacuolated Sertoli cells, intratubular mineralized proteinaceous rings, and widespread hyalinization with eosinophilic necrotic cells [61]. Inflammatory responses were also reported, including focal mononuclear infiltration in both testicular and epididymal tissues, neutrophilic infiltration, and calcification foci [61,65]. Moreover, three studies assessed testicular biometry. Vannucchi et al. [65] reported reductions in scrotal circumference and testicular volume, and Virendra et al. [66] observed a decrease in testicular weight. Soto et al. [61] found no significant changes in testicular size following treatment.

Zinc gluconate administration also affected semen quality and sperm function. Several studies have reported abnormal sperm morphology (teratozoospermia), low sperm motility and concentration, and the occurrence of azoospermia or aspermia [61-63,65,66]. In the latter, azoospermia was observed in four studies [62,63,65,66], whereas two studies found no reduction in sperm count [61,64]. Nevertheless, Brito et al. [64] observed the presence of motile sperm in the epididymis, indicating an incomplete suppression of spermatogenesis.

Hypertonic saline solution (n = 6 studies)

The efficacy of intratesticular injection of hypertonic saline solution for permanent sterilization was studied in rats, calves, cats, donkeys, and goats (Table 2). While three of them reported a significant reduction in testosterone levels [68,70,71], the others did not assess hormone concentrations. Intratesticular injection of hypertonic saline resulted in a sustained decrease in serum testosterone, lowering it to levels comparable to those observed after orchiectomy [68]. The authors did not report side effects after treatment.

Histopathological analyses consistently revealed severe degenerative changes across all studies. The findings included intratubular vacuolization and maturation arrest of spermatogenic cells, ultimately leading to testicular atrophy [69-72]. Extensive immune cell infiltration and degeneration of Leydig cells were frequently observed [67,71]. Baqerkhani et al. [71] also described vascular occlusion, edematous fluid accumulation, and dilation of the interstitial space, along with degenerated Leydig cells exhibiting pyknotic nuclei. Sertoli cells appeared disconnected from germ cells and displayed amorphous morphology with irregular, condensed nuclei. Neto et al. [68] further reported complete dehydration of testicular parenchymal cells and extensive fibrosis, accompanied by coagulative necrosis of Leydig cells and seminiferous tubules. Moreover, three studies assessed testicular biometry, with only one [70] reporting a significant reduction in the gonadosomatic index (the ratio of gonad weight to body weight of the animal). Regarding oxidative stress analyses, Baqerkhani et al. [71] reported low levels of testicular total antioxidant capacity, GPx, SOD, and GSH, along with increased concentrations of testicular MDA and the NADP⁺/NADPH ratio, indicating oxidative imbalance.

Sperm quality analyses revealed various impairments. Teratozoospermia, aspermia [68], reduced sperm count/concentration, decreased progressive motility and viability, and complete absence of live spermatozoa were reported [69-71]. None of the authors reported induction of azoospermia. Additionally, three studies performed DNA integrity analyses, all of which showed an increased percentage of sperm with fragmented DNA, indicating compromised nuclear integrity [67,70,71].

Chlorhexidine gluconate (n = 3 studies)

Chlorhexidine gluconate, as a permanent sterilizing agent, was evaluated through intratesticular (n = 1) or intraepididymal (n = 2) injection in horses and dogs (Table 2). Only one study assessed hormonal effects [73], with no significant changes in testosterone or estrone sulfate levels. Testicular morphometry was negatively affected, with reductions in scrotal circumference, paired testicular volume, and testicular weight [66]. In the epididymis, chlorhexidine induced inflammatory responses and structural disruption (destruction of the duct wall and development of sperm granulomas). Other damages included severe hemorrhagic, suppurative necrosis with fibrotic involvement of intra- and extratubular tissues [74]. Sperm analyses revealed clear evidence of azoospermia in two studies [66,74], as well as low semen volume and low sperm motility and viability [73]. One study reported side effects after intraepididymal injection, including scrotal edema, hemorrhage of the cauda epididymis, hydrocele, fibrosis of the tunica vaginalis and tunica albuginea with adhesions, and epididymal sclerosis [74].

Mannitol (n = 2 studies)

The intratesticular use of mannitol as a sterilizing agent in rats and donkeys caused reductions in serum testosterone levels [70,71]. Histopathological analyses described vacuolization, formation of multinucleated giant cells, tubular depletion, and consequent testicular atrophy (Table 2). A decrease in the gonadosomatic index was also reported. Semen analysis revealed extensive impairments, including reduced sperm count without causing azoospermia, reduced sperm motility and viability, increased incidence of DNA fragmentation, and the absence of viable sperm cells [70,71]. The authors stated that mannitol is free of side effects.

Glycerol (n = 2 studies)

The results regarding intratesticular injection of glycerol were partially contradictory. In cats, Madbouly et al. [76] found a significant reduction in plasma testosterone levels, whereas Wiebe et al. [75] reported no significant hormonal changes in monkeys over 22 months. Histological findings were consistent across both studies and included cystic dilation, widespread degeneration and

necrosis of seminiferous tubules, and complete depletion of spermatogenic cells (Table 2). The interstitial compartment displayed fibrous tissue proliferation and mononuclear cell infiltration. Testicular biometric parameters were also affected, with reductions in average length [76] and weight [75]. Epididymal histology revealed fibrosis, necrosis, and hyalinization of the tubular epithelium, as well as the absence of spermatozoa in the lumen. These morphological changes were reflected in marked reductions in sperm concentration, culminating in azoospermia [76] and diminished epididymal sperm count [75]. Madbouly et al. [76] reported no side effects, and Wiebe et al. [75] did not specify any adverse effects [75].

Chlorhexidine gluconate + cetrimide (n = 1 study)

Mohammed and James [77] evaluated the intratesticular injection of a chlorhexidine gluconate and cetrimide combination for chemical sterilization. The authors reported reductions in scrotal circumference and sperm motility and viability, along with the occurrence of azoospermia and aspermia in goats. Meanwhile, this combination caused pyrexia (40 ± 1.5 °C), reduced appetite, and depression in all six animals for the first three days post-injection.

Zinc acetate, zinc tannate, and zinc arginine (n = 1 study each)

Cavalieri et al. [78] investigated the efficacy of intratesticular zinc acetate in calves. In the testis, the substance induced chronic degeneration and atrophy of the seminiferous tubules, characterized by nuclear and cytoplasmic vacuolation of the Sertoli cells. Histological alterations also included multifocal granulomatous inflammation, mineralization, and interstitial fibrosis (Table 2). Biometric parameters were affected, with reductions in mean testicular diameter and total testicular weight. Alterations in the epididymis included a decrease in duct diameter and epithelial height, accompanied by underdeveloped microvilli, epithelial atrophy, the presence of embryonal-like mesenchymal tissue surrounding the ducts, and interstitial fibrosis, resulting in the complete absence of spermatozoa. Semen analysis revealed teratozoospermia and a reduction in the median maximum number of progressively motile sperm (Table 2). Side effects were not mentioned by the authors [78].

Zinc tannate was injected into the testis of rats to verify its potential as a sclerosing agent by Fahim et al. [79]. Hormonal analysis showed a significant decrease in plasma testosterone levels, leading to a profound decline in serum testosterone, reaching values consistent with those typically observed following surgical castration (Table 2). Histopathological evaluation revealed disruption of seminiferous cell layers, decreased populations of germ and Leydig cells, and widespread necrosis, including karyolysis, anucleate cytoplasmic remnants, nuclear fragmentation, and large lysosome-like vesicles. Organelles were either absent or undergoing disintegration, and nuclear condensation was lost. The application of zinc tannate caused a reduction in the weights of the testis, epididymis, prostate, and seminal vesicles. Epididymal alterations included attenuated cytoplasm, thick viscous secretion, sloughed epithelial debris, and loss of acinar boundaries. Also, the authors described vacuoles in the apical cytoplasm, intraluminal debris from the ciliated surface, and denaturation of the DNA-protein complex. The authors did not assess sperm count. Fertility assessment was conducted by mating 100 male Holtzman rats with proestrus females every 2 weeks, and the females remained with the males for a period of 10 days. At the end of one year, none of the females mated with treated males became pregnant. Side effects were not mentioned by the authors [79].

In the case of zinc arginine, one study investigated its efficacy in dogs using intraepididymal injection. Zinc arginine administration in the cauda region resulted in a reduced rete testis diameter and increased surrounding connective tissue [80]. The epididymis exhibited epithelial flattening (mainly in the mid-section) and increased fibrosis in the cauda region, with no sperm presence in the lumen (Table 2). Additional findings included a higher incidence of sperm abnormalities, azoospermia, decreased motility, and reduced semen volume [80]. The authors stated that zinc arginine has no side effects.

Benzalkonium chloride + cetrimide (n = 1 study)

The combination of benzalkonium chloride and cetrimide was investigated in dogs through intraepididymal injection [74]. The authors reported a marked inflammatory response in the epididymis, characterized by mononuclear cell infiltration and the formation of sperm granulomas, resulting from the destruction of the epithelial duct (Table 2). Severe hemorrhagic suppurative necrosis in intra-

and extratubular tissues, along with extensive fibrosis, was observed. This treatment also resulted in oligospermia progressing to azoospermia, reduced progressive sperm motility and viability, and decreased semen volume. Benzalkonium chloride combined with cetrimide was associated with severe local reactions, including hydrocele formation, fibrosis of the vaginal tunic and tunica albuginea, adhesions, hemorrhage, and a purulent inflammatory process in the cauda epididymis [74].

4-vinylcyclohexene 1,2-monoepoxide (VCM) (n = 1 study)

Sen et al. [53] evaluated the effects of VCM in inducing permanent sterilization in guinea pigs (Table 2), stating, in the end, that VCM is free of side effects. They observed low testosterone levels after the intratesticular injection, reaching concentrations like those found after orchiectomy, indicating a marked suppression of gonadal androgen production. Histological alterations included a high incidence of testicular atrophy, extensive fibrosis, and distinct necrotic and degenerative foci, including necrosis of Leydig cells. Animals also presented low sperm counts, resulting in azoospermia, diminished sperm motility, and a low percentage of viable sperm [53].

Ethanol and urea-ethanol mixture (n = 1 study)

Raman et al. [81] reported the effects of injecting ethanol and a urea-ethanol mixture into the vas deferens in rats. Both treatments led to occlusion of the vas deferens. In the urea-ethanol group, the testes exhibited necrotic areas and were severely atrophic, characterized by shrunken seminiferous tubules and extensive interstitial cell hyperplasia (Table 2). In both treatment groups, the epididymis presented necrotic areas and tubules containing sperm granulomas. Sperm count was not evaluated in this study. Fertility efficacy test was assessed two weeks following treatment, by mating six male Holtzman rats with two mature females. At the end of five months, all treated animals (100%) were infertile. The authors stated that ethanol is free of adverse effects.

Metallic copper particles (n = 1 study)

Xu et al. [82] investigated the effects of metallic copper particles on male rats' fertility. Hormonal assessment indicated no significant interference with

serum testosterone levels (Table 2). Histopathological examination revealed signs of cellular damage, including pyknosis, vacuolization, karyorrhexis, and cytolysis. Multinucleated giant cells, exfoliated cells, and acidophilic homogeneous masses were occasionally found in the tubular lumen. Some seminiferous tubules appeared markedly depopulated, retaining only a single layer of Sertoli cells and spermatogonia, and necrotic tubules were sometimes observed adjacent to morphologically normal ones.

In the epididymis, epithelial cytoplasm appeared pale with perinuclear vacuolization and loss of stereocilia. Sperm analysis demonstrated reduced sperm viability and a lower sperm count in the cauda epididymis, without reaching azoospermia. Although fertility was significantly impaired following treatment, partial recovery was noted 165 days after the injection. The intra-epididymal injection of oil or copper-oil mixtures resulted in abscess formation in the epididymides, suggesting a predisposition to localized inflammation following administration [82].

Methoxy poly(ethylene glycol)-modified gold nanorods (GNR) (n = 1 study)

Li et al. [83] investigated the effects of GNR combined with near-infrared irradiation (NIR; Table 2) in ICR (Institute of Cancer Research) male mice. FSH, LH, and testosterone levels remained unchanged after treatment. On the other hand, reductions in testis and epididymis indices (organ-to-body weight ratios) occurred in mice from groups exposed to the highest dose and temperature. Histological analysis revealed degeneration and disorganization of spermatogenic cells, particularly pachytene spermatocytes, early spermatids, and spermatozoa. Sperm were absent at day 7 but partially recovered by days 30 and 60, except in animals treated with the highest dose and temperature, which resulted in complete sperm depletion. Six males treated with GNR mated with two virgin untreated females on days 7 and 60 after GNR+NIR treatment. The results showed that fertility indices dropped to ~10% in some groups but recovered to 50% by study end. The highest treatment group, in turn, achieved complete infertility. Li et al. [83] did not clarify whether adverse reactions occurred.

Formalin (n = 1 study)

Moustafa et al. [84] evaluated an intratesticular injection of formalin solution as a sterilizing agent in dogs. They observed a significant reduction in serum testosterone levels, reaching values consistent with those associated with surgical castration. Oxidative stress markers were altered in the serum, with elevated cortisol and MDA levels and decreased GSH and nitric oxide levels (Table 2). Seminal fluid analysis revealed a complete absence of spermatozoa. Histopathological examination revealed coagulative necrosis of the seminiferous tubules, dystrophic calcification, interstitial hemorrhages, fibrosis, and dense lymphocytic infiltration. The authors did not assess sperm count [84] and did not clarify whether adverse reactions occurred.

2.3.3. *Risk of bias*

The methods applied to set up a random sequence generation were not mentioned in 36 articles and were often defined as 'unclear' (Fig. 3). The other two remaining studies described a random component in the sequence generation process ($n = 1$), and used biological characteristic of the animals to allocate them ($n = 1$), generating bias in the formation of groups. In 36 articles, the researchers described the relevant baseline characteristics of the animals. In contrast, the baseline characteristics were not mentioned in the other two studies (Fig. 3). Allocation concealment, random housing, and blinding assessment were also not mentioned in any of the papers (Fig. 3). Random outcome assessment was not mentioned in 32 studies, while in the remaining six articles, researchers used a random component in sequence generation to evaluate the results. In 32 studies, the authors did not provide details on specific blinding measures, making it not possible to assess whether there was a risk of bias in the analysis of the results (Fig. 3). Six authors mentioned that the analyses were carried out by a researcher who was unaware of the group to which each animal belonged, whereas, in one article, the evaluator knew which intervention each animal received (Fig. 3). Regarding incomplete outcome data, there is no information on how many animals were excluded from the experiment or the reasons for the exclusions, making it impossible to determine whether there was bias in the exclusion of animals or in the treatment of incomplete data (Fig. 3). In all of the articles, all primary and secondary outcomes were reported, without selective omissions, minimizing the risk of manipulation of results. Thirty-five articles did

not report animal replacements, ensuring the integrity of the original sample. In contrast, three articles did not clearly indicate whether animal replacements occurred during the study (Fig. 3).

	Sequence generation	Baseline characteristics	Allocation concealment	Random housing	Blinding (Performance bias)	Random outcome assessment	Blinding (Detection bias)	Incomplete outcome data	Selective outcome reporting	Other sources of bias
[47]	?	●	?	?	?	?	?	?	●	●
[48]	?	●	?	?	?	?	?	?	●	●
[49]	?	●	?	?	?	?	?	?	●	●
[50]	?	●	?	?	?	?	?	?	●	●
[51]	●	●	?	?	?	?	?	?	●	●
[52]	?	●	?	?	?	?	?	?	●	●
[53]	?	●	?	?	?	?	●	?	●	●
[54]	?	●	?	?	?	?	?	?	●	●
[55]	?	●	?	?	?	?	?	?	●	●
[56]	?	●	?	?	?	●	?	?	●	●
[57]	?	●	?	?	?	?	?	?	●	●
[58]	?	●	?	?	?	●	?	?	●	●
[59]	?	●	?	?	?	●	?	?	●	●
[60]	?	●	?	?	?	?	?	?	●	●
[61]	?	●	?	?	?	?	●	?	●	●
[62]	?	●	?	?	?	?	?	?	●	●
[63]	●	●	?	?	?	?	?	?	●	●
[64]	?	●	?	?	?	?	?	?	●	●
[65]	?	●	?	?	?	?	●	?	●	●
[66]	?	●	?	?	?	?	?	?	●	●
[67]	?	●	?	?	?	?	?	?	●	●
[68]	?	●	?	?	?	?	●	?	●	●
[69]	?	●	?	?	?	?	?	?	●	●
[70]	?	●	?	?	?	●	?	?	●	●
[71]	?	●	?	?	?	●	?	?	●	●
[72]	?	●	?	?	?	?	?	?	●	●
[73]	?	?	?	?	?	●	●	?	●	●
[74]	?	●	?	?	?	?	?	?	●	●
[75]	?	●	?	?	?	?	?	?	●	●
[76]	?	●	?	?	?	?	?	?	●	●
[77]	?	●	?	?	?	?	?	?	●	●
[78]	?	●	?	?	?	?	●	?	●	●
[79]	?	●	?	?	?	?	?	?	●	?
[80]	?	●	?	?	?	?	?	?	●	●
[81]	?	●	?	?	?	?	?	?	●	?
[82]	?	●	?	?	?	?	?	?	●	?
[83]	?	?	?	?	?	?	?	?	●	●
[84]	?	●	?	?	?	?	?	?	●	●

Figure 3. Risk of bias assessment and methodological quality indicators for the 38 studies included in this systematic review evaluating the effects of intratesticular and intraepididymal injection of sclerosing agents in animal

models. The SYRCLE Risk of Bias (RoB) tool was used, with items categorized as “yes” (indicating low risk of bias), “no” (indicating high risk of bias), or “unclear” (indicating insufficient reporting, and therefore an unclear risk of bias).

2.4. Discussion

This systematic review aimed to investigate the potential of sclerosing agents in promoting male sterilization in mammals, primarily through intratesticular and intraepididymal interventions. Extensive research efforts have focused on the development of an efficient method of chemical sterilization that not only causes long-term infertility but also ensures animal welfare and is economically viable [51]. A chemical approach could replace surgical castration, which, for many years, has held the position as the primary method for sterilizing animals, often regarded as the gold standard [6]. Despite its recognized effectiveness, surgical approaches have notable limitations. They are typically employed on a small scale, have substantial costs, demand significant time commitments, and necessitate the expertise of qualified professionals. Additionally, individualized postoperative care is imperative due to the potential for severe postoperative complications [5,18,47-49]. Herein, sixteen substances/mixtures were applied through intratesticular and intraepididymal vias, causing histological alterations and compromising sperm parameters. However, the intensity of testicular lesions occurred depending on the route. Moreover, only five out of the sixteen substances were evaluated under the fertility approach, which is critical for identifying the potential of chemicals to act as sterilizing agents. Fertility defines the rate of reproduction and the ability to propagate generations. It involves quantifying the number of sperm with the ability to fertilize the oocyte, both in *in vivo* and *in vitro* conditions [45]. This measurement is typically conducted through semen analysis, despite its inherent limitations [85]. Nevertheless, semen analysis provides insights into the normal functioning of the male reproductive organs, enabling the indirect assessment of sperm quality to predict fertility potential [86].

An ideal chemo-sterilizing agent should be safe, cost-effective, and logistically feasible for large-scale application, making it a practical and sustainable solution for population control and animal welfare programs [50]. It may effectively induce permanent male infertility by disrupting or inhibiting four

relevant reproductive processes, spermatogenesis and steroidogenesis in the testis, and sperm maturation and transport in the epididymis, without affecting the overall health and well-being of the treated animals [49,50]. Disturbances in spermatogenesis and steroidogenesis occur through functional and tissue damage of the tubular (seminiferous epithelium with Sertoli cells and germ cells) and intertubular compartment (mainly Leydig cells), respectively. In the epididymis, sperm maturation can be affected by lesions in the epididymal epithelium, which alter the luminal microenvironment in which sperm cells are exposed, thereby impairing the acquisition of sperm motility and fertility. Moreover, damage in the epididymal structure may provoke a blockage of the luminal duct, hampering the progress of sperm transit through the epididymal regions until reaching the cauda epididymis [87,88].

Mechanisms of cellular damage induced by sclerosing agents

Regardless of the administration route, the cellular damage caused by the sclerosing agents reviewed in this study occurs via diverse and often overlapping mechanisms, including osmotic stress, mitochondrial dysfunction, oxidative imbalance, and disruption of membrane integrity. Excessive calcium chloride, due to its high solubility and rapid dissociation into calcium and chloride ions [89], causes a significant osmotic imbalance. Calcium ions, pivotal in intracellular signaling and apoptosis, and chloride ions, essential for maintaining osmotic pressure, are tightly regulated in physiological conditions [90]. Their elevation induces the rapid efflux of water from cells, resulting in dehydration, shrinkage, and structural alterations [91,92], which in turn lead to changes in gene expression and the activation of apoptotic pathways [93-95].

Zinc-based compounds (e.g., gluconate, acetate, tannate, and arginine) similarly impair cellular integrity through oxidative and mitochondrial pathways. Their administration disrupts the mitochondrial membrane potential, depletes glutathione, and increases the production of reactive oxygen species (ROS), thereby initiating lipid peroxidation and nuclear damage. These effects are often mediated by the upregulation of the Bax protein, contributing to mitochondrial apoptosis [96,97]. Likewise, copper particles induce severe oxidative injury. Copper ions (Cu^{2+}) catalyze the formation of hydroxyl radicals via Fenton-like reactions, targeting amino acids, nucleic acids, and lipids, with the subsequent

formation of damaging adducts [98]. Moreover, Cu^{2+} overaccumulation is reduced to Cu^+ through the activity of ferredoxin 1 (FDX1), disrupting Fe-S cluster protein synthesis and protein thioesterification, ultimately triggering proteotoxic stress and cell death [99].

Hyperosmotic agents such as hypertonic saline, mannitol, and glycerol exert damage through local osmotic shock and subsequent cellular dehydration. This process impairs DNA repair, transcription, protein synthesis, and mitochondrial function, increasing ROS levels and inducing apoptosis [100,101]. Concurrently, osmotic stress activates Mitogen-Activated Protein Kinases (MAPKs), including p38 and c-Jun N-terminal kinase (JNK), and triggers inflammation through the release of Tumor Necrosis Factor (TNF) and interleukins (IL)-1 β , IL-6, IL-8, and IL-18 [70,102,103].

The antiseptic chlorhexidine gluconate disrupts actin cytoskeletal integrity and mitochondrial function, leading to ATP depletion and inhibition of succinate dehydrogenase [104,105]. These changes are accompanied by elevated intracellular calcium and ROS generation, promoting apoptosis and necrosis. Similarly, benzalkonium chloride, when combined with cetrimide, induces membrane disruption through hydrophobic and ionic interactions, elevates ROS levels, causes glutathione depletion, and contributes to mitochondrial fragmentation and ATP loss [106,107].

Other agents also act through pathways related to oxidative stress. The VCN alters redox homeostasis through ROS/reactive nitrogen species (RNS) generation and glutathione depletion, leading to widespread biomolecular damage [108,109]. Ethanol promotes mitochondrial dysfunction, apoptosis, and inflammation via ROS production, B-cell lymphoma 2 (Bcl-2) family activation, and disruption of intracellular calcium signaling [110-112]. GNR triggers oxidative damage and apoptosis through protein denaturation, membrane destabilization, and DNA impairment [113,114]. Formalin also increases ROS, suppresses antioxidant defenses, and causes inflammatory responses, contributing to tissue injury [98].

Impact of intratesticular and intraepididymal injections on male organs and gametes

Collectively, the toxic mechanisms of the sclerosing agents contribute to extensive damage to the testis and epididymis tissue after treatment. Interestingly, most studies employed a similar methodology, focusing on a single bilateral injection, as this procedure is desirable for controlling stray dog and cat populations and managing reproductive strategies of wildlife. Nonetheless, the hormonal, testicular, epididymal, and sperm effects varied according to the location of administration. Intratesticular injection of sclerosing agents (Fig. 2) elicited a reduction in the testosterone levels [47,48,50,53,65,68,70,71,76,79,84], which was not observed in studies testing the intraepididymal route [57,73,74,80,82]. In the latter, testicular alterations were different from those observed in treatments using intratesticular injection, with only modifications in testis biometry (weight, volume, scrotal circumference, and gonadosomatic index) [73,74,80,82] and histomorphometry [80] when the application is in the cauda epididymis, with a more pronounced testicular damage when the injection occurred in the caput epididymis [57,82].

The effect on hormone levels after intratesticular injections occurs because Leydig cells are responsible for producing androgens, including testosterone. The local injection within the testicular tissue causes degenerative changes [116] in the interstitial compartment of treated animals, resulting in necrosis of Leydig cells (Fig. 2), a reduction in the Leydig cell population [79], and fibrosis [50,52,53,70,71], acting directly on the source of hormone production. The main consequence is the dramatic drop in testosterone levels, reaching levels close to those observed in orchiectomized animals. Significant changes in sexual behavior were evident, including a noticeable loss of libido. These alterations may also be due to the considerable reduction in testosterone levels, as testosterone is the prime regulator of male sexual behavior [41,48]. Additionally, the direct damage to the testicular tissue may induce the release of chemotactic factors, leading to leukocyte infiltration into the interstitial spaces and the generation of large amounts of free radicals, thereby exacerbating testosterone suppression.

In light of the foregoing, low testosterone may cause germ cell degeneration driven by the presence of sclerosing agents in the testis. Testosterone plays a pivotal role in maintaining the structural morphology and normal physiology of the seminiferous epithelium. The diminished intratesticular concentration of testosterone triggers the activation of testicular caspase-3 activity, a key mediator

of germ cell apoptosis [48]. Another way by which germ cell loss may occur is through the generation of large amounts of intratesticular ROS (Fig. 2). The histological consequence elicited by this altered hormonal and oxidative environment in the male gonad is the presence of multinucleated giant cells in the seminiferous tubules [117,118], germ cell detachment from the basal lamina of testicular tissue [119], release of immature germ cells (shed cells) into the intraluminal space [118], appearance of exfoliated germ cells within the lumen and leukocyte infiltrates in the seminiferous tubules [119]. Calcium deposition and tubular atrophy were extreme effects caused by eight of the sixteen sclerosing agents tested in the testis (Fig. 2).

Free radicals have inhibitory effects on both spermatogenesis [120,121], as well as on testicular steroidogenesis [122]. They can also be generated by leukocytes, triggering the creation of an oxidative environment [48,123]. Moreover, the reduced GST and GSH/GSSG ratio in the testis represents another cause for the degenerative necrosis of the seminiferous tubules, as glutathione plays a crucial role in regulating the proliferation and differentiation of germ cells and providing protection to them against the harmful effects of free radicals [124,125]. These free radicals can lead to deterioration of cellular structures and may cause lipid damage by lipid peroxidation [47,49,50]. The chemical treatments are also linked to a decreased content of testicular reduced GSH, a significant non-enzymatic antioxidant in the testis [126]. This reduction may be attributed to the lowered levels of testosterone [127,128]. Therefore, some researchers may infer that the testicular degeneration observed in their experiment is, to some extent, a result of the generation of free radicals within the testis. Induction of testicular degeneration and tubular atrophy by these chemical agents is supported by the low testicular somatic index in treated animals, as this is an accepted measure of testicular damage [129]. Also, the significantly decreased gonadosomatic index observed is consistent with the histopathological findings, once the degenerative changes and atrophy caused severe testicular shrinkage and testicular weight loss [70].

The main consequence of spermatogenesis disturbance induced by sclerosing agents applied via intratesticular injection is the decreased sperm count in the epididymis of treated animals. Once the epididymis receives sperm cells produced by the testis through the efferent ductules, the quantity of gametes

within the epididymal regions depends directly on the daily sperm production [130]. Aside from this, the morphology of sperm cells may alter during their production and passage through the epididymal environment. The increase in morphological sperm defects and decreased viability, primarily due to the impairment of the sperm cell plasma membrane, is regarded as a potential factor behind sperm immobility [131]. Numerous researchers have demonstrated that substances that disrupt membrane integrity can have a detrimental impact on sperm motility [132]. When these substances interact with the testicular tissue or epididymis, they disrupt the delicate balance required for the normal development of sperm cells. This disruption can lead to structural abnormalities in sperm cells, which in turn affect their morphology. This damage interferes with the formation of healthy and functional sperm, impacting their ability to move and fertilize an egg effectively [131,133]. As mentioned earlier, exposure to these substances can potentially increase ROS production, induce oxidative stress, and disrupt cellular homeostasis, ultimately leading to sperm DNA damage. This oxidative stress can directly damage the DNA structure within sperm cells, leading to DNA fragmentation, base modifications, and strand breaks. Reportedly, DNA damages such as fragmentation and denaturation may adversely affect fertilization and can result in male infertility [70].

Furthermore, studies employing direct injection into the epididymis or vas deferens revealed effects restricted to the epididymis and sperm cells. Some of the histological alterations described included the presence of inflammatory infiltrates, formation of sperm granulomas, fibrosis, and necrosis. Injection into the caput or cauda epididymis achieves azoospermia, confirming the blockage and the absence of immature spermatozoa [57,74,80]. The local induction of ROS production may explain the azoospermia through the injection of a chemical agent, which can activate matrix metalloproteinases, allowing for the degradation of tight junction proteins and subsequent disruption of the blood-epididymis barrier. As such, changes that alter the structure or function of the blood-epididymis barrier inevitably also affect sperm maturation and may obstruct sperm transport within the ducts [81,88,131,134]. As a result, spermatozoa cannot progress through the epididymis, leading to their absence in the ejaculated semen, impacting male fertility and reproductive health. Moreover, impairment of epithelial integrity leads to deficiencies in sperm maturation, as the

principal cells are unable to produce an adequate milieu for promoting sperm maturation. Hence, sperm cells can be present in the cauda epididymis, but they will not have the ability to fertilize an egg [87, 131].

The observed infertility outcomes strongly suggest that the injected substances likely interfere with the normal functioning of spermatozoa. The impairment of testicular spermatogenesis and steroidogenesis, and the structure and functionality of the epididymis, negatively affect the number of viable and functional sperm available for successful fertilization [130,131,135]. In the epididymis, the damage and inflammation contribute to the obstruction of sperm cells and impairment of sperm maturation. Concomitantly, these alterations contribute to decreased sperm count and concentration, sometimes culminating in azoospermia, a condition characterized by the absence of sperm in the ejaculated seminal fluid [136,137,138]. These multifaceted changes collectively serve as pivotal elements in male infertility, ultimately hindering successful conception, though significant methodological constraints must be considered when interpreting these findings.

Beyond the site of administration, the concentration and volume of injection represent critical determinants of the severity and extent of the lesions induced by sclerosing agents. These variables are inherently dependent on the species, organ size, and body weight of the target animal. Notwithstanding, there is currently insufficient evidence to establish standardized concentrations and volumes that reliably induce sterilizing lesions without systemic or local side effects [139]. Moreover, we must emphasize the relevance of the temporal dimension of the observed effects. Short-term evaluations predominantly document early responses, such as alterations in semen parameters (motility, morphology, viability), acute inflammatory infiltrates, and oxidative stress markers, which provide mechanistic insights but cannot alone confirm sterility. Conversely, long-term assessments demonstrate persistent testicular and epididymal degeneration, culminating in irreversible azoospermia and infertility. The integration of both approaches is therefore essential. Short-term studies elucidate immediate toxicodynamics, whereas long-term investigations are indispensable for confirming the irreversibility of lesions and the permanence of infertility. Future research must systematically address these parameters to

optimize protocols and validate safe, effective, and field-applicable non-surgical sterilization strategies.

Practical suitability of sclerosing agents

Sclerosing agents suitable for practical application must fulfill two criteria: (1) absence of significant adverse effects, and (2) induction of irreversible azoospermia or infertility (confirmed by fertility efficacy test), after a single administration [5,8,49]. Across the included literature, the agents studied converge on a set of mechanistic pathways that underlie azoospermia/infertility induction: (I) cytotoxicity to Leydig cells, resulting in reduced testosterone production; (II) damage to Sertoli cells and the seminiferous epithelium, leading to spermatogenic arrest; (III) stromal remodeling (fibrosis, hyalinization, calcification), which abolishes regenerative capacity; and (IV) epididymal obstruction, which prevents sperm maturation and transport. The extent to which each agent consistently achieves these mechanisms determines its suitability for practical use in the field. The findings of this systematic review highlight that, although several sclerosing agents demonstrated potential to impair fertility, none of the compounds fully met the two criteria established in this study to be considered suitable for practical application. Nonetheless, calcium chloride and hypertonic saline emerge as potential candidates compared to the other compounds evaluated.

Calcium chloride was effective in inducing long-term infertility after being tested in several species with multiple reports. This agent induces extensive necrosis of the entire testicular tissue, including Leydig cells, resulting in reductions in intratesticular and serum testosterone levels to concentrations comparable to those achieved by surgical castration. This suppression disrupts the endocrine environment necessary for spermatogenesis and contributes to atrophy of the epididymis and accessory sex glands, such as the prostate, bulbourethral gland, and seminal vesicles. Concomitantly, Sertoli cell damage and degeneration of the seminiferous epithelium led to profound disorganization of the tubular architecture, as well as germ cell depletion and tubular atrophy, thereby compromising spermatogenesis [48,49,50]. Moreover, calcium chloride triggers a strong oxidative stress response, which amplifies germ cell apoptosis, impairs Sertoli cell function, and promotes fibrosis and hyalinization of the

interstitial stroma, thereby abolishing regenerative capacity [50]. The downstream effects of these processes are reflected in semen quality and fertility outcomes. Calcium chloride treatment was associated with necropermia, teratozoospermia, reduced semen volume, decreased sperm motility and viability, and significant reductions in sperm concentration. These alterations culminated in azoospermia in most studies, and crucially, the fertility efficacy trial available demonstrated a complete absence of implantation sites in mated females, directly confirming the induction of infertility [48]. Behavioral studies have further reported the suppression of sexual activity, an additional advantage for managing stray animal populations. Although adverse effects have been occasionally reported, their sporadic occurrence and strong association with preventable technical issues suggest that calcium chloride, with optimized protocols, may become a practical chemosterilizing agent.

Hypertonic saline has emerged as another promising candidate for chemical sterilization, with several studies documenting convergent endocrine, histological, oxidative, and functional alterations in the male reproductive tract. Importantly, across all available reports, no severe adverse effects were observed, suggesting a favorable safety profile compared to other compounds. Although none of the studies explicitly confirmed infertility through fertility efficacy tests, the combination of findings strongly supports its potential to induce permanent sterility. This agent has a mechanism of action similar to that of calcium chloride, causing degeneration and necrosis of Leydig cells, reductions in serum testosterone levels, and, in some cases, effects comparable to orchiectomy. Altogether, these effects lead to spermatogenic arrest and atrophy of the seminiferous tubules. Concomitant Sertoli cell injury, manifested as loss of germ cell attachment, further disrupts the structural and nutritional environment necessary for spermatogenesis [67,68]. Combined with stromal fibrosis, these alterations represent irreversible testicular remodeling that eliminates the gonads' regenerative potential. Hypertonic saline exposure was consistently associated with teratozoospermia, aspermia, reduced sperm counts and concentration, decreased motility and viability, and, critically, a complete absence of live spermatozoa in ejaculates [70,71]. Although no author explicitly reported azoospermia, the presence of necropermia and the total absence of viable sperm cells strongly suggest functional infertility [72]. Notably, the lack of reported

systemic or local adverse effects distinguishes hypertonic saline from most other agents, reinforcing its potential as a safe, low-cost, and minimally invasive alternative to surgical castration.

In contrast, the remaining compounds reviewed, despite showing potential to impair fertility, present significant limitations that compromise their reliability as a field protocol. While some authors reported good tolerability and suppression of spermatogenesis, sterility was not consistently achieved, with most results indicating only subfertility and lacking fertility efficacy tests. For instance, in the case of zinc gluconate, there is documented evidence of systemic adverse effects (vomiting, anorexia, lethargy) and severe local reactions (scrotal ulcers, draining tracts), with incomplete suppression of sperm and hormonal production. Despite other substances showing no side effects (e.g., mannitol and glycerol), azoospermia was not consistently achieved, and fertility was not investigated, which limits confidence in its applicability. The remaining compounds (including zinc acetate, zinc tannate, zinc arginine, benzalkonium chloride + cetrimide, VCM, ethanol/urea-ethanol mixtures, metallic copper particles, formalin, and GNR) were generally tested in single studies, thereby undermining their reliability. These agents also either failed to demonstrate azoospermia, did not assess fertility outcomes, caused significant side effects, or required impractical conditions (e.g., gold nanorods combined with near-infrared irradiation).

Taken together, while no compound yet fulfills all requirements for practical application, calcium chloride and hypertonic saline stand out as the most promising agents. Calcium chloride provides robust evidence of long-term efficacy and induces infertility, but it requires careful refinement to address safety concerns. Hypertonic saline, on the other hand, combines strong mechanistic plausibility and an excellent safety profile, but awaits confirmation of its permanent effects through fertility efficacy trials. Looking ahead, the future of chemosterilization research should focus on refining these two most promising agents, calcium chloride and hypertonic saline, toward practical field application. For calcium chloride, optimization of formulation, solvent choice, dosing, and injection protocols is required to minimize adverse effects while preserving its proven efficacy. For hypertonic saline, the main gap lies in the absence of fertility efficacy trials and long-term follow-up, despite its favorable safety profile and strong mechanistic plausibility. More broadly, standardized methodologies,

cross-species validation, and systematic reporting of adverse outcomes are urgently needed. Addressing these knowledge gaps will be essential to translate experimental evidence into safe, effective, low-cost, and welfare-friendly non-surgical sterilization protocols for large-scale population control.

Review limitations

Despite the compelling evidence regarding the reproductive disruption induced by these sclerosing substances, the SYRCLE's risk-of-bias tool revealed a considerable number of unclear responses across the included studies. This ambiguity largely stems from insufficient methodological details and inadequate reporting practices, which hinder accurate assessment of potential biases. In several cases, key aspects such as randomization, allocation concealment, blinding, and baseline comparability were either not clearly described or entirely omitted. Such deficiencies limit the reliability of the internal validity assessments and increase the likelihood of methodological bias.

Another limitation concerns the broad taxonomic range of animal models employed. While this diversity enhances the general scope of investigation, it poses challenges for cross-species extrapolation. Physiological differences among species may significantly influence the pharmacological response and reproductive outcomes following chemical sterilization. Therefore, although the review provides valuable insights into the potential efficacy and safety of these substances, the heterogeneity of animal models demands caution in generalizing the findings, particularly when aiming to apply results to companion animals such as dogs and cats. Further studies using standardized protocols and species-specific models are necessary to confirm the reproducibility and translatability of the observed effects.

Ultimately, most studies lacked comprehensive assessments of long-term fertility outcomes. The absence of fertility efficacy tests limits the ability to draw definitive conclusions regarding the permanency of infertility induced by these agents. Fertility is a multifaceted endpoint, and its proper evaluation requires long-term monitoring beyond immediate histopathological and biochemical alterations. Moreover, many studies were conducted over relatively short durations, raising concerns about delayed or reversible effects that may not have been captured. Extended longitudinal studies are essential to clarify the

sustained impact of intratesticular or intraepididymal injections on reproductive function and to ensure the long-term safety and effectiveness of chemical sterilization protocols.

2.5. Conclusion

This systematic review underscores the complex and multifaceted consequences of chemical sterilization via intratesticular or intraepididymal injection. The substances analyzed induce profound alterations in the male reproductive organs, mainly after intratesticular injection. Consistent evidence points to the degeneration of germ and Leydig cells, failed production of testosterone, generation of oxidative stress with concomitant inflammatory infiltration, and severe impairment of epididymal sperm transport, culminating in azoospermia and potential infertility. Among the evaluated compounds, calcium chloride and hypertonic saline emerge as the most promising candidates and should be prioritized in future translational research. Calcium chloride has the most robust evidence base, with fertility trial confirming long-term infertility and sporadic local adverse effects. Hypertonic saline, in turn, demonstrated a favorable safety profile, with no reports of severe adverse effects, and induced profound testicular degeneration and absence of live spermatozoa, all indicative of irreversible infertility although definitive fertility tests are lacking. In contrast, other compounds reviewed were limited by scarcity of studies, which compromises reliability, inconsistent outcomes, lack of fertility testing, insufficient long-term data, or significant adverse effects. Until such evidence is consolidated, the promise of chemical sterilization must be approached with both scientific enthusiasm and cautious responsibility.

CRedit authorship contribution statement

Iara Magalhães Ribeiro and Renner Philipe Rodrigues Carvalho performed the conceptualization, methodology, formal analysis, and wrote the original draft of the paper. Arabela Guedes de Azevedo Viana, Camilo Jose Ramirez-Lopez, and Tayná Bolsam da Silva performed the conceptualization, methodology, formal analysis, and writing—review and editing. Mariana Machado-Neves carried out the supervision, funding acquisition, project administration, conceptualization, methodology, writing—review and editing, and funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest.

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III. Capítulo 2 – Artigo 2: Preliminary insights into intratesticular and intraepididymal administration of eugenol: histological alterations and impact on sperm motility in Wistar rats

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motility in Wistar rats**

Abstract

This study assessed the histopathological and functional consequences of single bilateral intratesticular (InT; 200, 20, and 10 mg) and intraepididymal (InE; 50, 5, and 2.5 mg) eugenol administration in *Wistar* rats. Animals were monitored for up to 60 days, and testicular and epididymal tissues were examined for morphological alterations and sperm motility impairment. Both administration routes induced marked structural damage to the male reproductive tract. InT application led to progressive degeneration of the seminiferous epithelium, tubular atrophy, interstitial fibrosis, and focal mineralization, accompanied by reductions in both Leydig cell volumetric proportion and nuclear volume—hallmarks of impaired spermatogenesis and disrupted lumicrine signaling. In contrast, InE administration caused epithelial necrosis, intraluminal cellular debris accumulation, fibrotic remodeling, and ductal occlusion, ultimately leading to complete loss of sperm motility due to impaired maturation and obstructed transport. Although no overt systemic toxicity was observed, the findings indicate that local eugenol delivery exerts direct cytotoxic effects and promotes irreversible tissue remodeling. These results highlight eugenol's potential as a chemical sterilant and reinforce the need for further studies to evaluate the reversibility of reproductive damage, inflammatory sequelae, and interspecies variability in toxicological responses.

Keywords: chemical castration; *Syzygium aromaticum*; toxicology; morphology.

3.1. Introduction

Male reproductive health has garnered significant attention due to its pivotal role in human fertility (Khourdaji et al., 2018), wildlife conservation (Holt and Comizzoli, 2022) and livestock productivity (Thundathil et al., 2016). Moreover, the pursuit of novel male contraceptive approaches, both reversible and permanent, has intensified, encompassing not only pharmacological modulation but also chemical sterilization strategies as alternatives to surgical castration (Khourdaji et al., 2018; Hess et al., 2024). In parallel, the pharmacological exploitation of natural products has gained considerable traction, particularly regarding phytochemicals with potential to interfere with spermatogenesis, steroidogenesis, and epididymal function (Carvalho et al., 2022). Several plant-derived compounds have been reported to impair male fertility through endocrine disruption, germ cell apoptosis, or impairment of sperm maturation (Abshenas et al., 2013).

Among these phytochemicals, clove oil (*Syzygium aromaticum*) has attracted particular attention for its potential application in chemical sterilization protocols, owing to its high content of eugenol - a bioactive phenolic compound with potent pharmacological properties. Experimental studies in dogs (Ahmed, 2015; Aponte et al., 2024) and pigs (Trindade et al., 2023) have shown that InT administration of clove oil promotes pronounced testicular degeneration, suppression of steroidogenesis, and, in some cases, irreversible infertility. Nonetheless, these effects are markedly influenced by interspecies variability, dosage, and administration route. Eugenol (4-allyl-2-methoxyphenol; $C_{10}H_{12}O_2$), the predominant phenolic constituent of clove oil, exhibits a dual pharmacological profile: at low concentrations, it confers cytoprotective effects via antioxidant and anti-inflammatory pathways, whereas at higher doses, it exhibits cytotoxic effects,

functioning as a pro-oxidant and inducing oxidative stress, mitochondrial dysfunction, and apoptosis in various tissues (Aburel et al., 2021; Carvalho et al., 2022, Carvalho et al., 2025).

While the systemic pharmacodynamics of eugenol have been extensively characterized, its direct impact on male reproductive structures - particularly the testes and epididymal ductal system - remains insufficiently elucidated. Mechanistically, eugenol's high lipophilicity enables membrane partitioning and mitochondrial dysfunction (complex I inhibition/ $\Delta\Psi_m$ dissipation), with dose-dependent redox effects and modulation of inflammatory and cell-cycle pathways; however, how these processes affect spermatogenesis and epididymal function remains poorly characterized and not fully elucidated (e.g., Aburel et al., 2021; Taghipour et al., 2023). Given the epididymis's pivotal role in sperm maturation, acquisition of motility, and post-testicular sperm viability (Robaire & Hinton, 2006; Dacheux & Dacheux, 2014), the local effects of eugenol on this organ deserve further investigation. In this context, the objective of this study was to evaluate the histological alterations and effects on sperm motility resulting from intratesticular (InT) and intraepididymal (InE) administration of eugenol in Wistar rats at 15, 30, and 60 days post-injection.

3.2. Material and methods

Sixty-four *Wistar* rats (70 days old; 230–250 g) were obtained from the Central Animal Facility of the Universidade Federal de Viçosa (UFV) and housed individually in polypropylene cages under controlled photoperiod (12 h light/12 h dark) and temperature ($21 \pm 2^\circ\text{C}$). Food and water were provided ad libitum. The experimental design and procedures were approved by the Ethics Committee on Animal Use of UFV (CEUA/UFV, protocol no. 35/2023) and conducted in

accordance with the guidelines of the Brazilian National Council for the Control of Animal Experimentation (CONCEA). Throughout the study, animals were monitored daily for signs of distress, pain, or behavioral alterations, including changes in locomotion, feeding, and grooming behavior. The study was divided into two phases (Fig. 1). In phase one, 24 rats were randomly assigned to eight groups ($n = 3/\text{group}$) to evaluate the effects of different doses of eugenol (Sigma-Aldrich) (Fig. 1A). Four groups received bilateral InT injections: (1) Control: 200 μL of 2% Tween-20 in distilled water (used as vehicle); (2) Eugenol 200 mg (pure); (3) Eugenol 20 mg; and (4) Eugenol 10 mg, with the latter two diluted in 200 μL of vehicle. The remaining four groups received bilateral InE injections in the epididymal caput: (5) Control: 50 μL of vehicle; (6) Eugenol 50 mg (pure); (7) Eugenol 5 mg; and (8) Eugenol 2.5 mg, with the latter two diluted in 50 μL of vehicle. All injections were performed on day 0 (D0) following sedation with ketamine (75 mg kg^{-1}) and xylazine (10 mg kg^{-1}). A scrotal approach was used in all animals. For the InT group, the needle was inserted through the scrotal skin at the caudal pole and gently advanced into the middle third of the testicular parenchyma, avoiding the rete testis and epididymal tail. For the InE group, injections were directed into the caput epididymidis, which is readily palpable and well defined in Wistar rats. In both cases, solutions were delivered slowly using insulin syringes (29G; $0.33 \text{ mm} \times 13 \text{ mm}$) to minimize tissue trauma and reflux. Animals were monitored daily, and euthanasia was performed on day 15 (D15) via deep anesthesia with xylazine (45 mg Kg^{-1}) and ketamine hydrochloride (240 mg Kg^{-1}). Testes and epididymides were collected and fixed in Bouin's solution for 24 hours.

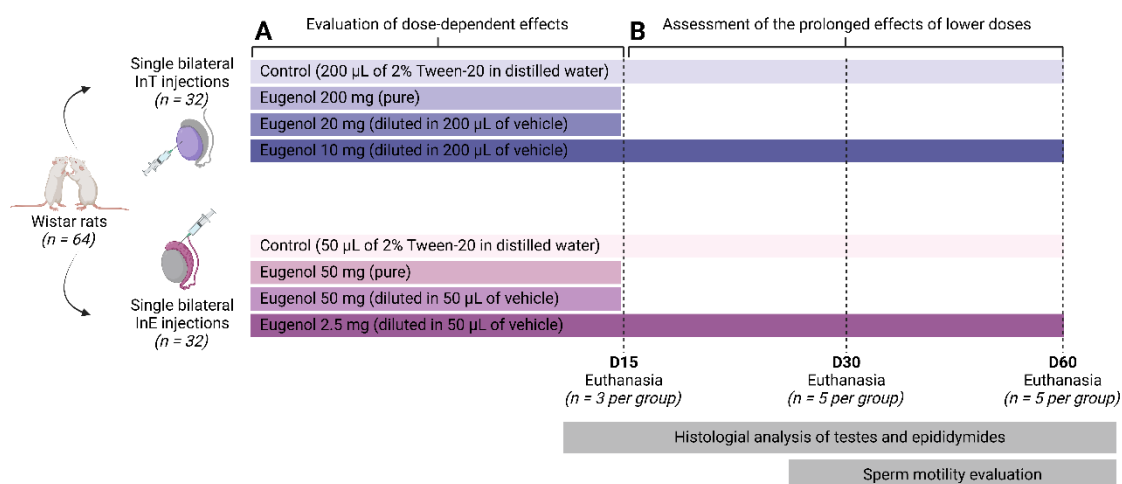


Fig. 1. Experimental design for intratesticular (InT) and intraepididymal (InE) eugenol administration in *Wistar* rats. A total of 64 *Wistar* rats were divided into two experimental phases. (A) In the first phase, 24 rats ($n = 3$ per group) were randomly assigned to evaluate the dose-dependent effects of eugenol. Animals received a single bilateral injection of either the vehicle (2% Tween-20 in distilled water) or different doses of eugenol in the testes (InT) or epididymal caput (InE). InT groups included: (1) Control (200 μ L of the vehicle), (2) Eugenol 200 mg (pure), (3) Eugenol 20 mg (diluted in 200 μ L of the vehicle), and (4) Eugenol 10 mg (diluted in 200 μ L of the vehicle). InE groups included: (5) Control (50 μ L of the vehicle), (6) Eugenol 50 mg (pure), (7) Eugenol 5 mg (diluted in 50 μ L of the vehicle), and (8) Eugenol 2.5 mg (diluted in 50 μ L of the vehicle). Euthanasia was performed on day 15 (D15), and testes and epididymides were collected for histological analysis. (B) In the second phase, 40 rats ($n = 10$ per group) were used to assess the prolonged effects of the lowest eugenol doses. The experimental groups were: (1) Control (InT; 200 μ L of the vehicle), (2) Eugenol 10 mg (InT; diluted in 200 μ L of the vehicle), (3) Control (InE; 50 μ L of the vehicle), and (4) Eugenol 2.5 mg (InE; diluted in 50 μ L of the vehicle). Animals were euthanized at D30 and D60 ($n = 5$ per timepoint), and tissues were collected for histological analysis and sperm motility evaluation.

Phase two focused on the prolonged effects of the lowest eugenol doses. Forty rats were divided into four groups ($n = 10$ /group; Fig. 1B). Two groups received InT injections: (1) Control: 200 μ L of vehicle; (2) Eugenol 10 mg diluted

in 200 µL of vehicle. The other two groups received InE injections: (3) Control: 50 µL of vehicle; (4) Eugenol 2.5 mg diluted in 50 µL of vehicle. Injections were administered on D0, and animals were monitored daily. Five animals per group were euthanized on days 30 (D30) and 60 (D60) for tissue collection. Testes and epididymides were fixed in Bouin's solution for 24 hours, and cauda epididymal samples were collected for sperm motility evaluation.

Testicular and epididymal tissues were dehydrated in ethanol, embedded in 2-hydroxyethyl methacrylate (Historesin®, Leica), sectioned at 3 µm using a rotary microtome (RM 2255, Leica), stained with hematoxylin and eosin, and mounted with Entellan (Merck). Qualitative analyses were performed under a light microscope at 10x, 20x, and 40x magnifications (Carvalho et al., 2022). Digital images of testicular parenchyma were acquired using a photomicroscope (Olympus BX-53, Tokyo, Japan) and analyzed using Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD, USA). The volumetric proportion (%) of Leydig cells was obtained by counting 1,000 points projected onto ten images per animal from the intertubular compartment. Moreover, 50 nuclei of Leydig cells were chosen randomly in either circular or elliptical form, and their volume was measured using image analysis software Leica Q-win (version 3) with the aid of the software ImageJ 1.48. The nuclear volume of Leydig cells was determined by the mathematical formula: $[\text{Diameter}^3 \times \pi \times 1/6]$ (Barros et al., 2018). These morphometric analyses were performed on testicular samples from animals subjected to InT eugenol administration and evaluated at 30 and 60 days.

For sperm motility evaluation, epididymal cauda fragments were incubated in 1 mL of Biggers-Whitten-Whittingham medium at 37°C for 5 min. Aliquots of the supernatant were placed between a pre-warmed slide and coverslip. Sperm

motility was assessed by analyzing 200 spermatozoa per animal under a phase-contrast microscope (L-1000B, Bioval) at 400x magnification, classifying them as motile or immotile (Carvalho et al., 2022). Statistical analysis was performed exclusively on sperm motility and Leydig cell morphometric data. The Shapiro–Wilk test was used to assess the normality of the data distribution. Comparisons between control and eugenol-injected groups were conducted using *Student's T-test*. Differences were considered statistically significant at $p < 0.05$. All analyses and graphical representations were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean $\pm$ standard deviation (SD).

3.3. Results and Discussion

This study offers novel insights into the histopathological and functional repercussions of single-dose bilateral administration of eugenol via InT and InE routes in *Wistar* rats. Both approaches elicited pronounced alterations in the structural integrity of testicular and epididymal tissues, resulting in the disruption of spermatogenesis and/or impairment of sperm maturation. The observed degenerative and necrotic changes within the seminiferous epithelium and epididymal ductal epithelium underscore the direct cytotoxic potential of eugenol upon contact with male reproductive structures. Notably, the complete absence of sperm motility in animals receiving InE injections strongly suggests epididymal ductal obstruction or collapse, ultimately precluding sperm maturation and transport. Throughout the observation period, none of the eugenol-injected animals exhibited overt clinical signs of discomfort, behavioral changes, gastrointestinal distress, or systemic toxicity. Nevertheless, the possibility of subclinical or delayed - onset adverse effects - such as chronic inflammation or

immune-mediated responses - warrants further investigation. Collectively, these findings reinforce eugenol's reproductive toxicity and inform ongoing efforts to develop chemical sterilants with defined safety profiles for potential application in fertility control protocols.

Histological examination of the testes and caput epididymis showed significant time-dependent morphological changes following InT eugenol administration. At 15 days post-injection, control animals that received vehicle application alone exhibited well-organized seminiferous tubules with a structured epithelium, germ cells at various stages of development, abundant luminal spermatozoa, and a preserved intertubular compartment containing Leydig cells and blood vessels (Fig. 2A, 2B, 2C). Conversely, animals that received InT injections of eugenol exhibited pronounced histopathological alterations. In the 200 mg and 20 mg groups, seminiferous tubules showed extensive degeneration and necrosis, polymorphonuclear leukocyte infiltration, the presence of residual spermatozoa, exfoliation of germ cells, and the formation of multinucleated giant cells (Fig. 2D, 2E, 2F, 2F', 2G, 2H, 2I, 2I'). Even at the lowest dose (10 mg), InT administration of eugenol induced significant architectural disruption, marked by necrosis, degeneration, atrophy, vacuolization, increased vascular congestion, and inflammatory infiltration. At the same time, some seminiferous tubules still harbored resilient spermatozoa (Fig. 2J, 2K, 2K', 2L, 2L'). In a few tubules, some cells compatible with undifferentiated spermatogonia were still distinguishable near the basement membrane, although they appeared reduced in number compared with controls.

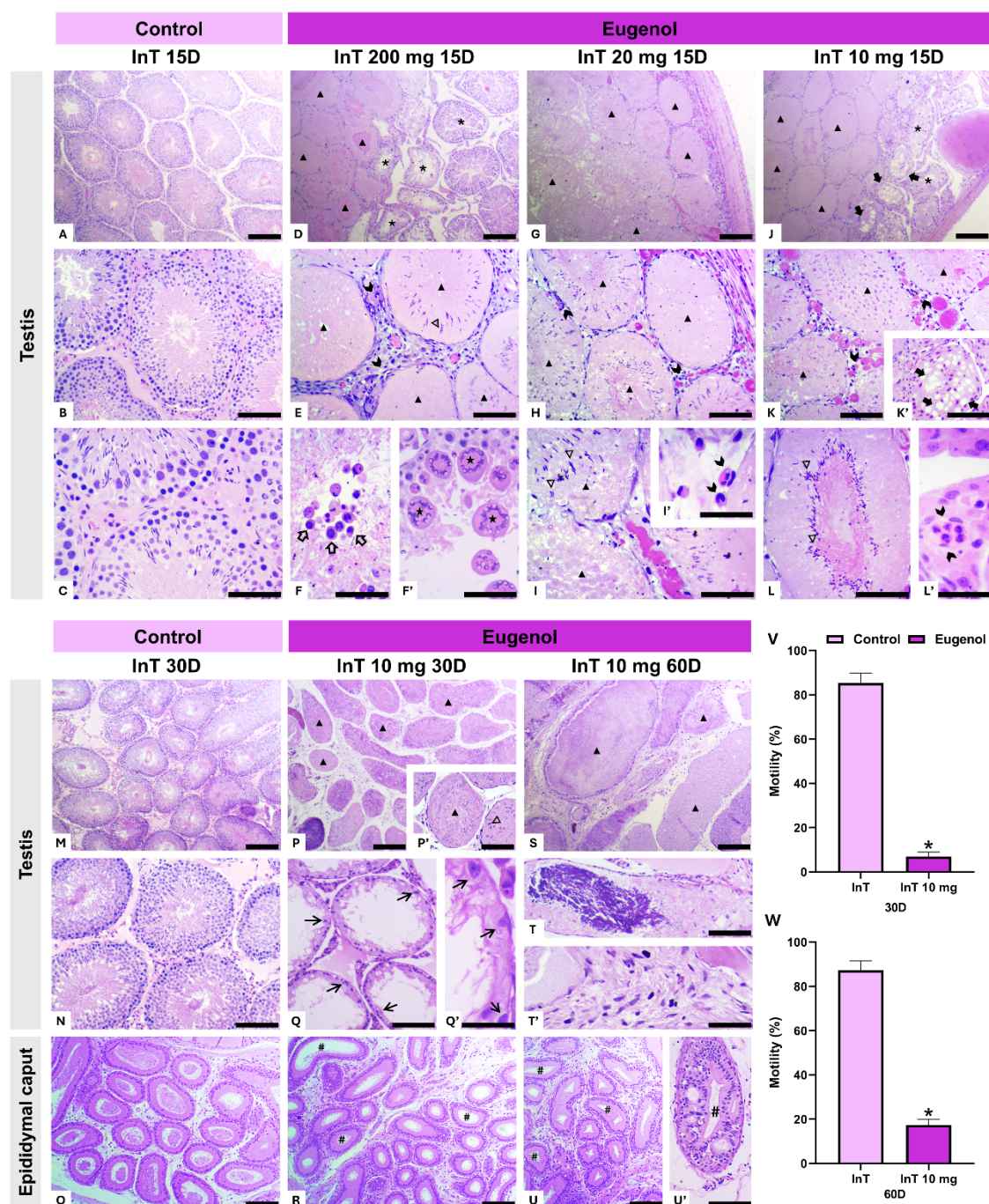


Fig. 2. Histological sections of testis and caput epididymis and sperm motility of *Wistar* rats, at 15, 30, and 60 days (D) after single bilateral intratesticular (InT) administration of different doses of eugenol (200, 20, and 10 mg). A, B, and C show the typical histological architecture of the control group 15 days after the InT administration of the vehicle, characterized by seminiferous tubules with an organized seminiferous epithelium, germ cells at different stages of development, and spermatozoa in the lumen, as well as a preserved intertubular compartment with Leydig cells and blood vessels. D, E, F, and F' show seminiferous tubules

undergoing degeneration (*) and partial necrosis (▲), polymorphonuclear leukocytes in the intertubular compartment (►), residual spermatozoa in the seminiferous tubules (Δ), exfoliation of germ cells (⇔), and multinucleated giant cells in the seminiferous tubules (★) at 15 days after the InT administration of 200 mg of eugenol. G, H, I, and I' show necrotic seminiferous tubules (▲), polymorphonuclear leukocytes in the intertubular compartment (►), and residual spermatozoa in the seminiferous tubules (Δ) at 15 days after the InT administration of 20 mg of eugenol. J, K, K', L, and L' show necrotic seminiferous tubules (▲), seminiferous tubules undergoing degeneration and atrophy (*), vacuolization of seminiferous tubules (□), increased congestion of blood vessels, polymorphonuclear leukocytes in the intertubular compartment (►), and residual spermatozoa in the seminiferous tubules (Δ) at 15 days after the InT administration of 10 mg of eugenol. M and N show the typical histological architecture of the control group after 30 days of InT vehicle administration, with the preserved organization of the seminiferous tubules and intertubular compartment. In O, the typical histological architecture of the caput epididymis is observed, characterized by a pseudostratified columnar epithelium, with spermatozoa in the lumen and preserved connective stroma. P, P', Q, and Q' show necrotic seminiferous tubules (▲), residual spermatozoa in the seminiferous tubules (Δ), and seminiferous tubules completely devoid of germ cells lined only by Sertoli cells (→). In R, a complete absence of spermatozoa in the lumen of the caput epididymis ducts (#) is observed 30 days after the InT administration of 10 mg of eugenol. In S, necrotic seminiferous tubules (▲) are observed; in T, a fine granular to amorphous laminated basophilic material and possible mineralization are noted, and in T', the presence of fibers indicates fibrosis. In U and U', a complete absence of spermatozoa in the lumen of the caput epididymis (#) is observed, as well as epithelial vacuolization at 60 days after the InT administration of 10 mg of eugenol. Scale bars: 100 μm. Sperm motility percentages of *Wistar* rats at 30 and 60 days after InT eugenol administration (10 mg) are shown in graphs V and W, respectively. Significant difference ($p < 0.05$) between control and eugenol-injected groups by *Student's t-test*. Control group: 2% Tween-20 (60 days); Eugenol groups: eugenol diluted in 2% Tween-20. (n = 5 animals/group).

These testicular histopathological alterations align with previously reported toxic effects of eugenol. Although eugenol is generally considered safe as a food additive (Carvalho et al., 2022), its cytotoxic potential increases when administered directly into tissues (Aburel et al., 2021). Upon direct application, eugenol may act similarly to sclerosing agents (Oliveira et al., 2012; Paranzini et al., 2018), compromising the blood testis barrier and potentially triggering an autoimmune response, where anti-sperm antibodies are generated, further exacerbating tissue damage. Additionally, due to its lipophilic nature, eugenol may increase cell membrane permeability, disrupt ionic homeostasis, and induce oxidative stress via reactive oxygen species production (Carvalho et al., 2022). These effects are comparable to the cytotoxic mechanisms observed in zinc oxide-eugenol materials, widely used in dentistry. These have been reported to induce chronic inflammation, degeneration, and necrosis, particularly when in direct contact with vital tissues (Nejad et al., 2017). Despite extensive testicular necrosis, spermatozoa were still detected in some tubules, suggesting that sperm produced before the injury had not been fully degraded, likely due to the structural stability conferred by their highly condensed chromatin (Wu et al., 2008). These findings indicate that eugenol-induced toxicity extends beyond direct chemical disruption, potentially initiating a systemic immunological response that perpetuates tissue damage over time.

At 30 days post-InT injection, control animals maintained preserved testicular architecture, with intact seminiferous tubules and an undisturbed intertubular compartment (Fig. 2M, 2N). The caput epididymis exhibited a pseudostratified columnar epithelium, with spermatozoa in the lumen and

preserved connective stroma (Fig. 2O). Conversely, animals that received 10 mg of eugenol via InT displayed severe histopathological damage, characterized by necrotic seminiferous tubules, the presence of residual spermatozoa, and complete depletion of germ cells, with some tubules still retaining a few Sertoli cells lining the basement membrane (Fig. 2P, 2P', 2Q, 2Q'). These structural findings were corroborated by the morphometric analysis of the intertubular compartment, which revealed a significant reduction in Leydig cell volumetric proportion, as well as decreases in nuclear and cytoplasmic fractions and smaller nuclear diameters when compared with control animals ($p < 0.05$; Table 1). At this point, only the lowest dose was used, as earlier observations indicated that InT application of 10 mg eugenol induced testicular damage comparable to higher doses.

By 60 days, while the testicular and epididymal architecture remained intact in control animals (data not shown), InT-administered eugenol resulted in further tissue deterioration, with seminiferous tubules largely disorganized and only rarely showing any cells morphologically compatible with germ or Sertoli cells. Besides extensive necrosis, laminated basophilic material suggestive of dystrophic mineralization (Foster, 2016) was present in the testes, along with fibrotic fibers (Fig. 2S, 2T, 2T'). These findings indicate progressive tissue injury, characterized by fibrosis, epithelial vacuolization, and mineralization - reparative processes that fail to restore standard functionality. Fibrosis typically replaces necrotic testicular parenchyma with fibroblasts and collagen deposition (Xu et al., 2024). At the same time, vacuole formation likely represents spaces left by lost germ cells during germinal atrophy (Cesta et al., 2014). Additionally, mineralization—frequently associated with sperm stasis—was observed in the

capsule, blood vessels, and seminiferous tubules, further compromising tissue architecture (Cesta et al., 2014). Consistent with these histological alterations, morphometric evaluation also revealed a significant reduction in Leydig cell volumetric proportion, as well as decreased nuclear and cytoplasmic fractions and smaller nuclear diameters compared with controls ($p < 0.05$; Table 1), reinforcing the progressive impairment of the testicular damage at this later time point.

Table 1. Morphometric parameters of Leydig cells of Wistar rats at 30 and 60 days after intratesticular (InT) administration of eugenol (10 mg).

	Leydig cell volumetric proportion (%)	Nuclear proportion (%)	Cytoplasmic proportion (%)	Nuclear diameter (μm)
<i>30 days</i>				
InT Control	9.52 ± 0.78	1.74 ± 0.22	7.78 ± 0.54	165.48 ± 11.85
InT Eugenol	3.82 ± 0.46*	0.72 ± 0.10*	3.10 ± 0.38*	133.54 ± 10.7*
10 mg				
<i>60 days</i>				
InT Control	8.04 ± 0.67	1.60 ± 0.20	6.44 ± 0.42	158.62 ± 13.88
InT Eugenol	2.14 ± 0.31*	0.40 ± 0.07*	1.74 ± 0.24*	128.36 ± 11.72*
10 mg				

Mean ± SD. Control group: Rats receiving vehicle (2% Tween-20) by intratesticular injection; Eugenol groups: Rats receiving eugenol (10 mg; diluted in 2% Tween-20) by intratesticular injection. Asterisks (*) indicate significant difference ($p < 0.05$) between control and eugenol-treated groups by Student's t-test. (n = 5 rats/group).

At 15 days post-InE administration, a similar pattern of tissue damage was observed as in the InT injections, reinforcing the toxic effects of eugenol on male reproductive structures (Ahmed, 2015; Trindade et al., 2023; Aponte et al., 2024). Control animals displayed a normal caput epididymis, with well-differentiated ducts lined by a pseudostratified columnar epithelium, abundant luminal spermatozoa, and a preserved connective stroma (Fig. 3A, 3B, 3C). In contrast, animals that received InE injections exhibited acute structural damage. Ductal necrosis, epithelial vacuolization, and leukocyte infiltration were prominent in all groups, with increasing severity at higher doses (Fig. 3D, 3E, 3F, 3F', 3G, 3H, 3I, 3I', 3J). In several regions, pronounced immune cell infiltration and accumulation of cellular debris within the ductal lumen were evident, indicating a robust inflammatory response (Cesta et al., 2014; Fig. 3K, 3L, 3L'). Similar findings were described by Leoci et al. (2019), who reported necrotic epididymal tubules surrounded by dense collagen fiber encapsulation and calcium deposits following InE injection of calcium chloride in dogs. The fibrotic response and encapsulation observed in the surrounding connective tissue in our study (Fig. 3K, 3L) suggest ongoing tissue remodeling, consistent with the chronic inflammatory and necrotizing action of sclerosing agents. As observed in InT injections, the lowest InE dose (2.5 mg) induced comparable damage to the higher doses, leading to its selection for evaluation at 30 and 60 days.

At 30 days post-InE administration, control animals maintained a well-organized epididymal architecture, with a pseudostratified epithelium, preserved lumen filled with spermatozoa, and intact connective stroma (Fig. 3M, 3N, 3O). Conversely, in animals receiving InE eugenol injections, tissue damage became more extensive, with generalized ductal necrosis and loss of tubular structure.

Clusters of residual spermatozoa were still observable in some regions (Fig. 3P, 3Q). Still, the lumen contained significant amounts of cellular debris and fibrous-like material in many areas, particularly in the cauda epididymis (Kempinas and Klinefelter, 2015; Fig. 3R). This accumulation of debris suggests that the local inflammatory response and tissue degradation may have contributed to ductal obstruction, impeding sperm transport (Azenabor et al., 2015; Colpi and Caroppo, 2024).

By 60 days post-InE administration, while the caput and cauda epididymal architecture remained intact in control animals (data not shown), the extent of caput epididymal damage had increased significantly, with widespread fibrotic deposition, persistent leukocyte infiltration, and severe structural disruption (Fig. 3S, 3T). Although spermatozoa could still be retrieved from the cauda epididymis, they exhibited complete immotility, reinforcing that epididymal dysfunction rather than sperm absence was responsible for the reproductive impairment. This observation supports the well-established role of the epididymis in the acquisition of motility and fertilizing ability, which depends on proper epithelial function, luminal fluid composition, and intercellular signaling (Robaire & Hinton, 2006; Dacheux & Dacheux, 2004). Structural or functional disruption of the epididymal epithelium can impair sperm maturation, leading to the release of immotile or non-functional sperm despite their presence (Gatti et al., 2004; Oliveira et al., 2016; Fig. 3U, 3U', 3U"). Given the progressive fibrotic remodeling observed, it is plausible that these spermatozoa originated from spermatogenesis prior to the InE injection, remaining stored in the cauda epididymis, as previously demonstrated in ligated rabbits where spermatozoa persisted for weeks without numerical loss despite degeneration (Jones, 2004). At the same time, new sperm

production was prevented from reaching the epididymal lumen due to ductal obstruction. These findings align with reports that epididymal obstruction, often a consequence of inflammation or fibrotic remodeling, can disrupt sperm transport and contribute to post-testicular infertility by blocking the excurrent duct system, leading to azoospermia or severe oligozoospermia (Jequier, 2011; Azenabor et al., 2015; Colpi and Caroppo, 2024). Functionally, this severe structural alteration corresponded with a total loss of sperm motility (Fig. 3W), a stark contrast to the residual motility observed following InT administration. Given the critical role of the epididymis in sperm maturation, these findings indicate that InE administration not only interferes with sperm transport but also compromises the epididymal microenvironment, which is extremely necessary for sperm viability (Dacheux & Dacheux, 2004; Robaire & Hinton, 2006; Zhou et al., 2019). Further long-term studies are warranted to determine whether chronic inflammatory responses or sperm granuloma formation may occur in the epididymal caput following chemical insult, potentially leading to persistent tissue remodeling and reproductive complications. Previous studies have demonstrated that disruption of the epididymal epithelium, particularly under conditions of testosterone depletion, can lead to extravasation of spermatozoa and round spermatids into the interstitium, triggering granulomatous inflammation, immune cell infiltration, and local fibrosis (Dutta et al., 2019). These pathological processes are especially relevant in the context of non-surgical sterilization strategies targeting the epididymis, and their potential long-term consequences should be carefully evaluated.

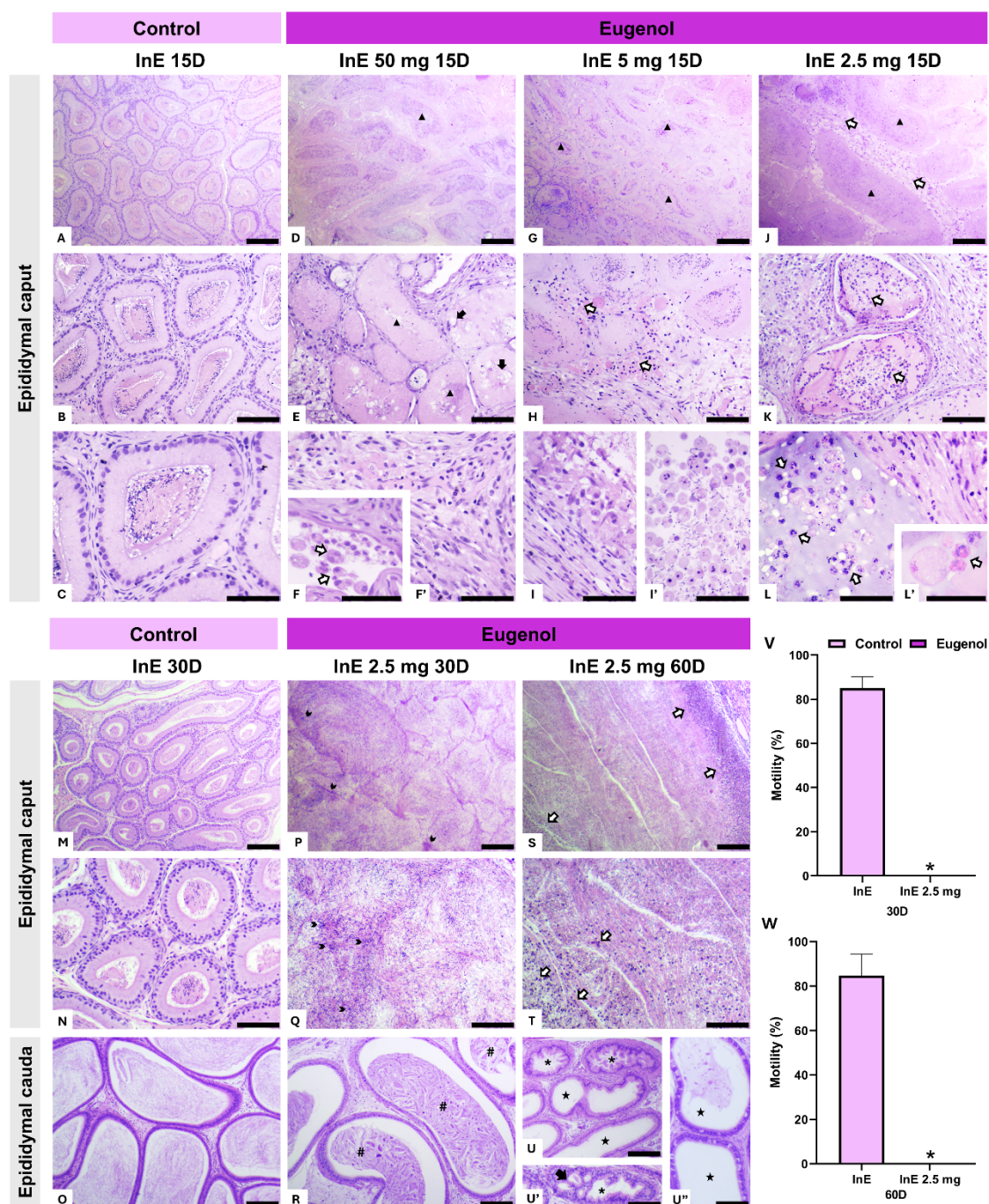


Figure 3. Histological sections of the epididymis and sperm motility of *Wistar* rats at 15, 30, and 60 days (D) after a single bilateral intraepididymal (InE) administration of different doses of eugenol (50, 5, and 2.5 mg). A, B, and C show the typical histological architecture of the caput epididymis in the control group 15 days after InE administration of the vehicle, characterized by a pseudostratified columnar epithelium, the presence of spermatozoa in the lumen, and a preserved connective stroma. D and E show widespread degeneration and necrosis with structural deterioration of the ducts (▲) and epithelial vacuolization

(□). F and F' show polymorphonuclear leukocytes in the duct lumen (⇒), while in F', an increase in blood vessels and connective fibers, characteristic of fibrosis, is observed 15 days after the InE administration of 50 mg of eugenol. G and H show necrotic epididymal ducts (▲) and polymorphonuclear leukocytes in the interductal compartment (⇒). I and I' show massive infiltration of immune cells and cellular debris in the duct lumen 15 days after the InE administration of 5 mg of eugenol. J, K, L, and L' show necrotic epididymal ducts (▲) and polymorphonuclear leukocytes both in the interductal compartment and within the epididymal ducts (⇒) 15 days after the InE administration of 2.5 mg of eugenol. M, N, and O show the typical histological architecture of the control group after 30 days of InE vehicle administration, with a preserved organization of the caput and cauda epididymis, characterized by a pseudostratified columnar epithelium, the presence of spermatozoa in the lumen, and a preserved connective stroma. P and Q show generalized ductal necrosis with loss of tubular structure, but clusters of residual spermatozoa (➤) are still observed. In R, the sections of the cauda epididymis exhibit an intact epithelium, and the lumen contains cellular debris and fibrous-like material (#) 30 days after the InE administration of 2.5 mg of eugenol. S and T demonstrate fibrous deposition in the caput epididymis, consistent with fibrosis, as well as dispersed and aggregated polymorphonuclear leukocytes (⇒). In U, U', and U'', sections of the cauda epididymis show ducts devoid of spermatozoa (★), a cribriform appearance (*), and epithelial vacuolization (□) at 60 days after the InE administration of 2.5 mg of eugenol. Scale bars: 100 µm. Sperm motility percentages of *Wistar* rats at 30 and 60 days after InE eugenol administration (2.5 mg) are shown in graphs V and W, respectively. Significant difference ($p < 0.05$) between control and eugenol-injected groups by *Student's t-test*. Control group: 2% Tween-20 (60 days); Eugenol groups: eugenol diluted in 2% Tween-20. (n = 5 animals/group).

While the findings of this study highlight eugenol's potential as a chemical sterilant, important considerations regarding its long-term safety and reversibility must be addressed. One critical concern is whether the histopathological damage observed in the testes and epididymides is permanent or reversible over

extended periods. The extensive fibrosis and mineralization detected in the testicular parenchyma, along with epididymal ductal necrosis and obstruction, suggest that tissue remodeling might lead to irreversible infertility (Leoci et al., 2019). Potential systemic inflammatory responses cannot be ruled out, as eugenol-induced tissue damage could trigger prolonged immune activation, with implications for overall reproductive and endocrine function (Carvalho et al., 2022). Another key aspect to consider is species-specific variability in response to eugenol exposure. Although the results obtained in *Wistar* rats provide valuable insights, differences in reproductive physiology across species (Hess et al., 2024) necessitate further studies to evaluate the safety and efficacy of eugenol in larger animal models, particularly those used in livestock production or as contraceptive targets in wildlife management. Addressing these uncertainties will be crucial for determining whether eugenol-based formulations could be safely and effectively implemented in long-term reproductive control strategies.

3.4. Conclusion

In summary, both InT and InE administration of eugenol severely damaged male reproductive structures through distinct mechanisms. InT administration disrupted testicular architecture, causing seminiferous tubule degeneration and altering key morphometric parameters of Leydig cells, which likely compromised androgen synthesis and contributed to the observed decline in sperm motility. InE administration targeted the epididymis, inducing ductal necrosis, epithelial vacuolization, and sperm transport obstruction, resulting in complete loss of motile sperm in the cauda. Taken together, these outcomes indicate that eugenol acts directly on both germ cells and somatic components - particularly Sertoli,

Leydig, and epididymal epithelial cells - promoting local cytotoxicity, structural destabilization, and secondary inflammatory–fibrotic remodeling. These findings support the feasibility of eugenol as a sterilization agent, though concerns remain regarding irreversibility, systemic inflammation, and species-specific variability. Because this study focused on morphological and functional endpoints, the precise molecular pathways and cell-specific targets remain unresolved.

3.5. References

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IV. Capítulo 3 – Artigo 3: Intraepididymal injection of eugenol and magnetite nanoparticles reduces male fertility through localized, non-endocrine epididymal remodeling

Abstract

The development of safe, effective, and low-cost non-surgical contraceptive methods remains a major challenge for large-scale population control of free-roaming animals. This study evaluated the contraceptive effects and biological responses following intraepididymal injection of eugenol (Eug), magnetite nanoparticles (Fe_3O_4), and eugenol-coated magnetite nanoparticles ($\text{Eug}\cdot\text{Fe}_3\text{O}_4$) in adult male Wistar rats. Animals received a single bilateral intraepididymal injection ($5\text{ }\mu\text{g}/50\text{ }\mu\text{L}$ per epididymis) and were evaluated 24 hours, 14 days, and 80 days after treatment. Histopathological analysis revealed a temporal progression from inflammatory infiltrates at 24 h to nodular epididymal remodeling at 14 days. These lesions persisted at 80 days and were accompanied by luminal narrowing and absence of luminal spermatozoa, whereas testicular architecture remained preserved. Oxidative and nitrosative stress markers increased transiently only at 14 days and returned to control levels by 80 days. Proteomic analysis of epididymal fluid revealed distinct group-specific protein profiles. All treatments reduced sperm motility, whereas normal sperm morphology was reduced in the Eug and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ groups and sperm plasma membrane integrity only in the Eug group. Mating behavior was preserved, but the male fertility index declined to 0% after Eug treatment and to 10% after Fe_3O_4 or $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ treatment, relative to the control group. Testosterone, FSH, LH, and physiological stress indicators were not affected by treatment. These findings demonstrate that intraepididymal administration induced persistent epididymal remodeling associated with impaired sperm quality and reduced fertility through a localized, non-endocrine mechanism. Functionalization of Fe_3O_4 with eugenol attenuated the biological effects observed with free eugenol while maintaining a reduction in male fertility, supporting further investigation of this localized, non-hormonal contraceptive approach.

Keywords: Chemical sterilization; male contraception; epididymis; cravo-da-índia; magnetite nanoparticles; nanotechnology

4.1. Introduction

Population control of animals represents a challenge for both domestic and wild species. Among domestic animals, the overpopulation of free-roaming dogs and cats remains a major global challenge, with important consequences for public health, animal welfare, and environmental conservation (Robertson, 2008; Abshenas et al., 2013; Abu-Ahmed, 2015). Large populations of stray animals increase human–animal conflicts and disrupt ecological balance through wildlife predation and competition for resources (Slater, 2001, 2005; Jackman & Rowan, 2007; Forzán et al., 2014; Jivago et al., 2021; Dayanti et al., 2021). They also serve as reservoirs for several zoonotic pathogens, including rabies, cystic hydatidosis, leishmaniasis, leptospirosis, trypanosomiasis, toxoplasmosis, neosporosis, dirofilariasis, and brucellosis (Forzán et al., 2014; Lessa et al., 2016; Doherty et al., 2017; Ellwanger & Chies, 2019). Among wild species, invasive species such as marmosets of the genus *Callithrix*, which are widely distributed in urban and peri-urban areas of Brazil, can threaten native species through competition, hybridization, and changes in ecosystem dynamics (da Silva et al., 2017; Moraes et al., 2019). Although surgical castration remains the gold standard for male sterilization, its application in large-scale population control of domestic animals is limited by costs, infrastructure requirements, the need for trained personnel, and the risk of complications such as hemorrhage, infection, herniation, and death (Levy et al., 2008; Ibrahim et al., 2016; Fesseha, 2019; Hamed et al., 2023). Similar constraints apply to wildlife population management, where surgical sterilization, despite being an effective tool, is limited on a large scale by the costs associated with surgery and anesthesia (Leary et al., 2021).

Non-surgical approaches have been investigated as alternatives to surgical castration since the 1970s (Dubé et al., 1987; Jana & Samanta, 2007; Abshenas et al., 2013). Hormonal and immunological strategies have shown promising results in some species; however, their contraceptive effects are often temporary (Dubé et al., 1987; Tremblay and Bélanger, 1984; Junaidi et al., 2009; Goericke-Pesch et al., 2011), inconsistent (Gonzalez et al., 1989; Dowsett et al., 1991; Kutzler and Wood, 2006; Ahn et al., 2021; Wang et al., 2023), or require repeated administration to maintain efficacy (Ponglowhapan, 2002; Junaidi et al., 2003; Trigg et al., 2006; Ponglowhapan, 2011; Romagnoli et al., 2012). These limitations have encouraged the development of chemical sterilization strategies

aimed at inducing permanent infertility through localized alterations of reproductive tissues.

Direct administration of sclerosing or cytotoxic agents into the testes, epididymis, or vas deferens has emerged as a promising non-surgical approach. A recent systematic review of 38 studies evaluating sixteen chemosterilizing agents showed that these compounds act through a limited number of mechanisms, including Leydig cell cytotoxicity with reduced testosterone production, damage to Sertoli cells and the seminiferous epithelium leading to spermatogenic arrest, stromal remodeling (fibrosis, hyalinization, and calcification), and epididymal obstruction that prevents sperm maturation and transport (Ribeiro et al., 2026). Although these mechanisms differ, no agent has yet combined the absence of adverse effects with consistent induction of irreversible infertility, highlighting the need for a safe, low-cost contraceptive that can be administered in a single application (Ribeiro et al., 2026).

Most studies on chemical sterilization have focused on the testis (Leoci et al., 2014; Abshenas et al., 2013; Jana & Samanta, 2007), whereas the epididymis has received comparatively little attention despite its recognized susceptibility to toxicant-induced injury (Hinton et al., 2010; Machado-Neves, 2022). In addition to sperm transport, the epididymis is essential for sperm maturation, membrane remodeling, acquisition of motility, concentration, and storage (James et al., 2020; Breton et al., 2019). Spermatozoa leaving the testis are incapable of fertilization until epididymal transit is complete (James et al., 2020). Consequently, structural or functional alterations in the epididymis may impair fertility even when spermatogenesis and endocrine function remain largely preserved (Elbashir et al., 2021). Previous studies have shown that localized epididymal injury induces epithelial degeneration, inflammation, fibrosis, and obstruction of sperm transport, resulting in long-term reductions in fertility (Colpi & Caroppo, 2024; Ribeiro et al., 2026). These characteristics support the epididymis as a promising target for the development of non-surgical contraceptive strategies based on local tissue remodeling rather than systemic endocrine suppression (Hinton & Cooper, 2010).

Among the candidate compounds, eugenol, a phenolic constituent of clove oil with antimicrobial, anti-inflammatory, antioxidant, and cytotoxic properties (Nejad et al., 2017; Nisar et al., 2021), has attracted considerable interest. The

route of administration appears to influence its reproductive effects. Prolonged oral administration of eugenol (10–40 mg/kg for 60 days) reduces serum testosterone concentrations and impairs sperm motility and membrane integrity without altering testicular or epididymal histoarchitecture (Carvalho et al., 2022). This lack of structural alterations was subsequently confirmed after oral administration of a substantially higher dose (200 mg/kg for 28 days), which produced no detectable effects on testicular weight, testosterone concentrations, antioxidant status, or histology (Reddy et al., 2026). In contrast, our group's previous study demonstrated that a single intraepididymal injection of eugenol (2.5–50 mg) induces marked epithelial degeneration, inflammatory infiltration, tissue remodeling, and obstruction of the excurrent ducts (Carvalho et al., 2026). These findings indicate that local administration, rather than systemic exposure, is necessary to produce the localized and structurally irreversible tissue alterations required for a single-administration contraceptive strategy.

Advances in nanobiotechnology have expanded the potential of nanomaterials for reproductive applications, including strategies to improve the efficacy and safety of male contraception (Iftikhar et al., 2021; Jivago et al., 2021; Lokman et al., 2022; Coimbra et al., 2023; Ferraz et al., 2024). Besides serving as drug delivery systems, metal and metal-oxide nanoparticles can directly affect the male reproductive tract when administered locally, primarily through oxidative stress mechanisms similar to those described for heavy metals (Machado-Neves, 2022). A systematic review of rodent studies reported recurrent structural and functional alterations in Sertoli, Leydig, germ, and sperm cells following exposure to several inorganic nanoparticles, including silver, gold, iron oxide, zinc oxide, titanium dioxide, and nickel. These effects were mainly associated with oxidative stress, apoptosis, and necrosis, although the underlying molecular mechanisms and the influence of the administration route remain poorly understood (Dantas et al., 2022).

Consistent with these observations, intratesticular injection of superparamagnetic iron oxide nanoparticles impairs Leydig cell steroidogenesis and reduces daily sperm production and sperm motility in mice (Ferraz et al., 2024). Similar reproductive toxicity has also been reported after intratesticular administration of gold nanoparticles, including increased testicular blood flow, histological damage, and reduced sperm concentration (Coimbra et al., 2023,

2025). Together, these findings suggest that magnetite nanoparticles may act as chemosterilizing agents rather than simply serving as inert carriers. Functionalization of metal nanoparticles with bioactive molecules may further modify their biological effects. For example, a gold nanoparticle–cetuximab nanocomplex preferentially accumulated in testicular tissue while producing lower systemic toxicity than uncoated nanoparticles (Coimbra et al., 2025). Whether eugenol coating similarly alters the local effects or systemic toxicity of Fe_3O_4 nanoparticles after intraepididymal administration, and whether $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ differs from eugenol or Fe_3O_4 administered alone, remains unknown.

Based on the available evidence, we hypothesized that localized intraepididymal chemosterilization could induce long-term male infertility through selective epididymal injury while avoiding systemic toxicity. We also hypothesized that nanoformulation would modify the biological effects of eugenol following intraepididymal administration. To test these hypotheses, we compared the long-term effects of a single intraepididymal administration of eugenol, Fe_3O_4 nanoparticles, and eugenol-coated Fe_3O_4 nanoparticles ($\text{Eug}\cdot\text{Fe}_3\text{O}_4$) in a rat model by evaluating reproductive performance, sperm quality, hormone concentrations, histopathological alterations, oxidative and nitrosative stress, proteomic changes, and multiple indicators of systemic safety at short-, medium-, and long-term time points.

4.2. Material and methods

4.2.1. Synthesis and characterization of Fe_3O_4 and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ nanoparticles

4.2.1.1. Reagents

For the synthesis of Fe_3O_4 nanoparticles, the following reagents were employed (high purity grade): iron (III) chloride hexahydrate ($\text{FeCl}_3\cdot 6\text{H}_2\text{O}$, Sigma-Aldrich, Riedel-de Haen, Søborg, Denmark); iron (II) sulfate heptahydrate ($\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, Sigma-Aldrich, Riedel-de Haen, Søborg, Denmark); sodium sulfite (Na_2SO_3 , Sigma-Aldrich, Riedel-de Haen, Søborg, Denmark); ammonium hydroxide (NH_4OH , Fluka, Seelze, Germany); and hydrochloric acid (HCl , Sigma-Aldrich, Riedel-de Haen, Søborg, Denmark). Eugenol ($\text{C}_8\text{H}_{10}\text{O}_2$, Sigma-Aldrich Co., St. Louis, MO) was used for nanoparticle coating.

4.2.1.2. Synthesis of Fe_3O_4 and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ nanoparticles

The synthesis and eugenol coating of Fe_3O_4 nanoparticles were performed according to previously described methods (Ferreira et al., 2009; Andrade et al., 2020). Briefly, 15 mL of a 2 mol L⁻¹ $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ solution (dissolved in 0.5 mol L⁻¹ HCl) was mixed with 10 mL of a 1 mol L⁻¹ Na_2SO_3 solution, resulting in a color change from dark yellow to light yellow. Subsequently, 400 mL of diluted NH_4OH solution was added, leading to the formation of a black precipitate, which was stirred for an additional 30 minutes. The solution was decanted, and the particles were washed three times with degassed deionized water and magnetically separated using a permanent magnet. The resulting magnetite particles were collected and designed as " Fe_3O_4 ". For eugenol functionalization, 25.4 mL of NH_4OH solution was diluted in 400 mL of deionized water, followed by the addition of 1.01 g or 0.10 g of eugenol. The precursor solution containing ferric and sulfite ions was then added to the NH_4OH -eugenol solution under magnetic stirring, resulting in the formation of a black precipitate. Resulting samples were designated as $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (1g) and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (0.1g), respectively.

4.2.1.3. Characterization of Fe_3O_4 and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ nanoparticles

Different techniques were employed to identify and characterize the material properties. X-ray diffraction (XRD) and Fourier-transform infrared (FTIR) spectroscopy were used to identify the crystalline phases. Powder XRD analysis was carried out using an Empyrean Panalytical diffractometer using Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$), operated at 45 kV/40 mA. Phase identification was performed using Jade software (Materials Data, Inc.) and the PDF 4+ database from the International Center for Diffraction Data (ICDD). Rietveld structural refinement was performed using the FULLPROF 2013 software. FTIR spectroscopy was performed using a VERTEX 70V BRUKER spectrometer equipped with a vacuum system and a Platinum ATR accessory with a diamond crystal. The analysis was conducted in attenuated total reflection (ATR) mode, with a spectral resolution of 4 cm⁻¹ and a spectral range from 300 to 4000 cm⁻¹.

Thermogravimetric analysis (TGA) and differential TGA (DTGA) were also carried out to calculate the composition of the eugenol coating on Fe_3O_4 nanoparticles. The experiments were conducted using a simultaneous

thermogravimetric analyzer, model SDT 650 from TA Instruments, within a temperature range of 40°C to 1000°C, at a heating rate of 10°C/min, in an alumina sample holder. The analyses were performed under an oxidizing (synthetic air) atmosphere. The measurement uncertainty for mass was 0.3 mg, while the temperature uncertainty was 2°C. The pre-purge time was 15 minutes. The magnetic properties of the samples were characterized using a vibrating sample magnetometer (VSM, Lake Shore 7400 Series) at room temperature. Magnetic hysteresis loops were measured up to a maximum applied field of 1.8 T (18 kOe). The zeta potential was evaluated using the Particle Analyzer Litesizer™ 500 AntonPaar, using diluted colloidal suspensions in filtered deionized water. At least thirty measurements were obtained for each nanoconjugate system, and the average and standard deviation (SD) values were calculated. The measurements were obtained at room temperature. For dynamic light scattering (DLS) analysis, the samples were diluted in water at a nanoparticle/water ratio of 1:100.

The quality of the Rietveld refinement was evaluated from the profile factor (R_p), weighted profile factor (R_{wp}), goodness of fit indicator (S), Bragg Factor (RB), and crystallographic RF-factor (RF) parameters (Table 1).

The crystallite size can be calculated by using the Scherrer equation (1) as:

$$d = \frac{k \lambda}{\beta \cos \phi} \quad \text{Equation 1}$$

where d is the crystal size (nm), K is the shape factor, λ is the X-ray wavelength (nm), β is the width at half-maximum of the strongest peak (3 3 1) (radian), θ is the Bragg's angle (radian). The crystallite sizes of the Fe_3O_4 , $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (0.1g), and $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (1g) were estimated to be 7.5, 7.2 and 7.8 nm, respectively.

4.2.2. *In vitro* cytotoxicity assay

Before *in vivo* experiments, an *in vitro* cytotoxicity assay was conducted to evaluate the general biocompatibility of Eug, Fe_3O_4 , and $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ in non-target mammalian cells. VERO (African green monkey kidney epithelial cells) and MELAN-A (murine non-tumor melanocytes) cell lines were selected as representative epithelial cell models, as they are widely employed in preliminary

toxicological screening to detect general cytotoxic effects in non-target mammalian cells.

4.2.3. Cell culture

The MELAN-A cell line was kindly donated by Dr. Roger Chammas (Laboratory of Experimental Oncology, University of São Paulo, USP, São Paulo, Brazil). The VERO cell line was obtained from the Laboratory of Molecular Animal Infectiology (LIMA), Universidade de Viçosa (UFV, Brazil). Both cell lines were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum (Gibco), 1% penicillin (Sigma-Aldrich), 1% streptomycin (Sigma-Aldrich), 0.025% amphotericin B (Sigma-Aldrich). The pH was adjusted to 7.4, and the cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

4.2.4. Cytotoxicity assay

The cytotoxicity of Eug, Fe₃O₄, and Eug·Fe₃O₄ was evaluated in VERO and MELAN-A cells using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich] reduction assay according to Mosmann (1983). For each independent experiment, concentrated solutions of Eug, Fe₃O₄, and Eug·Fe₃O₄ were prepared and diluted in dimethyl sulfoxide (DMSO; Sigma-Aldrich). The treatment concentrations tested (100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 µg/mL) were obtained by serial dilution. Cells were seeded in 96-well plates at a density of 1 × 10⁴ cells per well in 100 µL of culture medium and incubated for 24 hours under the conditions described above. Subsequently, the culture medium of each well was replaced with 100 µL of medium containing Eug, Fe₃O₄, or Eug·Fe₃O₄ at the indicated concentrations. Control cells received medium containing DMSO only (vehicle control). The final concentration of DMSO was maintained below 0.5% (v/v) in all experimental and control wells. Cells were then incubated for an additional 48 hours. Thereafter, the experimental solutions were replaced by 100 µL of MTT solution, and cells were incubated for an additional 4 hours. The supernatant was subsequently removed, and the resulting formazan crystals formed during the MTT reaction were dissolved in 100 µL of DMSO. Absorbance was measured at 570 nm using a SpectraMax microplate reader. All treatments were performed in triplicate wells and each experiment was independently repeated at least three times. Cell

viability was expressed as a percentage relative to the vehicle-treated control group.

4.2.5. *In vivo* experimental procedures

4.2.5.1. *Animals and ethics statement*

Eighty adult male Wistar rats (70 days old; 200 ± 50 g) and 40 adult female Wistar rats (70 days old; 150 ± 50 g) were obtained from the Central Animal Facility of the Biological and Health Sciences of the Universidade Federal de Viçosa (UFV, Brazil). Animals were housed individually in polypropylene cages under controlled environmental conditions ($21\text{ }^{\circ}\text{C}$; 12 h light:12 h dark photoperiod) with free access to commercial rat chow (Nuvilab CR-1; Quimtia S.A; Paraná, Brazil) and drinking water. All experimental procedures were reviewed and approved by the Ethics Committee for Animal Use of UFV (CEUA; protocol 35/2023), and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

4.2.5.2. *Experimental design*

After a 10-day acclimatization period, male rats were randomly allocated into four experimental groups ($n = 20$ /group): (1) Control, injected with $50\text{ }\mu\text{L}$ of 2% Tween-20 diluted in distilled water (vehicle); (2) Eug, injected with eugenol; (3) Fe_3O_4 , injected with magnetite nanoparticles; and (4) Eug- Fe_3O_4 , injected with eugenol-coated magnetite nanoparticles. Each epididymis received $5\text{ }\mu\text{g}$ of the respective formulation in $50\text{ }\mu\text{L}$, administered as a single bilateral intraepididymal injection (total dose per animal: $10\text{ }\mu\text{g}$ in $100\text{ }\mu\text{L}$). The selected dose was based on previous studies demonstrating biological activity of nanoparticles administered locally to the male reproductive tract within the range of $2\text{--}25\text{ }\mu\text{g}$ per testis (Ferraz et al., 2024; Coimbra et al., 2023; Coimbra et al., 2025). Additionally, previous studies using intraepididymal eugenol demonstrated that higher doses induce extensive epithelial damage, fibrosis, and ductal obstruction, resulting in severe impairment of sperm maturation and transport (Carvalho et al., 2026). Therefore, a lower dose ($5\text{ }\mu\text{g}$) was selected to remain within the biologically active range reported for nanoparticle-based approaches while minimizing extensive tissue damage associated with high-dose eugenol exposure.

Animals were further allocated into three independent cohorts, each used exclusively for a single experimental time point: 24 hours ($n = 5/\text{group}$; 20 animals total), 14 days ($n = 5/\text{group}$; 20 animals total), and 80 days ($n = 10/\text{group}$; 40 animals total). No animal was evaluated at more than one time point. The 80-day cohort was also used for the fertility assessment described in section 2.3.4, with mating initiated on D70 and tissue collection performed 10 days later, at D80.

For the intraepididymal application, the animals were weighed and anesthetized with xylazine (10 mg kg^{-1} , intraperitoneally) and ketamine hydrochloride (80 mg kg^{-1} , intraperitoneally). The injection was performed on experimental day 0 (D0), and animals were evaluated at 24 hours (D1), 14 days (D14), and 80 days (D80) after administration, corresponding to short-, medium-, and long-term experimental endpoints, respectively. The D1 time point was designed to investigate the initial biodistribution of the nanoparticles, whereas D14 was used to evaluate acute toxicity and post-injection inflammatory responses. The D80 time point was chosen to encompass one complete spermatogenic cycle in rats (approximately 56 days), and the additional period required for fertility assessment.

4.2.5.3. *Observation of behavioral changes and signs of toxicity*

Throughout the experimental period, body weight, food intake, water consumption, and clinical signs of toxicity (e.g., diarrhea, vomiting, piloerection, alopecia) were monitored. In addition, the Rat Grimace Scale and body and scrotal temperatures were monitored during the first 14 days following intraepididymal application to detect potential signs of discomfort or local inflammation. Body and scrotal temperatures were measured using a digital infrared laser thermometer, and pain assessment was performed according to the Rat Grimace Scale as described by Turner et al. (2019).

4.2.5.4. *Fertility indexes*

Male fertility was evaluated in 5 male rats per group. Males were cohoused with healthy adult females (1:1) beginning on D70, for a 72-hour period. Vaginal smears were collected after 12, 24, and 72 h to confirm mating by the presence of spermatozoa. The detection of sperm cells in vaginal cytology was considered gestational day 0 (GD0) (Lima et al., 2018; Coimbra et al., 2023). Following the

mating period, males were separated and maintained for an additional 7 days before euthanasia to reestablish the sperm reservoir in the cauda epididymis. Females were maintained until GD19 (Lima et al., 2018), when they were anesthetized (xylazine 30 mg kg⁻¹ and ketamine hydrochloride 300 mg kg⁻¹, intraperitoneally) and euthanized; pregnancy was confirmed by the presence of implantation sites in the uterine horns. From this protocol, two indices were calculated: the male mating index [(number of males mated/number of males) × 100] and the male fertility index [(number of males fertilizing females/number of males mated) × 100].

4.2.5.5. *Euthanasia and sample collection*

Male rats were euthanized at D1, D14, or D80, according to cohort assignment. Prior to euthanasia, animals were weighed and subjected to a 10-hour fasting period. Animals were then euthanized under deep anesthesia with ketamine hydrochloride (150 mg kg⁻¹ intraperitoneally) and xylazine (10 mg kg⁻¹ intraperitoneally), followed by blood collection via cardiac puncture. Blood samples were centrifuged at 700 × g for 15 minutes to obtain serum, which was used for the determination of reproductive hormones [testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH)], physiological stress indicators (cortisol, glucose, and urea nitrogen), and nanoparticle biodistribution analyses. The testes, epididymides, heart, kidneys, spleen, liver, and epididymal adipose tissue were excised, dissected, and weighed. Spermatozoa recovered from the cauda epididymis were collected for the evaluation of sperm motility, viability, and morphology.

Representative fragments of all organs were fixed in 4% paraformaldehyde for histopathological evaluation, while additional tissue samples were snap-frozen in liquid nitrogen and stored at -80 °C for nanoparticle biodistribution. Testes were also collected for determination of tissue testosterone concentration. Furthermore, epididymal tissue was used to evaluate antioxidant enzyme activity and oxidative/nitrosative stress biomarkers. All frozen samples were stored at -80 °C until biochemical analyses were performed.

For proteomic analysis, epididymal fluid was collected by microperfusion: 20 µL of phosphate-buffered saline (PBS) was injected into the epididymal lumen

to recover the luminal contents, which were centrifuged at 600 x g for 10 minutes at 4 °C to remove cellular debris. The resulting supernatant was subsequently centrifuged at 15,000 x g for 20 min at 4 °C to obtain a clarified epididymal fluid fraction (Dacheux et al., 1983; Belleannee et al., 2015). Protease inhibitor cocktail (#8830; Sigma-Aldrich, USA) was added to all samples at a 1:1000 dilution, and samples were stored at -80 °C until proteomic analysis. Proteomic analysis was performed on cauda epididymal fluid samples from the 80-day cohort (n = 5/group).

4.2.5.6. Serum and tissue hormone levels

Serum concentrations of LH, FSH, and testosterone were determined using commercial ELISA kits according to the manufacturers' instructions (LH: EEL122, Thermo Fisher Scientific; FSH: EEL125, Thermo Fisher Scientific; Testosterone: AccuBind ELISA, Monobind Inc.). Serum samples (n = 10 per group) were diluted 1:10 prior to LH and FSH analyses, and final concentrations were corrected by the dilution factor. The analytical sensitivities of the assays were 0.94 mIU/mL for LH, 1.88 ng/mL for FSH, and 0.0576 ng/mL for testosterone. Intratesticular testosterone concentrations were measured from approximately 50 mg of frozen testicular tissue by ELISA. The assays were preceded by steroid enrichment based on lipid extraction with HPLC-grade ethyl ether, as previously described (Hany et al., 1999).

4.2.5.7. Magnetite nanoparticles biodistribution

To evaluate the biodistribution of magnetite nanoparticles, iron (Fe) concentration was quantified in biological tissues and blood by graphite furnace atomic absorption spectrometry (GFAAS). Tissue samples were first dried in a forced-air oven at 50–60 °C until constant weight was achieved. Approximately 100 mg of each dried tissue sample was transferred to digestion tubes. Blood samples (1 mL) were dried under the same conditions prior to digestion. Mineral extraction was performed by wet acid digestion using a nitric acid/perchloric acid mixture (HNO₃:HClO₄, 2:1, v/v). Briefly, 2.0 mL of the acid mixture was added to each sample, and digestion was carried out in a digestion block with gradual heating. Samples were initially maintained at approximately 90 °C to allow nitric acid evaporation and predigestion, followed by a progressive increase in

temperature up to a maximum of 220 °C until complete oxidation of the organic matrix was achieved, as indicated by a clear digest. Blank samples containing only the acid mixture were processed simultaneously.

After cooling, the digests were quantitatively transferred to 10-mL volumetric flasks, diluted to volume with deionized water, filtered, and stored at 4 °C until analysis. Iron concentrations were determined using a graphite furnace atomic absorption spectrometer (Varian SpectrAA 220Z, Varian Inc., Palo Alto, CA, USA) equipped with an automatic sampler. Calibration curves were prepared from certified iron standard solutions (1000 mg L⁻¹; Merck, Darmstadt, Germany), and results were expressed as µg g⁻¹ dry weight for tissues and µg mL⁻¹ for blood.

4.2.5.8. *Physiological stress indicators*

Serum samples (n = 10 per group) were obtained after clotting and centrifugation (2000 × g for 10–15 min) and stored at –20 °C until biochemical analysis. Physiological stress indicators were evaluated by measuring serum concentrations of total protein, glucose, and urea nitrogen. Total protein concentration was measured by the biuret method, whereas urea nitrogen and glucose levels were measured using enzymatic colorimetric assays. All analyses were performed using commercial diagnostic kits in an automated biochemical analyzer following the manufacturers' protocols.

4.2.5.9. *Sperm motility, viability, and morphology*

Cauda epididymal samples (n = 5 per group) were minced into small fragments and incubated in 1 mL of Biggers–Whitten–Whittingham (BWW) medium at 37 °C for 5 min to allow sperm dispersion. The resulting suspension was used for all subsequent sperm analyses. Sperm motility was evaluated using a 10 µL aliquot of the epididymal fluid placed on a pre-warmed microscope slide. A total of 200 spermatozoa per animal were examined under a phase-contrast microscope (L-1000B, Bioval, São Paulo, Brazil) at 400× magnification and classified as either motile or immotile. Sperm motility was expressed as the percentage of motile spermatozoa relative to the total number of cells evaluated.

Sperm membrane integrity and morphology were assessed using eosin–nigrosin staining (BotuVital). Stained cells were considered non-viable, whereas

unstained cells were classified as viable. Morphological abnormalities affecting the head, midpiece, and tail regions were recorded and expressed as percentages.

4.2.5.10. Histological processing and analysis

Tissue samples (n = 5 per group) were dehydrated in graded ethanol solutions (70%, 80%, 90%, 95%, and absolute), embedded in paraffin, sectioned at 5 µm thickness using a rotary microtome (RM 2255, Leica, Nussloch, Germany), and stained with hematoxylin and eosin (HE). Slides were mounted with Entellan® (Merck, Germany) for histopathological evaluation. Histological examination and image acquisition were performed using a photomicroscope (Olympus BX53, Tokyo, Japan) coupled to a digital camera (Olympus DP73, Tokyo, Japan). Testicular, efferent duct, and epididymal lesions were evaluated by a blinded observer and classified descriptively according to type, extent, and severity (absent, mild, moderate, or severe), using a semi-quantitative 0–5 severity scale as an internal reference framework to standardize the qualitative classification reported in the Results. The evaluated parameters included inflammatory infiltrates, epithelial degeneration, epithelial disorganization, vacuolization, luminal debris accumulation, tubular or ductal collapse, mineralization, and other tissue-specific alterations.

4.2.5.12. Antioxidant enzymes and oxidative/nitrosative stress markers

Epididymal samples (n = 5/group; ~100 mg) were homogenized in 1 mL of phosphate buffer (pH 7.4) and centrifuged at 10,000 × g for 10 min at 4 °C. The resulting supernatants were used to determine the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione S- transferase (GST), as well as to quantify oxidative and nitrosative stress markers, including nitric oxide (NO) and malondialdehyde (MDA) levels. The pellets were used for the determination of protein carbonyl (PC) content. All analyses were performed using an ELISA microplate reader (Multiskan SkyHigh; Thermo Scientific).

Superoxide dismutase activity was determined by the pyrogallol autoxidation method, based on the enzyme's ability to inhibit pyrogallol oxidation (Dieterich et al., 2000). Catalase activity was measured by monitoring the rate of hydrogen peroxide decomposition, according to Aebi (1984). Glutathione S-

transferase activity was determined spectrophotometrically according to Habig et al. (1974) by monitoring the formation of the glutathione conjugate with 1-chloro-2,4-dinitrobenzene (CDNB). Absorbance was measured at two time points (30 and 90 s), and enzyme activity was calculated using a molar extinction coefficient of $\epsilon_{340} = 9.6 \text{ mmol L}^{-1} \text{ cm}^{-1}$. Results were expressed as units (U) per mg of protein.

Nitric oxide levels were quantified using the Griess reaction, according to Tsikas (2007). Briefly, 50 μL of each sample was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine, and 2.5% phosphoric acid [H_3PO_4]) and incubated at room temperature for 10 min. Absorbance was recorded at 570 nm using a microplate reader, and nitric oxide concentrations ($\mu\text{mol/L}^{-1}$) were calculated using a sodium nitrite standard curve (0-125 μM). Lipid peroxidation was evaluated by measuring malondialdehyde concentrations using the thiobarbituric acid reactive substances (TBARS) assay. Briefly, the supernatant was incubated with TBARS reagent (15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.25 N HCl) for 15 min, and the formation of thiobarbituric acid-reactive products was quantified at 535 nm, according to Buege and Aust (1978). Protein oxidation was assessed by quantifying protein carbonyl content. Pellets were treated with 0.5 mL of 2,4-dinitrophenylhydrazine (DNPH) solution, and the resulting carbonyl derivatives were measured spectrophotometrically in the final supernatant at 370 nm, as described by Levine et al. (1994). All measurements were performed using a microplate reader (Multiskan SkyHigh, Thermo Scientific).

4.2.5.13. Proteomic analysis

Frozen samples were precipitated overnight with cold acetone (1:4, v/v) at -20°C , followed by three consecutive centrifugation steps at $14,000 \times g$ for 10 minutes at 4°C (Viana et al., 2025). The resulting protein pellets were resuspended in 0.02 M triethylammonium bicarbonate (TEAB), and soluble protein content was quantified using QubitTM assay (Thermo Fisher Scientific, Waltham, MA, USA). For protein digestion, 60 μg of protein from each sample was resuspended in 50 mM ammonium bicarbonate containing 500 mM dithiothreitol (DTT) and incubated at 55°C for 25 minutes for protein reduction. Subsequently, iodoacetamide (IAA) was added to reach a final concentration of

0.014 M, and the samples were incubated in the dark at 21°C in for 40 minutes for protein alkylation. Calcium chloride (0.001 M) was then added prior to trypsin digestion (Promega, Fitchburg, WI, USA), which was performed at an enzyme-to-substrate ratio of 1:50 (w/w) for 18 hours at 37°C. The reaction was stopped by adding trifluoroacetic acid (TFA) to a final concentration of 1% (Viana et al., 2018). The resulting peptides were desalted using C18 Zip-Tips (ZTC18S096, Millipore, Burlington, USA) according to the manufacturer's instructions, lyophilized, and stored at -20 °C until analysis.

Prior to mass spectrometry analysis, the lyophilized peptides were resuspended in 20 µl of 0.1% formic acid, and the peptide concentration was determined using the Qubit™ Assay. An aliquot of 2 µg of tryptic peptides from each sample was analyzed using an Easy-1000 nLC system (Thermo Scientific, USA) coupled to an Orbitrap Q-Exactive Plus mass spectrometer (Thermo Scientific, USA). Samples were initially loaded onto a trapping column (HPLC Acclaim PepMap C18 100 ×20 mm, Thermo Scientific, EUA) at a flow rate of 7 µL/ min for 3 min. Peptides were subsequently separated using an Easy-Spray analytical column (2 µm particle size, 75 µm ×500 mm; Thermo Scientific, EUA), operating at a flow rate of 0.2 µL/min. The mobile phase of the chromatographic process consisted of 0.1% formic acid, 5% acetonitrile, and 95% water (solvent A) and 0.1% formic acid, 95% acetonitrile, and 5% water (solvent B). Chromatographic separation occurred according to the following schedule: 6–25% B for 85 min, 25–45% B for 22 min, 45–95% B for 5 min, and 95% B for 8 min.

The raw data was processed using FragPipe 21.1 software (<https://fragpipe.nesvilab.org/>) for protein identification, validation and quantification through the MSFragger, Philosopher, and IonQuant tools (Kong et al., 2017). The protein mass lists were compared with the *Rattus norvegicus* (Proteome ID: UP000002494) databases deposited in the UniProt Knowledgebase database. Search parameters were a maximum of two missed cleavages and full-length tryptic peptides: carbamidomethylation (C) as fixed modification and oxidation (M) and acetylation (N-terminal protein) as variable modifications, a precursor mass tolerance of 10 ppm and 0.1 Da fragment ion mass tolerance. Mass calibration and parameter optimization have been enabled.

The false discovery rate (FDR) was <1% at the peptide and PSM levels using the Percolator algorithm (Käll et al., 2007).

To account for variations in sample loading and instrument sensitivity, raw spectral counts (SpC) for each protein were normalized. Total spectral count normalization was applied to the dataset according to standard proteomic workflows (Griffin et al., 2010). KEGG Pathway analysis was carried out using DAVID platform (<https://davidbioinformatics.nih.gov/>, p-value ≤ 0.05) for the unique proteins of each group. Sample classification was performed using pairwise Linear Discriminant Analysis (LDA) to identify protein profiles that maximize the separation between distinct experimental groups (Witten & Tibshirani, 2011). To explore the functional organization of the proteomic landscape, a protein-protein interaction (PPI) network was constructed based on data retrieved from public repositories. Topological analysis of the network was performed to identify key regulatory nodes, specifically hubs and bottlenecks (Yu et al., 2007).

4.2.6. Statistical analysis

The Shapiro–Wilk test was used to assess the normality of the data distribution. Comparisons among experimental groups for continuous variables were performed using one-way analysis of variance (one-way ANOVA), followed by *post hoc* Tukey's multiple comparison test when significant differences were detected. Differences between groups were considered statistically significant at $p < 0.05$. All analyses and graphical representations were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean $\pm$ standard deviation (SD).

4.3. Results

4.3.1. Structural characterization of the synthesized nanoparticles

The crystalline structure of the synthesized iron oxide nanoparticles was identified by the Rietveld refinement of the XRD patterns. This identification involving the Fe_3O_4 , $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (1g) and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (0.1g) samples was performed with space groups $Fd-3m$. The pseudo-Voigt function was used and the resulting Rietveld profiles are displayed in Fig. 1A. Diffraction peaks were

observed at (220), (311), (222), (400), (422), (333), and (440), consistent with the cubic spinel structure of magnetite (Liu et al., 2006). No diffraction peaks characteristic of maghemite or hematite [(113), (210), (213), (210)] (Murbe et al., 2008) were detected.

Table 1. Structural parameters, Rietveld agreement factors, mean particle size diameter (Z-Ave), polydispersity index (PDI), and zeta potential (ZP) for the samples.

Parameter	Samples		
r			
	Fe ₃ O ₄	Eug·Fe ₃ O ₄ (0.1g)	Eug·Fe ₃ O ₄ (1g)
Magnetite (a = b = c)	8.356 ± 0.001	8.347 ± 0.002	8.361 ± 0.002
<i>RP</i>	0.314	6.27	5.36
<i>RWP</i>	0.396	8.16	6.92
<i>S</i>	0.88	0.80	0.81
<i>RB</i>	0.139	2.55	1.90
<i>RF</i>	0.151	2.12	1.51
Z-Ave/nm	3322	564	432
PDI	0.23	0.20	0.22
ZP/Mv	5.6	-56.7	-60.8

R_p: Profile factor; *Rwp*: Weighted Profile factor; *S*: Goodness of fit; *RB*: Bragg factor; *RF*: Crystallographic RR-factor.

The FTIR spectra of Fe₃O₄, Eug, Eug·Fe₃O₄ (1g) and Eug·Fe₃O₄ (0.1g) samples are shown in Fig. 1B. The presence of magnetite was confirmed in the Fig. 1Ba. Fig. 1Bb shows the spectrum of eugenol. Due to the presence of double bonds among the functional groups of eugenol, an intense band is observed in the range of 1640-1500 cm⁻¹, corresponding to the C=C stretching mode. As illustrated in Fig. 1Bb, eugenol displays a characteristic and a well-defined peak at 1520 cm⁻¹, attributed to the C=C stretching of the aromatic ring, providing unique spectral information that is highly relevant for substance identification (de Oliveira et al., 2019; Pramod et al., 2015; Yang et al., 2019).

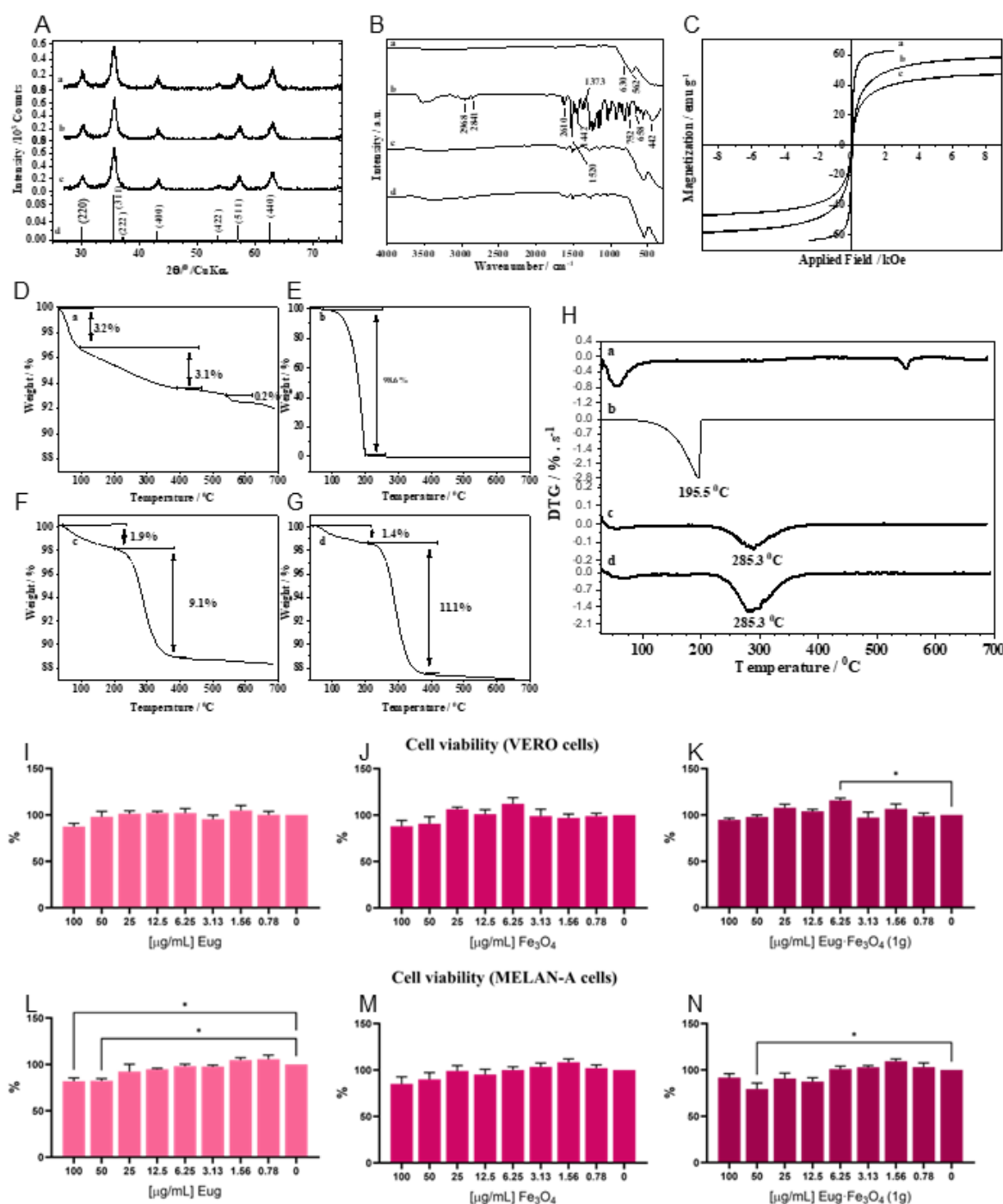


Fig. 1. Physicochemical characterization and *in vitro* cytotoxicity of eugenol-loaded Fe_3O_4 nanoparticles. (A) Powder X-ray diffraction (XRD) patterns of Fe_3O_4 (curve a), $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (0.1g) (curve b), $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (1g) (curve c), and the reference magnetite pattern (ICDD #19-0629) (d). (B) Fourier-transform infrared (FTIR) spectra of Fe_3O_4 (curve a), Eug (curve b), $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (0.1g) (curve c), and $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (1g) (curve d). (C) Magnetization hysteresis curves measured at 296 K for Fe_3O_4 (curve a), $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (0.1g) (curve b), and $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (1g) (curve c). (D–G) Thermogravimetric analysis (TGA) curves of Fe_3O_4 (D), Eug (E), $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (0.1g) (F), and $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (1g) (G), respectively. (H) Derivative thermogravimetric (DTG) curves of Fe_3O_4 (curve a), Eug (curve b), $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (1g) (curve c), and $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (0.1g) (curve d). (I–K) Cell viability of VERO cells after injection with Eug, Fe_3O_4 , and $\text{Eug} \cdot \text{Fe}_3\text{O}_4$ (1g), respectively. (L–

N) Cell viability of MELAN-A cells after injection with Eug, Fe_3O_4 , and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (1g), respectively. Cell viability was determined after exposure to concentrations ranging from 0.78 to 100 $\mu\text{g/mL}$. Data are expressed as the mean $\pm$ standard deviation (SD) of independent experiments. Statistical significance relative to the untreated control is indicated by $p < 0.05$.

The surface charge of the nanoparticles was assessed through zeta potential (ZP) measurements (Table 1). Zeta potential values were +5.6 mV for Fe_3O_4 , -56.7 mV for $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (0.1g), and -60.8 mV for $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (1g). Hydrodynamic diameters (Z-Ave) were 3322 nm, 564 nm, and 432 nm, respectively, with polydispersity index (PDI) values of 0.23, 0.20, and 0.22.

Magnetization curves obtained for Fe_3O_4 , $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (0.1g), and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (1g) samples at 298 K are presented in Fig. 1C. There was no hysteresis in the magnetization for all samples, suggesting the magnetic particles produced are superparamagnetic. The saturation magnetization (M_s) was obtained from moment versus magnetic field (B) curves. For the Fe_3O_4 sample, the value is 62.8 emu g⁻¹, being higher than values reported in the literature (46.3 emu g⁻¹) for samples prepared by the same method (Sato et al., 1987), whereas for the samples coated with eugenol, $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (0.1g), and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (1g), the corresponding values are 59.2 and 47.2 emu g⁻¹.

The TGA and DTA profiles of Fe_3O_4 , Eug, $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (0.1g), and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (1g) are shown in Fig. 1D- G. The TGA curve of the Fe_3O_4 sample shows a well-defined thermal event with a mass loss of approximately 3.2 % in the temperature range of 30-90 °C (Fig. 1D), followed by an additional mass loss (~3.1% between 90-370 °C) (Mohammed et al., 2021). A further thermal event was observed between 370-600 °C ($\text{Fe}_3\text{O}_4(g) + \frac{1}{4}\text{O}_{2(g)} \rightarrow \frac{3}{2} - \text{Fe}_2\text{O}_{3(s)}$), accompanied by a small mass gain (Mohammed et al., 2021).

The TGA curve obtained for Eug (Fig. 1E) presents a unique step of mass corresponding to evaporation of the eugenol at 200 °C. The TGA plot of the $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (1g) (Fig. 1F) and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (0.1g) (Fig. 1G) sample exhibits two mass loss steps. The first mass loss (1.9 and 1.4%) which occurs in the range of 30–210 °C, corresponds to the loss of water adsorbed on the surface of the samples. The second (9.1 and 11.1%) stage, between 210–380 °C, corresponds to the decomposition of Eug adsorbed on the surface of the magnetite nanoparticles, and to the loss of physically adsorbed water molecules. The

eugenol coating content was estimated at 9% and 11% w/w for Eug·Fe₃O₄ (1g) and Eug·Fe₃O₄ (0.1g) sample, respectively.

The DTGA curves (Fig. 1H) showed that the Eug decomposition peak at about 195 °C was shifted to 285 °C in Eug·Fe₃O₄ (1g), and Eug·Fe₃O₄ (0.1g) samples. Based on these physicochemical results, Eug·Fe₃O₄ (1g) was selected for subsequent *in vitro* and *in vivo* biological evaluation, hereafter referred to simply as Eug·Fe₃O₄.

4.3.2. Cytotoxicity of eugenol, Fe₃O₄, and Eug·Fe₃O₄ in cell cultures

Cell viability results are shown in Fig. 1I–N. In VERO cells, neither Eug nor Fe₃O₄ affected cell viability at any tested concentration ($P > 0.05$). Likewise, Eug·Fe₃O₄ did not reduce cell viability at any tested concentration ($P > 0.05$). A significant increase in cell viability was observed only at 6.25 µg/mL compared with the control ($P < 0.05$). In MELAN-A cells, Eug significantly reduced cell viability at the highest concentrations tested (100 and 50 µg/mL, $P < 0.05$). Similarly, Eug·Fe₃O₄ decreased cell viability only at 50 µg/mL ($P < 0.05$), whereas no significant differences were detected at the remaining concentrations ($P > 0.05$). Fe₃O₄ did not affect MELAN-A cell viability at any tested concentration ($P > 0.05$).

To reflect the temporal progression of the epididymal response, results are presented sequentially according to the three evaluation periods: 24 hours, 14 days, and 80 days after intraepididymal injection.

4.3.3. Effects of intraepididymal application of Eug, Fe₃O₄, and Eug·Fe₃O₄ after 24 hours

4.3.3.1. Magnetite nanoparticles biodistribution

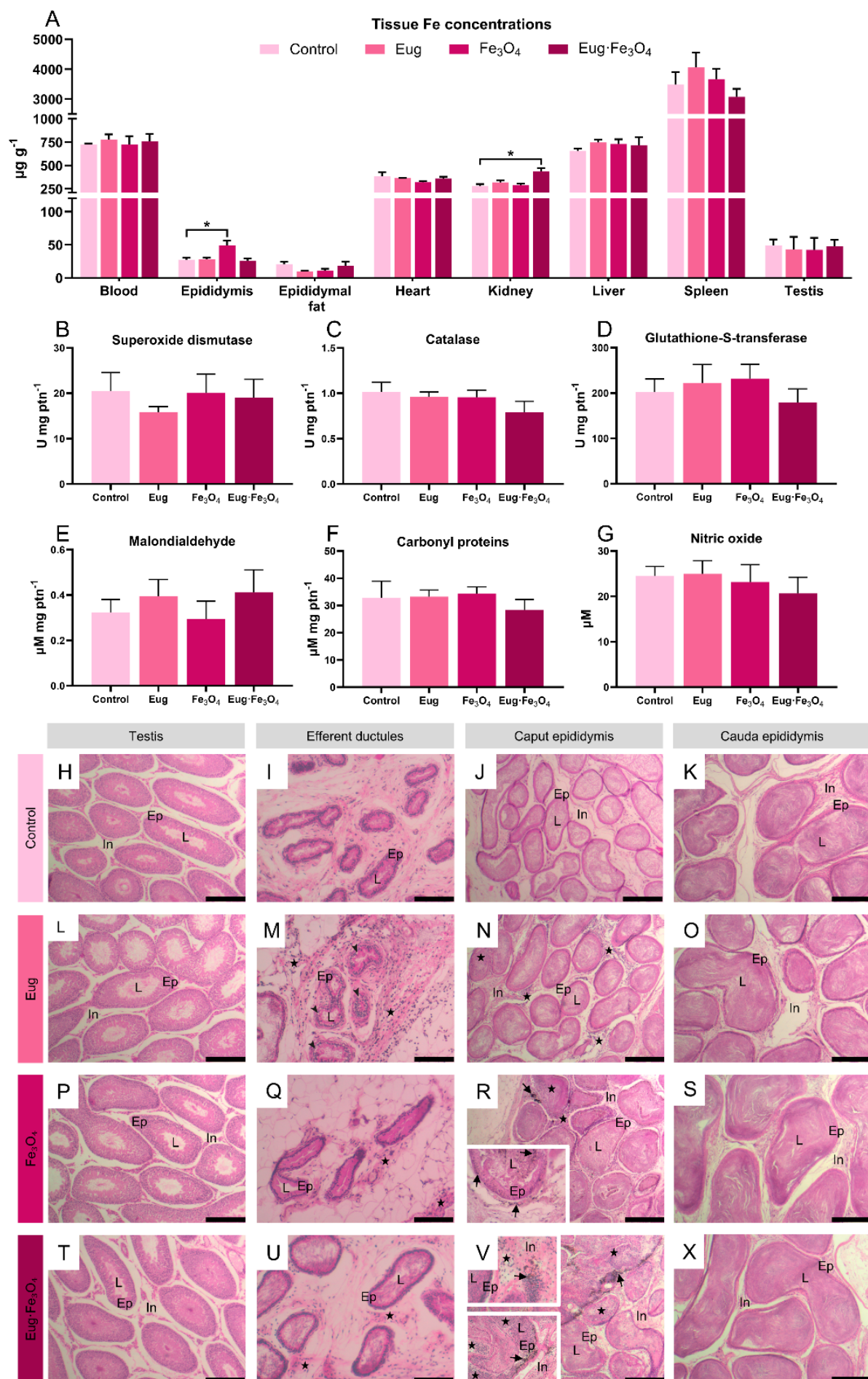


Fig. 2. Tissue Fe concentrations, epididymal antioxidant enzymes and oxidative/nitrosative stress markers, and representative histological sections of the testes, efferent ducts, caput epididymis, and cauda epididymis of adult male rats 24 h after intraepididymal injection. (A) Tissue Fe concentrations in blood, epididymis, epididymal adipose tissue, heart, kidney, liver, spleen, and testis. (B–G) Activity of antioxidant enzymes and oxidative/nitrosative stress markers in the epididymis, including superoxide dismutase (B), catalase (C), glutathione-S-transferase (D), malondialdehyde (E), carbonyl proteins (F), and nitric oxide (G). Data are expressed as mean $\pm$ standard deviation (SD). Control: 50 μ L of 2% Tween-20 in distilled water; Eug, Fe_3O_4 , and Eug· Fe_3O_4 : 5 μ g/50 μ L/epididymis. Asterisks indicate statistically significant differences between the indicated groups ($p < 0.05$; Tukey's test; $n = 5$ animals/group). Fe concentrations are expressed as $\mu\text{g g}^{-1}$. (H–K) Control group showing preserved architecture of the testis (H), efferent ductules (I), caput epididymis (J), and cauda epididymis (K). (L–O) Eug group showing preserved testicular architecture (L), marked periductal inflammatory infiltrate (★) associated with focal epithelial vacuolization (►) in the efferent ductules (M), marked interstitial inflammatory infiltrate (★) in the caput epididymis (N), and preserved morphology of the cauda epididymis (O). (P–S) Fe_3O_4 group showing preserved testicular architecture (P), mild interstitial inflammatory infiltrate (★) in the efferent ductules (Q), and discrete interstitial, intraepithelial, and intraluminal pigmented granular deposits compatible with the injected iron-oxide nanoparticles (→) associated with mild interstitial inflammatory infiltrate (★) in the caput epididymis (R), whereas the cauda epididymis remained morphologically preserved (S). (T–X) Eug· Fe_3O_4 group showing preserved architecture of the testis (T), mild interstitial inflammatory infiltrate (★) in the efferent ductules (U), together with interstitial pigmented granular deposits compatible with the injected iron-oxide nanoparticles (→) and mild inflammatory infiltrate (★) in the caput epididymis (V), while the cauda epididymis remained morphologically preserved (X). Insets in panels R and V show additional representative fields highlighting the pigmented granular deposits compatible with the injected iron-oxide nanoparticles. Ep, epithelium; L, lumen; In, interstitium. ★, inflammatory infiltrate; ►, epithelial vacuolization; →, pigmented granular deposits compatible with the injected iron-oxide nanoparticles. Hematoxylin and eosin stain. Scale bar = 200 μm .

Iron biodistribution 24 h after intraepididymal injection is summarized in Fig. 2A. A significant increase in Fe concentration was detected in the epididymis of animals treated with Fe_3O_4 nanoparticles compared with the other experimental groups ($p < 0.05$). In contrast, Fe concentrations in the testes and epididymal adipose tissue did not differ among groups ($p > 0.05$). Among non-

reproductive tissues, a significant increase in renal Fe concentration was observed in the Eug·Fe₃O₄ group compared with all other administrations ($p < 0.05$). No significant differences were detected in Fe concentrations in the liver, heart, blood, or spleen ($p > 0.05$).

4.3.3.2. *Antioxidant enzymes and oxidative/nitrosative stress markers*

The activity of antioxidant enzymes and oxidative/nitrosative stress markers in the epididymis is shown in Fig. 2B (superoxide dismutase), Fig. 2C (catalase), Fig. 2D (glutathione S-transferase), Fig. 2E (malondialdehyde), Fig. 2F (protein carbonyls), and Fig. 2G (nitric oxide), 24 h after intraepididymal administration of Eug, Fe₃O₄, or Eug·Fe₃O₄. No significant differences were observed among the experimental groups for any of these parameters ($p > 0.05$).

4.3.3.3. *Histopathology of testes, efferent ducts, and epididymis*

Twenty-four hours after intraepididymal injection, the testis showed preserved architecture in all groups, with regularly outlined seminiferous tubules, complete spermatogenesis and an unremarkable interstitium. No evidence of degeneration, inflammation, or interstitial alterations was observed. Sertoli and Leydig cells displayed normal morphology, and abundant spermatozoa were present within the tubular lumen (Fig. 2H,L,P,T). The efferent ductules and the caput epididymis of the control group (Fig. 2I,J) remained morphologically preserved, with no evidence of inflammatory infiltrate, epithelial degeneration, or luminal abnormalities. The cauda epididymis was similarly preserved in all groups, with lumina densely filled with spermatozoa (Fig. 2K,O,S,X).

In the Eug group, the efferent ductules exhibited a marked periductal inflammatory infiltrate associated with focal epithelial vacuolization (Fig. 2M). The caput epididymis showed marked interstitial and intraluminal inflammatory infiltrate (Fig. 2N).

The Fe₃O₄ group exhibited mild periductal inflammatory infiltrate in the efferent ductules (Fig. 2Q). The caput epididymis showed mild interstitial, intraepithelial, and intraluminal pigmented granular deposits compatible with the injected iron-oxide nanoparticles, accompanied by mild interstitial inflammatory infiltrate (Fig. 2R).

Similar findings were observed in the Eug·Fe₃O₄ group, including mild periductal inflammatory infiltrate in the efferent ductules (Fig. 2U) and pigmented granular deposits in the caput epididymis compatible with the injected iron-oxide nanoparticles, accompanied by mild interstitial inflammatory infiltrates (Fig. 2V).

4.3.4. Effects of intraepididymal application of Eug, Fe₃O₄, and Eug·Fe₃O₄ after 14 days

4.3.4.1. Magnetite nanoparticles biodistribution

Tissue iron concentration 14 days after intraepididymal injection are presented in Fig. 3A. No significant differences were observed among the experimental groups in the epididymis, epididymal adipose tissue, testes, liver, heart, kidneys, blood, or spleen ($p > 0.05$).

4.3.4.2. Behavioral changes and signs of toxicity

No clinical signs of systemic toxicity were observed in any of the experimental groups throughout the study. Water and food consumption remained within the expected range and did not differ between experimental groups. Likewise, no clinical abnormalities such as diarrhea, vomiting, piloerection or alopecia were observed during the experimental period. Evaluation using the Rat Grimace Scale revealed scores consistent with normal behavior in all groups with no evidence of injection-related pain or discomfort following intraepididymal injection. Body weight remained stable throughout the study, and no abnormal variations were observed among the groups. Similarly, body and scrotal temperatures remained stable during the first 14 days after injection and did not differ from those of the control group (Table 2).

Table 2. Daily assessment of pain-related behaviors, physiological parameters, and body weight during the first 14 days of the experimental period.

Parameters		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Grimace scale (event frequency)	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Eug	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Fe ₃ O ₄	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Eug·Fe ₃ O ₄	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Behavioral assessment (event frequency)	Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Eug	0	0	2 (LB)	0	1 (LB)	0	2 (LB)	0	0	0	0	0	0	0
	Fe ₃ O ₄	0	0	1 (LB)	0	1 (LB)	0	1 (LB)	0	0	0	0	0	0	0
	Eug·Fe ₃ O ₄	0	0	2 (LB)	0	0	0	1 (LB)	0	0	0	0	0	0	0
Body temperature (°C)	Control	36.65	36.43	36.48	37.08	36.78	36.95	37.08	36.88	36.95	37.00	36.80	36.73	36.85	36.88
		± 0.24	± 0.21	± 0.85	± 0.38	± 0.19	± 0.27	± 0.61	± 0.15	± 0.21	± 0.25	± 0.16	± 0.37	± 0.27	± 0.13
		36.96	36.76	36.92	36.90	36.68	37.50	37.42	36.70	37.24	37.06	37.10	36.62	36.76	36.84
	Eug	± 0.29	± 0.42	± 0.19	± 0.26	± 0.18	± 0.26	± 0.18	± 0.19	± 0.30	± 0.38	± 0.12	± 0.13	± 0.17	± 0.26
		37.26	37.06	36.88	36.82	36.76	37.42	37.52	36.68	37.46	37.70	37.24	36.42	36.78	36.72
		± 0.46	± 0.46	± 0.13	± 0.13	± 0.31	± 0.36	± 0.34	± 0.34	± 0.29	± 0.19	± 0.36	± 0.35	± 0.45	± 0.31
	Fe ₃ O ₄	37.08	36.68	36.66	36.70	36.62	36.92	36.94	36.74	36.88	37.08	37.20	36.66	36.52	36.94
		± 0.37	± 0.42	± 0.11	± 0.28	± 0.17	± 0.38	± 0.19	± 0.36	± 0.41	± 0.58	± 0.41	± 0.27	± 0.55	± 0.90
		0.37	0.42	0.11	0.28	0.17	0.38	0.19	0.36	0.41	0.58	0.41	0.27	0.55	0.90

Scrotal temperat ure (°C)	Control	35.85	33.90	32.95	34.53	33.15	32.75	33.80	33.83	33.28	34.43	34.95	35.08	34.00	33.05
		±	±	±	±	±	±	±	±	±	±	±	±	±	±
		0.35	0.66	0.30	0.82	0.77	0.53	0.87	0.43	0.82	0.43	0.98	0.82	0.70	0.64
	Eug	36.00	33.60	33.42	33.22	32.96	33.00	34.06	34.32	33.26	34.00	33.94	34.38	33.12	32.58
		±	±	±	±	±	±	±	±	±	±	±	±	±	±
		0.22	0.92	0.72	0.55	0.55	0.43	0.49	0.95	0.86	0.86	0.65	1.23	0.55	0.32
	Fe ₃ O ₄	35.58	34.64	34.32	33.70	32.64	33.08	34.38	33.72	33.88	33.82	33.74	34.36	33.28	34.00
		±	±	±	±	±	±	±	±	±	±	±	±	±	±
		0.71	1.01	0.83	0.09	0.35	0.56	0.74	0.24	0.74	0.78	0.96	0.36	0.19	0.83
	Eug· Fe ₃ O ₄	35.30	33.32	33.40	33.42	32.78	32.66	34.06	34.22	34.10	34.00	35.18	34.78	33.66	34.54
		±	±	±	±	±	±	±	±	±	±	±	±	±	±
		0.61	1.36	0.95	1.41	0.48	0.21	0.55	0.74	0.55	1.27	0.39	1.39	0.65	0.64
Body weight (g)	Control	350.2	353.5	355.2	356.7	359.7	361.2	364.2	364.5	366.0	366.7	366.7	373.2	369.5	372.3
		5 ±	0 ±	5 ±	5 ±	5 ±	5 ±	5 ±	0 ±	0 ±	5 ±	5 ±	5 ±	0 ±	0 ±
		32.57	34.92	35.82	31.23	33.89	32.88	36.10	35.03	35.19	35.54	36.65	31.45	35.80	32.30
	Eug	378.0	375.0	373.8	377.0	377.4	378.8	381.8	378.8	380.2	381.4	388.2	375.2	386.2	389.2
		0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±
		17.79	21.21	20.71	21.87	19.40	20.47	18.67	18.03	17.22	17.39	16.71	29.56	15.63	17.31
	Fe ₃ O ₄	365.2	368.6	367.0	369.8	371.4	373.6	378.0	375.6	379.0	374.8	378.8	379.2	381.0	391.4
		0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±
		13.63	14.88	17.61	13.37	13.52	13.09	12.29	15.27	16.39	15.27	13.48	14.25	10.05	15.25
	Eug· Fe ₃ O ₄	358.6	366.2	363.2	358.8	361.6	362.8	366.2	367.8	368.2	371.2	353.6	375.0	377.8	388.7
		0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±	0 ±
		5.18	4.92	11.05	10.26	13.59	12.44	10.13	12.74	13.22	13.33	38.93	15.30	12.66	14.27

Values are expressed as mean ± standard deviation (SD). Grimace scale and behavioral parameters are presented as the number of observed events per day. Animals were evaluated daily during the first 14 days of the experimental period (n = 5/group). Body and scrotal temperatures are expressed in degrees Celsius (°C), and body weight in grams (g). LB = Licking or biting behavior

4.3.4.3. *Physiological stress indicators*

Serum physiological stress indicators measured 14 days after injection are presented in Fig. 3B-D. No significant differences among the experimental groups were observed for total protein (Fig. 3B), glucose (Fig. 3C), or urea concentrations (Fig. 3D) ($p > 0.05$).

4.3.4.4. *Antioxidant enzymes and oxidative/nitrosative stress markers*

At 14 days after intraepididymal injection, significant changes were observed in several antioxidant and oxidative stress parameters ($p < 0.05$) (Fig. 3E-J). Superoxide dismutase activity was significantly higher in animals treated with Fe_3O_4 nanoparticles compared with the control, eugenol, and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ groups (Fig. 3E). A similar pattern was observed for catalase, with increased activity detected only in the Fe_3O_4 -treated group relative to the other experimental groups (Fig. 3F). Glutathione-S-transferase activity did not differ significantly among the groups ($p > 0.05$) (Fig. 3G). In contrast, markers of oxidative damage were significantly elevated in treated animals. Malondialdehyde levels were higher in all treated groups compared with the control group ($p < 0.05$) (Fig. 3H). Likewise, carbonyl protein concentrations were significantly increased in the eugenol, Fe_3O_4 , and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ groups relative to controls (Fig. 3I). Nitric oxide levels did not differ significantly among the experimental groups at this time point ($p > 0.05$) (Fig. 3J).

4.3.4.5. *Histopathology of testes, efferent ducts, and epididymis*

Fourteen days after intraepididymal injection, testicular architecture and spermatogenesis remained intact across all experimental groups (Fig. 3K,O,S,W). In the control group, the efferent ductules and the caput epididymis (Fig. 3L,M) were morphologically unaltered, with no evidence of inflammatory infiltrate or structural abnormalities. The cauda epididymis remained morphologically preserved in control, Fe_3O_4 , and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ groups (Fig. 3N,V,Z).

In the Eug group, the efferent ductules exhibited a mild periductal inflammatory infiltrate associated with epithelial disorganization, characterized by loss of the normal columnar arrangement with cellular stratification (Fig. 3P). The caput epididymis showed an injection-site nodular, with collagen fibers

surrounding the epididymal ducts and the nodular lesion, as demonstrated by Sirius Red staining (Fig. 3Q). The cauda epididymis remained morphologically preserve, with luminal spermatozoa present (Fig. 3R).

The Fe_3O_4 group exhibited a mild periductal inflammatory infiltrate in the efferent ductules (Fig. 3T). The caput epididymis showed an injection-site nodular lesion, accompanied by collagen fibers around the epididymal ducts and the nodular lesion on Sirius Red staining (Fig. 3U).

Similar findings were observed in the $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ group, including a mild periductal inflammatory infiltrate in the efferent ductules (Fig. 3X) and an injection-site nodular lesion in the caput epididymis, with collagen fibers around the epididymal ducts and the nodular lesion on Sirius Red staining (Fig. 3Y).

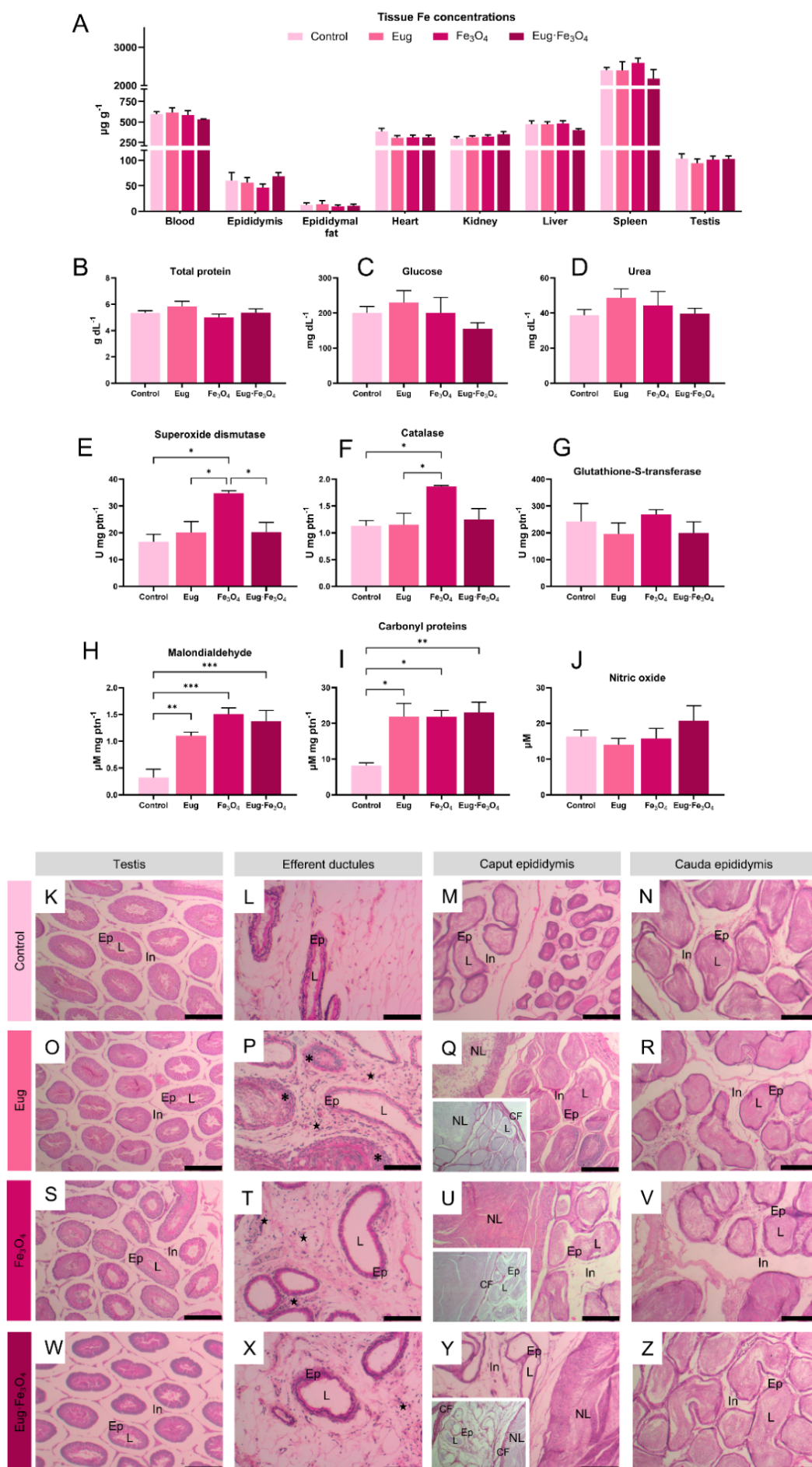


Fig. 3. Tissue Fe concentrations, serum physiological stress indicators, epididymal antioxidant enzymes and oxidative/nitrosative stress markers, and representative histological sections of the testes, efferent ducts, caput epididymis, and cauda epididymis of adult male rats 14 days after intraepididymal injection. (A) Tissue Fe concentrations in blood, epididymis, epididymal adipose tissue, heart, kidney, liver, spleen, and testis. (B) Total protein, (C) Glucose, (D) Urea. (E–J) Activity of antioxidant enzymes and oxidative/nitrosative stress markers in the epididymis, including superoxide dismutase (E), catalase (F), glutathione-S-transferase (G), malondialdehyde (H), carbonyl proteins (I), and nitric oxide (J). Data are expressed as mean $\pm$ standard deviation (SD). Control: 50 μ L of 2% Tween-20 in distilled water; Eug, Fe_3O_4 , and Eug· Fe_3O_4 : 5 μ g/50 μ L/epididymis. Asterisks indicate statistically significant differences between the indicated groups ($p < 0.05$; Tukey's test; $n = 5$ animals/group; $n = 10$ animals/group for serum physiological stress indicators). Fe concentrations are expressed as $\mu\text{g g}^{-1}$. (K–N) Control group showing preserved architecture of the testis (K), efferent ductules (L), caput epididymis (M), and cauda epididymis (N). (O–R) Eug group showing preserved testicular architecture (O), mild interstitial inflammatory infiltrates (★), epithelial disorganization (*) in the efferent ductules (P), nodular lesion (NL) in the caput epididymis (Q), and preserved cauda epididymis (R). (S–V) Fe_3O_4 group showing preserved testicular architecture (S), mild interstitial inflammatory infiltrates (★) in the efferent ductules (T), nodular lesion (NL) in the caput epididymis (U), and preserved morphology of the cauda epididymis (V). (W–Z) Eug· Fe_3O_4 group showing preserved testicular architecture (W), mild interstitial inflammatory infiltrates (★) and epithelial disorganization (*) in the efferent ductules (X), nodular lesion (NL) in the caput epididymis (Y), while the cauda epididymis remained morphologically preserved (Z). The insets in panels Q, U, and Y show the corresponding Sirius Red-stained sections, highlighting collagen fiber deposition within the nodular lesions and epididymal ducts. Ep, epithelium; L, lumen; In, interstitium; NL, nodular lesion; CF, collagen fibers; ★, inflammatory infiltrate; *, epithelial disorganization; Hematoxylin and eosin stain. Scale bar = 200 μm .

4.3.5. Effects of intraepididymal application of Eug, Fe_3O_4 , and Eug· Fe_3O_4 after 80 days

4.3.5.1. Antioxidant enzymes and oxidative/nitrosative stress markers

At 80 days after intraepididymal injection, the activity of antioxidant enzymes and oxidative/nitrosative stress markers in the epididymis was not significantly affected by the injection (Fig. 4). Animals receiving Eug, Fe_3O_4 , or Eug· Fe_3O_4 showed values comparable to the control group for superoxide dismutase (Fig. 4A), catalase (Fig. 4B), and glutathione-S-transferase activities (Fig. 4C), with no significant differences among groups. Likewise, markers of oxidative damage, including malondialdehyde (Fig. 4D), and carbonyl proteins

(Fig. 4E), as well as nitric oxide levels (Fig. 4F), remained similar across all experimental groups.

4.3.5.2. Serum and tissue hormone levels

Serum and intratesticular testosterone, serum FSH and LH concentrations at 80 days post-injection are shown in Fig. 4G-J. No marked variations were observed among the experimental groups for any of the evaluated hormonal parameters. Serum testosterone (Fig. 4G) and intratesticular testosterone concentrations (Fig. 4H) remained within a similar range across injection. Likewise, FSH and LH levels showed no evident alterations (Fig. 4I,J).

4.3.5.3. Histopathology of testes, efferent ducts, and epididymis

At 80 days after intraepididymal injection, all experimental groups retained normal testicular morphology and spermatogenesis (Fig. 4K,O,S,W). The control group displayed unaltered efferent ductules (Fig. 4L), caput epididymis (Fig. 4M) and cauda epididymis (Fig. 4N), devoid of inflammatory infiltrate, epithelial degeneration or luminal abnormalities, with lumina densely packed with spermatozoa.

In the Eug group, the efferent ductules exhibited narrowing of the ductular lumen (Fig. 4P). The caput epididymis showed an injection-site nodular lesion with collagen fibers surrounding the epididymal ducts and the nodular lesion on Sirius Red staining, together with narrowing of some epididymal duct lumina and absence of luminal spermatozoa (Fig. 4Q). The cauda epididymis showed narrowing of some epididymal duct lumina, presence of debris in the epididymal duct lumina, and absence of luminal spermatozoa (Fig. 4R).

The Fe_3O_4 group exhibited narrowing of the efferent ductules lumen associated with focal epithelial vacuolization (Fig. 4T). The caput epididymis showed an injection-site nodular lesion with collagen fibers around the epididymal ducts and the nodular lesion on Sirius Red staining, accompanied by absence of luminal spermatozoa (Fig. 4U). The cauda epididymis showed narrowing of some epididymal duct lumina, and absence of luminal spermatozoa (Fig. 4V).

In the Eug· Fe_3O_4 group, the efferent ductules exhibited epithelial disorganization in some ductules, characterized by loss of the normal columnar arrangement with cellular stratification, associated with focal epithelial

vacuolization and narrowing of some ductular lumina (Fig. 4X). The caput epididymis showed an injection-site nodular lesion with collagen fibers around the epididymal ducts and the nodular lesion on Sirius Red staining, accompanied by absence of luminal spermatozoa (Fig. 4Y). The cauda epididymis showed

narrowing of some epididymal duct lumina, and absence of luminal spermatozoa (Fig. 4Z).

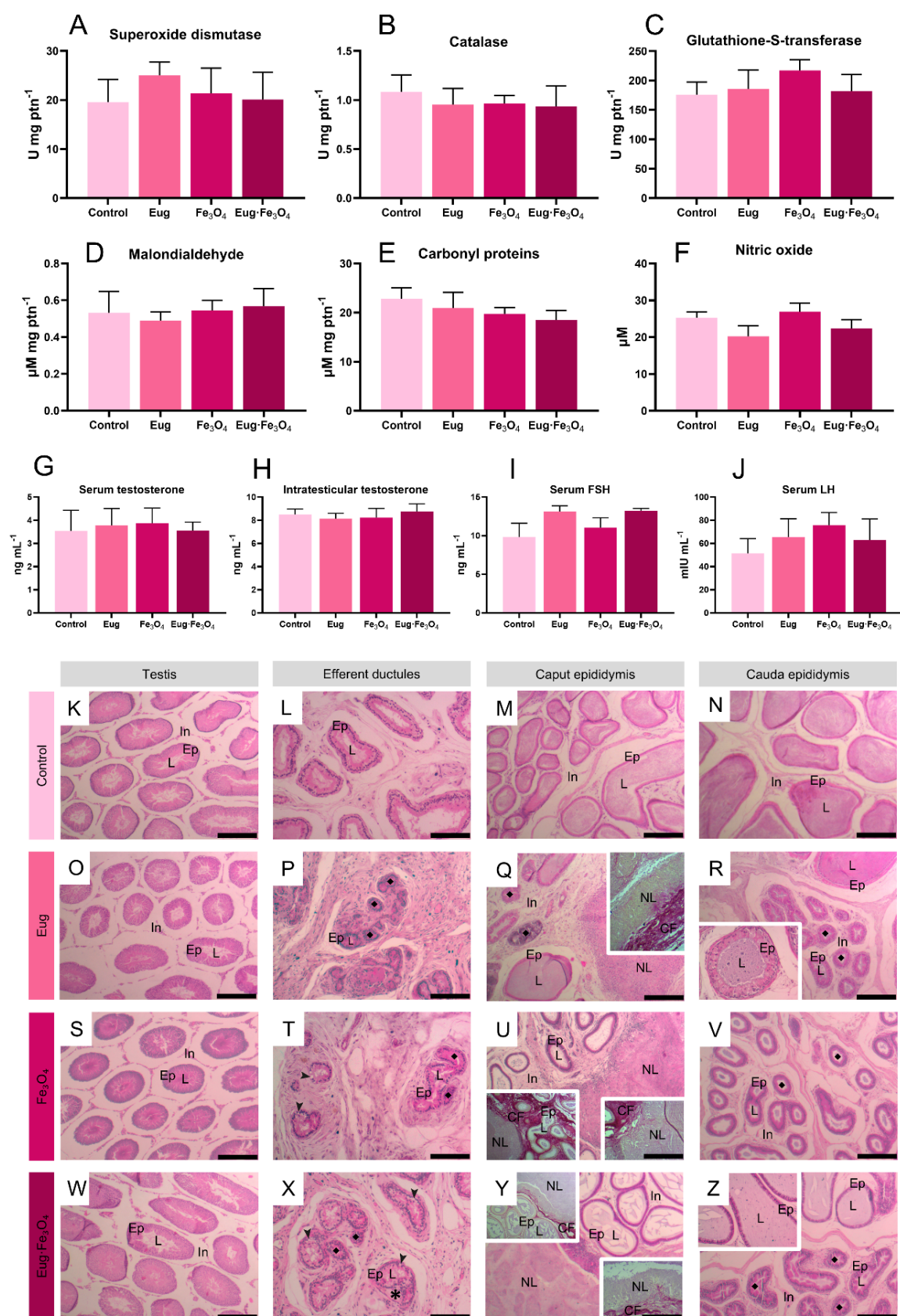


Fig. 4. Epididymal antioxidant enzymes and oxidative/nitrosative stress markers, reproductive hormone concentrations, and representative histological sections of the testes, efferent ducts, caput epididymis, and cauda epididymis of adult male rats 80 days after intraepididymal injection.

(A–F) Activity of antioxidant enzymes and oxidative/nitrosative stress markers in the epididymis, including superoxide dismutase (A), catalase (B), glutathione-S-transferase (C), malondialdehyde (D), carbonyl proteins (E), and nitric oxide (F). (G–J) Reproductive hormone concentrations, including serum testosterone (G), intratesticular testosterone (H), serum FSH (I), and serum LH (J). Data are expressed as mean $\pm$ standard deviation (SD). Control: 50 μ L of 2% Tween-20 in distilled water; Eug, Fe_3O_4 , and Eug· Fe_3O_4 : 5 μ g/50 μ L/epididymis. Asterisks indicate statistically significant differences between the indicated groups ($p < 0.05$; Tukey's test; $n = 5$ animals/group; $n = 10$ animals/group for serum reproductive hormones). (K–N) Control group showing preserved testicular architecture (K), efferent ductules (L), caput epididymis (M), and cauda epididymis (N); (O–R) Eug group showing preserved testicular architecture (O), luminal narrowing of the efferent ductules ($\blacklozenge$) (P), nodular lesion, and luminal narrowing of the caput epididymal ducts ($\blacklozenge$) (Q), luminal narrowing of the cauda epididymal ducts ($\blacklozenge$) associated with absence of luminal spermatozoa; inset panel highlights the presence of luminal debris (R); (S–V) Fe_3O_4 group showing preserved testicular architecture (S), focal epithelial vacuolization ($\blacktriangleright$), luminal narrowing of the efferent ductules ($\blacklozenge$) (T), nodular lesion, and absence of luminal spermatozoa in the caput epididymis (U), luminal narrowing of the cauda epididymal ducts ($\blacklozenge$), together with absence of luminal spermatozoa (V); (W–Z) Eug· Fe_3O_4 group showing preserved testicular architecture (W), focal epithelial vacuolization ($\blacktriangleright$), epithelial disorganization (*), and luminal narrowing of the efferent ductules ($\blacklozenge$) (X), nodular lesion, and absence of luminal spermatozoa in the caput epididymis (Y), luminal narrowing of the cauda epididymal ducts ($\blacklozenge$) associated with absence of luminal spermatozoa (Z). Insets in panels Q, U, and Y show the corresponding Sirius Red-stained sections, highlighting collagen fiber deposition within the nodular lesions and the surrounding epididymal connective tissue. Ep, epithelium; L, lumen; In, interstitium; NL, nodular lesion; CF, collagen fiber deposition. $\star$, inflammatory infiltrate; *, epithelial disorganization; $\blacklozenge$, luminal narrowing. Hematoxylin and eosin stain. Insets: Sirius Red stain. Scale bar = 200 μ m.

4.3.5.4. Proteomic analysis

A total of 1,601 proteins were identified in the epididymal fluid across the four experimental groups ($n = 5$ animals/group). Venn analysis revealed that 614 proteins (38,4%) were common to all groups, representing the core epididymal proteome. The number of proteins exclusively identified in a single group was 80 for Control, 208 for Eug, 12 for Fe_3O_4 , and 71 for Eug· Fe_3O_4 (Fig. 5A). The Eug

group therefore showed the largest set of exclusive proteins, whereas the Fe_3O_4 group showed the smallest. Among pairwise overlaps, 265 proteins were shared exclusively between Fe_3O_4 and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$, and 102 proteins were shared exclusively between Eug , Fe_3O_4 and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$.

KEGG enrichment analysis of the group-exclusive proteins was performed using the DAVID platform (Fig. 5B), based on statistical significance expressed by the $-\log(\text{p-value})$. In the Control group, the enriched pathways comprised baseline physiological processes and pathways of neurodegeneration – multiple diseases, thermogenesis, chemical carcinogenesis – reactive oxygen species, oxidative phosphorylation, motor proteins, and cardiac muscle contraction. The Eug group showed the highest number of enriched pathways, including one carbon pool by folate, nicotinate and nicotinamide metabolism, purine metabolism, biosynthesis of amino acids, alanine, aspartate and glutamate metabolism, nucleotide metabolism, pyrimidine metabolism, cysteine and methionine metabolism, biosynthesis of cofactors, and protein processing in the endoplasmic reticulum. In the $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ group, three pathways were enriched: carbon metabolism, citrate cycle (TCA cycle) and propanoate metabolism. No significantly enriched pathway ($p > 0.05$) was identified for the proteins exclusive to the Fe_3O_4 group.

Functional protein–protein interaction networks were constructed for each experimental group (Fig. 5). A total of 80 differentially expressed proteins were identified and grouped according to their biological processes. In all groups, the identified proteins were distributed across functional macro-clusters corresponding to proteolysis (protease/peptidase and protease/peptidase inhibitor subclusters), immune response (immunoglobulins and complement activation subclusters), metabolism (including a lipid metabolism subcluster), cytoskeleton, redox, heat shock proteins (HSP), spermatogenesis and iron transport/binding. The overall network topology and cluster organization were comparable among groups. The control group represents the baseline biological state (Fig. 5C). Differences were observed in the relative abundance profile of the constituent nodes: the Eug group (Fig. 5D) displayed a predominance of higher-abundance (red) nodes (upregulation) within the proteolysis and immune response clusters, whereas the Fe_3O_4 (Fig. 5E) and $\text{Eug}\cdot\text{Fe}_3\text{O}_4$ (Fig. 5F) groups displayed a greater proportion of lower-abundance (blue) (downregulation) nodes

within the metabolism and redox clusters. Nodes assigned to the iron transport/binding cluster were present in all groups.

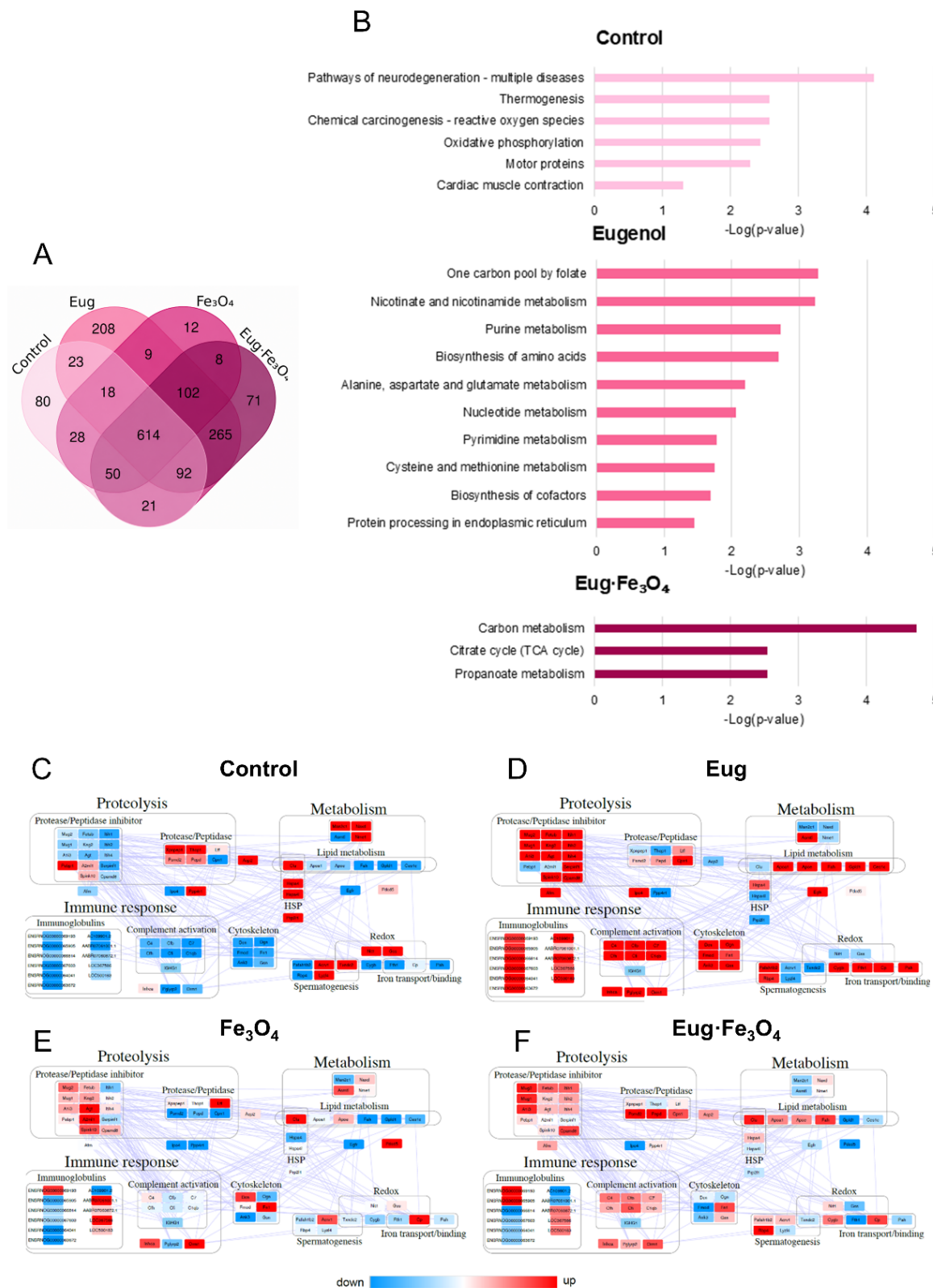


Fig. 5. Distribution and functional enrichment of the epididymal fluid proteome of adult male rats 80 days after intraepididymal injection. (A) Four-way Venn diagram showing the number of proteins shared and exclusively identified among Control, Eug, Fe₃O₄, and Eug-Fe₃O₄ groups. **(B)** KEGG

pathway enrichment analysis of group-exclusive proteins for Control, Eugenol, and Eug·Fe₃O₄, ranked by statistical significance ($-\log[p\text{-value}]$; DAVID platform, $p \leq 0.05$); no pathway reached statistical enrichment among the 12 proteins exclusive to the Fe₃O₄ group. (C) Functional protein–protein interaction (PPI) networks constructed from the differentially abundant proteins of each experimental group, organized into functional clusters (proteolysis, metabolism, immune response, cytoskeleton, redox, spermatogenesis, iron transport/binding, and heat shock proteins [HSP]). Node color indicates the direction of relative protein abundance change compared with control (blue, downregulated; red, upregulated). Samples were obtained from cauda epididymal fluid at 80 days after intraepididymal injection ($n = 5$ animals/group).

4.3.5.5. *Sperm motility, sperm plasma membrane integrity, and sperm morphology*

At 80 days after injection, treatment affected several sperm quality parameters. All treated groups exhibited markedly reduced sperm motility compared with the control group (Fig. 6A). Likewise, the percentage of sperm with normal morphology was significantly lower in the Eug and Eug·Fe₃O₄ groups than in the control group ($p < 0.05$) (Fig. 6C). In contrast, sperm plasma membrane integrity was significantly reduced only in the Eug group, whereas the Fe₃O₄ and Eug·Fe₃O₄ groups did not differ from the control group (Fig. 6B). The frequency of sperm head defects was significantly higher in the Eug and Eug·Fe₃O₄ groups than in the control group (Fig. 6D). Similarly, the frequency of sperm midpiece defects was significantly higher in the Eug and Eug·Fe₃O₄ groups than in the control group (Fig. 6E). The frequency of sperm tail defects was also significantly higher in the Eug and Eug·Fe₃O₄ groups than in the control group (Fig. 6F).

4.3.5.6. *Fertility indexes*

At 80 days after intraepididymal injection, male mating behavior was not affected by any treatment, with all treated groups maintaining a mating index equivalent to 100% of the control value (Fig. 6G). Male fertility, however, differed among groups (Fig. 6H). Five of the ten control males sired offspring, compared with none in the Eug group and one of ten in both the Fe₃O₄ and Eug·Fe₃O₄ groups. To facilitate comparison among groups, fertility was normalized to the

control group (100%), resulting in relative fertility indexes of 0%, 10%, and 10% for the Eug, Fe_3O_4 , and Eug· Fe_3O_4 groups, respectively.

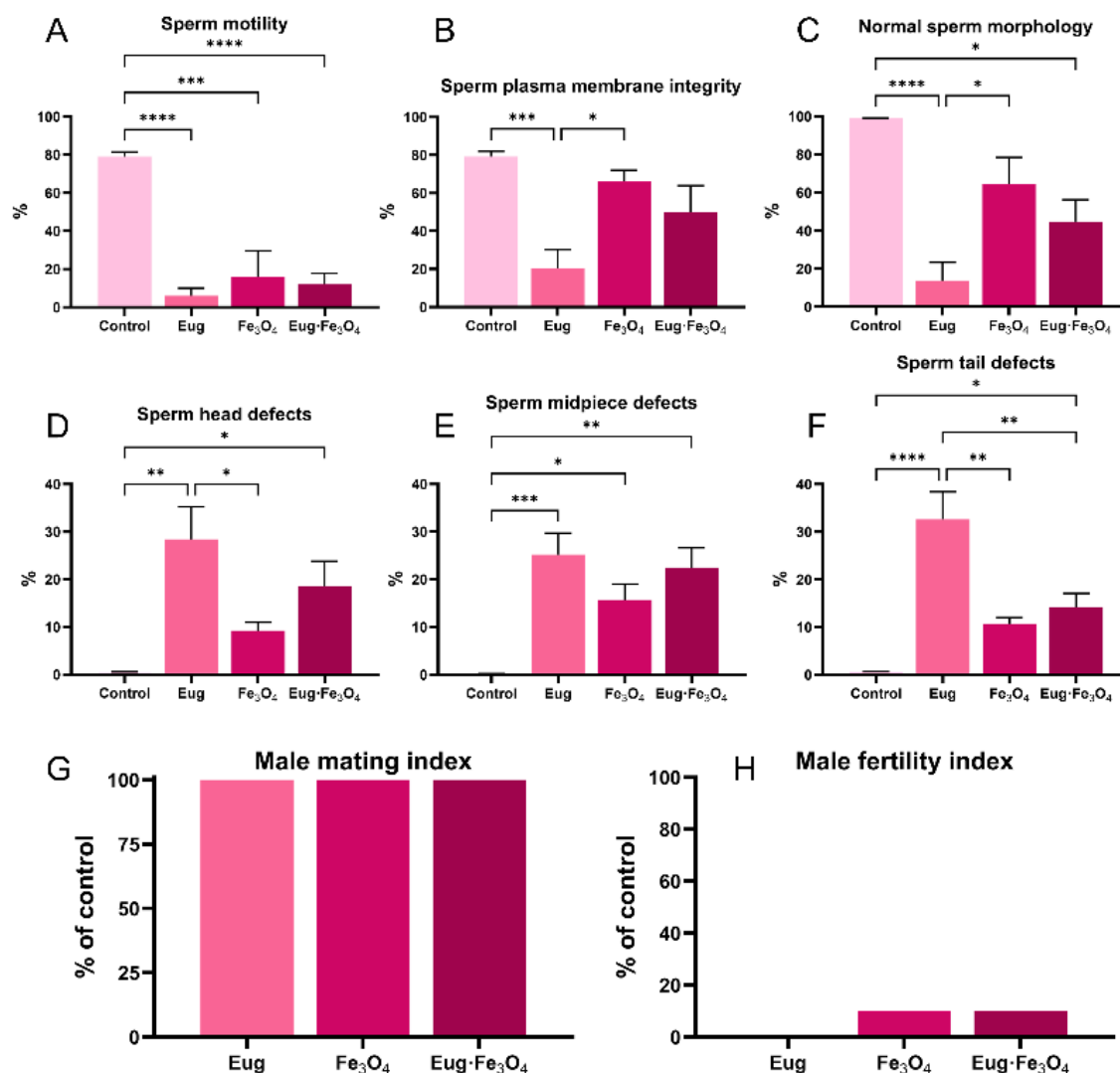


Fig. 6. Sperm quality parameters and reproductive indices of adult male rats 80 days after intraepididymal injection. (A) Sperm motility. (B) Sperm plasma membrane integrity. (C) Normal sperm morphology. (D) Sperm head defects. (E) Sperm midpiece defects. (F) Sperm tail defects. Data in A–F are expressed as percentage (%) and represent mean $\pm$ standard deviation (SD). Control: 50 μL of 2% Tween-20 in distilled water; Eug, Fe_3O_4 , and Eug· Fe_3O_4 : 5 $\mu\text{g}/50 \mu\text{L}$ /epididymis. Asterisks indicate statistically significant differences between the indicated groups (Tukey's test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; $n = 5$ animals/group). (G) Male mating index and (H) male fertility index, expressed as percentage relative to the control group (100%).

4.4. Discussion

This study demonstrates that a single bilateral intraepididymal injection of eugenol, Fe₃O₄ nanoparticles, or eugenol-coated Fe₃O₄ nanoparticles impairs male fertility in Wistar rats through a localized process that progresses from acute inflammation to nodular lesion formation. These structural changes were accompanied by alterations in sperm quality, the epididymal proteome, and the male fertility index, indicating that the local lesion was associated with functional and molecular changes beyond the histological findings. Conjugation of eugenol to Fe₃O₄ modified its local biological effects, supporting our hypothesis that nanoparticle association modulates eugenol bioavailability and, consequently, the tissue response. Interestingly, Fe₃O₄ nanoparticles alone also impaired fertility, indicating that they exert biological effects at the injection site rather than acting solely as an inert carrier. Overall, these findings reinforce the epididymis as a suitable target for inducing infertility through a local, non-endocrine mechanism and demonstrate that the biological effects of eugenol can be modulated, although not abolished, by nanoparticle functionalization.

Eugenol has been investigated as a non-surgical chemical contraceptive because of its concentration-dependent cytotoxic effects (Abshenas et al., 2013; Nejad et al., 2017; Nisar et al., 2021; Carvalho et al., 2026). At low concentrations, eugenol scavenges free radicals and inhibits cyclooxygenase and prostaglandin H synthase, reducing the release of pro-inflammatory mediators by macrophages (Thompson & Eling, 1989; Lee et al., 2007; Pramod et al., 2010). At higher local concentrations, however, eugenol undergoes oxidation to form an electrophilic quinone methide (Thompson et al., 1990; Han et al., 2007; Bolton et al., 2018; Carvalho et al., 2022b), which binds to nucleophilic sites on cellular macromolecules, inactivates proteins, and promotes local tissue injury (Thompson et al., 1993; Medeiros et al., 1996; Nisar et al., 2021). Accordingly, the inflammatory response induced by eugenol appears to be secondary to its cytotoxic action (Fujisawa et al., 2002; Lopes et al., 2018). Intratesticular administration of clove oil in dogs induces diffuse tubular necrosis associated with oedema, haemorrhage, vascular congestion, and inflammatory infiltrate (Abshenas et al., 2013; Kumar et al., 2023; Poormohammad et al., 2025). Likewise, clove oil produces testicular degeneration and testosterone suppression comparable to calcium chloride, although with less acute pain and

swelling (Abshenas et al., 2013; Abu-Ahmed, 2015; Poormohammad et al., 2025), indicating that its cytotoxic action coexists with its anti-inflammatory and analgesic properties. Previous findings from our group (Carvalho et al., 2026) further demonstrated that the epididymis is a more suitable target than the testis for eugenol-based chemosterilization. Unlike intratesticular administration, which damages the seminiferous epithelium and impairs endocrine function regardless of the sclerosing agent, intraepididymal administration induces infertility while preserving testicular architecture and hormone production (Xu et al., 1985; Fahim et al., 1993; Kutzler, 2015). However, free eugenol produces an extensive necrotizing lesion in the epididymis, suggesting that reducing local tissue damage without compromising contraceptive efficacy may improve this strategy. Based on this rationale, we investigated whether conjugation of eugenol to Fe_3O_4 nanoparticles could modulate its local biological effects.

The synthesized nanoparticles were characterized before the biological assays to confirm their crystalline structure, colloidal stability, and successful surface functionalization with eugenol. X-ray diffraction confirmed the preservation of the cubic spinel structure of magnetite without detectable secondary iron oxide phases. FTIR spectra retained the characteristic vibrational bands of both magnetite and eugenol, indicating successful surface functionalization while preserving the crystalline core. Coating markedly altered the physicochemical properties of the nanoparticles, shifting the zeta potential from +5.6 mV in uncoated Fe_3O_4 to -56.7 and -60.8 mV in the coated formulations, reducing the hydrodynamic diameter, and changing the thermal decomposition profile of eugenol, consistent with its interaction with the nanoparticle surface. These findings are consistent with previous studies from our group and collaborators using iron oxide and gold nanoparticles (Batista et al., 2026; Andrade et al., 2026). Iron oxide nanoparticles combine biodegradability, biocompatibility, and relatively low intrinsic toxicity among inorganic nanomaterials evaluated in male reproductive models (Dantas et al., 2022; Yang et al., 2022; Verma et al., 2022). In addition, surface functionalization has been shown to modify the biological behavior of both iron oxide (Ferraz et al., 2024) and gold nanoparticles (Coimbra et al., 2023, 2025). Fe_3O_4 nanoparticles functionalized with natural or synthetic coatings have also been explored to improve drug delivery and modulate release kinetics (Shen et al.,

2018; Nordin et al., 2023; Sharma et al., 2025). Their superparamagnetic properties further allow externally triggered drug release under alternating magnetic fields (Liu et al., 2020; Wang et al., 2023; Desai et al., 2025), representing a potential strategy for optimizing eugenol delivery. Before the *in vivo* experiments, the formulations were evaluated in VERO and MELAN-A cell lines. None of the formulations affected VERO cell viability. In MELAN-A cells, reductions in viability were restricted to the highest concentrations of Eug and Eug·Fe₃O₄, whereas Fe₃O₄ alone had no effect. These physicochemical and *in vitro* findings supported the progression of the nanoformulation to the *in vivo* experiments.

Twenty-four hours after intraepididymal injection, Fe₃O₄ nanoparticles remained predominantly associated with the epididymis, as demonstrated by the increased tissue iron concentration. The presence of pigmented granular deposits in the caput epididymis was consistent with the tissue iron measurements and further supported local retention of the nanoparticles. Although the Eug·Fe₃O₄ group showed higher renal iron concentrations, no increase was detected in the testes, liver, heart, spleen, or blood, indicating limited systemic distribution during the first 24 h after administration. The biodistribution of magnetic iron oxide nanoparticles is strongly influenced by their physicochemical properties, including particle size, surface charge, coating molecules, and route of administration, all of which affect tissue distribution and clearance (Jain et al., 2008; Alphan  ry, 2019; Nowak-Jary & Machnicka, 2023). In the present study, FTIR analysis together with the changes in hydrodynamic diameter and zeta potential confirmed the successful functionalization of Fe₃O₄ nanoparticles with eugenol. These modifications may have contributed to the distinct biodistribution profile observed for Eug·Fe₃O₄, including the higher renal iron concentration detected at 24 h. However, additional pharmacokinetic studies are required to determine whether this finding reflects transient renal distribution, clearance of a fraction of the nanoformulation, or another mechanism.

The localized distribution of the formulations was accompanied by an inflammatory response restricted to the efferent ductules and caput epididymis, whereas the testicular architecture, spermatogenesis, and cauda epididymis remained preserved. Similar early inflammatory responses have been described following administration of sclerosing agents for chemical sterilization, in which

local tissue injury represents the initial biological response and may precede fibrosis or ductal obstruction, depending on the agent and experimental conditions (Ribeiro et al., 2026). A comparable sequence has also been reported after intratesticular administration of zinc gluconate in cats, where inflammatory cell infiltration preceded seminiferous epithelial degeneration and germ cell loss (Fagundes et al., 2014). Despite the localized inflammatory infiltrate, antioxidant enzyme activity and oxidative and nitrosative stress markers remained unchanged 24 h after treatment. These findings indicate that the initial tissue response occurred in the absence of detectable oxidative imbalance, suggesting that the early biological effects were predominantly associated with local tissue injury. Persistent inflammatory stimuli are recognized as important drivers of fibroblast activation and extracellular matrix deposition during the progression of fibrosis (Xu et al., 2025; Liang & Liu, 2026; Liu et al., 2026).

Fourteen days after intraepididymal injection, the acute inflammatory response observed at 24 h had evolved into a localized nodular lesion in the caput epididymis of the Eug, Fe₃O₄, and Eug·Fe₃O₄ groups, accompanied by early collagen deposition demonstrated by Sirius Red staining. Mild inflammatory infiltrates persisted in the efferent ductules, and epithelial disorganization was observed in the Eug and Eug·Fe₃O₄ groups, whereas the testicular architecture and the morphology of the cauda epididymis remained preserved. This progression from an acute inflammatory response to localized tissue remodeling is consistent with the expected sequence of events described after administration of sclerosing agents to the male reproductive tract and with the onset of fibrosis, as evidenced by collagen deposition (Jana & Samanta, 2011; Hess et al., 2024; Xu et al., 2025; Ribeiro et al., 2026).

This stage of tissue remodeling was accompanied by oxidative damage, as demonstrated by increased malondialdehyde and protein carbonyl levels in the Eug, Fe₃O₄, and Eug·Fe₃O₄ groups. In contrast, superoxide dismutase and catalase activities increased only in the Fe₃O₄ group, whereas glutathione-S-transferase activity and nitric oxide levels remained unchanged. The selective increase in SOD and catalase activity in the Fe₃O₄ group is consistent with the ability of iron oxide nanoparticles to activate antioxidant defenses through redox-active mechanisms (Jiang et al., 2022; Ferraz et al., 2024; Islam et al., 2026). In contrast, the Eug and Eug·Fe₃O₄ groups showed oxidative damage without a

corresponding increase in these enzymes, a response similar to previous reports describing suppression of antioxidant enzymes by eugenol despite increased lipid and protein oxidation (Carvalho et al., 2022b, 2023). Because oxidative stress markers were measured in whole epididymal homogenates, part of the observed signal may reflect reactive oxygen species generated by infiltrating inflammatory cells rather than exclusively oxidative changes within the epididymal epithelium (Machado-Neves, 2022).

Unlike the findings at 24 h, tissue iron concentrations no longer differed among the experimental groups after 14 days, suggesting that the early differences in tissue distribution were no longer detectable at this stage. Likewise, no clinical signs of systemic toxicity, pain-related behavior, changes in body weight, body or scrotal temperature, or alterations in serum glucose, total protein, and urea concentrations were detected throughout the experimental period. These findings indicate that, despite the expected local tissue remodeling induced by the formulations, no detectable systemic adverse effects were observed during the first two weeks after administration. This profile is desirable for the development of chemical sterilization protocols, in which the induction of localized reproductive tract lesions should ideally occur without clinically relevant systemic effects (Ribeiro et al., 2026).

Eighty days after intraepididymal injection, the localized nodular lesion identified at 14 days persisted in the caput epididymis of the Eug, Fe_3O_4 , and Eug· Fe_3O_4 groups. Sirius Red staining demonstrated denser collagen deposition than at the previous timepoint, accompanied by narrowing of the efferent ductules and epididymal ducts and absence of luminal spermatozoa within the affected segments. Epithelial disorganization remained evident in the Eug and Eug· Fe_3O_4 groups, whereas the testicular architecture remained preserved throughout the experimental period. Together, these findings indicate progressive epididymal remodeling, with increasing fibrosis while maintaining structural preservation of the testis, a pattern consistent with the obstructive mechanism described for chemical sterilization of the male reproductive tract (Hess et al., 2024; Ribeiro et al., 2026; Carvalho et al., 2026).

Unlike the findings at 14 days, oxidative and nitrosative stress markers had returned to values comparable to the control group by 80 days, indicating that oxidative stress was associated with the early phase of tissue remodeling

rather than with maintenance of the chronic lesion. Likewise, serum and intratesticular testosterone concentrations, as well as FSH and LH levels, remained unchanged throughout the experimental period, indicating preservation of endocrine function. This endocrine profile distinguishes the present approach from intratesticular administration of eugenol and metal nanoparticles, in which direct testicular injury is accompanied by reduced testosterone production and disruption of Leydig cell function (Liu et al., 2020; Ferraz et al., 2024; Coimbra et al., 2023, 2025; Brito et al., 2025; Carvalho et al., 2026). These findings reinforce that the contraceptive effects observed after intraepididymal administration resulted primarily from localized epididymal remodeling rather than endocrine disruption.

Because sperm maturation depends on the specialized luminal environment established by the epididymal epithelium, structural alterations in this tissue are expected to affect the composition of epididymal fluid (Hinton & Paladino, 1995; Dacheux & Dacheux, 2014; James et al., 2020; Breton et al., 2020). Consistent with this concept, proteomic analysis revealed distinct molecular signatures among the experimental groups. Although 614 proteins were shared by all groups, the Eug group showed the largest number of exclusive proteins (208) and enrichment of pathways related to one-carbon metabolism, nucleotide metabolism, purine and pyrimidine metabolism, cysteine and methionine metabolism, and protein processing in the endoplasmic reticulum. In contrast, the Fe_3O_4 group exhibited only 12 exclusive proteins and no significantly enriched pathways, whereas the Eug· Fe_3O_4 group presented an intermediate profile, with enrichment of carbon metabolism, the tricarboxylic acid cycle, and propanoate metabolism. Together, these findings indicate that the extent of epithelial remodeling was accompanied by proportional changes in the molecular composition of the epididymal fluid.

The proteomic profile also provides insight into the mechanisms underlying the persistent epididymal lesion (Cornwall, 2009; Skerrett-Byrne et al., 2022; Trigg et al., 2026). Enrichment of cysteine and methionine metabolism in the Eug group is particularly relevant because these pathways provide the cysteine required for glutathione synthesis, one of the major antioxidant defense systems of epididymal epithelial cells (Wang et al., 1997; Lu, 2013; Chiang et al., 2022; Margret & Jain. 2024). Likewise, the predominance of proteolysis- and

immune-related protein interaction networks agrees with the persistent inflammatory infiltrate and tissue remodeling (Zhang et al., 2026) observed histologically, whereas the limited proteomic alterations in the Fe₃O₄ group are consistent with its milder biological effects across the evaluated endpoints. Since the epididymal epithelium establishes the luminal environment required for sperm maturation through the secretion and absorption of proteins, ions, and metabolites (Hinton & Palladino, 1995; Dacheux & Dacheux, 2014; Gervasi & Visconti, 2017; Marchiani et al., 2017; Zhou et al., 2018), these molecular alterations likely reflect functional changes in epididymal epithelial activity rather than simply representing secondary consequences of tissue injury. As a result, the altered luminal environment may have compromised the acquisition of sperm fertilizing capacity, providing a plausible mechanistic link between persistent epithelial remodeling and the functional impairment observed at 80 days.

These molecular alterations provide a plausible explanation for the functional impairment observed at 80 days. The reduction in sperm motility, increased morphological abnormalities, and marked decline in fertility are consistent with disruption of the epididymal luminal environment required for sperm maturation. Previous studies demonstrated that eugenol impairs sperm motility by disrupting calcium homeostasis through increased Ca²⁺-ATPase activity and interaction with TRPA1 channels, even in the absence of histological lesions (Carvalho et al., 2022). However, fertility in the present study was assessed approximately one complete spermatogenic cycle after treatment, making it unlikely that the reproductive impairment resulted solely from direct effects on spermatozoa present in the epididymis at the time of injection. Because sperm acquire progressive motility, membrane stability, and fertilizing capacity during epididymal transit through sequential interactions with the luminal microenvironment established by the epididymal epithelium (Cooper & Yeung, 2006; Dacheux & Dacheux, 2014; Gervasi & Visconti, 2018; Agudo-Rios et al., 2026), the persistent structural remodeling and proteomic alterations observed in the present study provide a more plausible explanation for the sustained impairment of sperm function and fertility.

The preservation of testicular architecture together with the absence of detectable endocrine alterations distinguishes intraepididymal administration from intratesticular chemosterilization approaches. Previous studies using

intratesticular eugenol (Carvalho et al., 2026), superparamagnetic iron oxide nanoparticles (Ferraz et al., 2024), and gold nanoparticles (Coimbra et al., 2023, 2025) demonstrated that direct testicular exposure compromises Leydig cell function and reduces testosterone production as part of the contraceptive mechanism. In contrast, the present findings indicate that infertility resulted primarily from persistent epididymal remodeling and disruption of sperm maturation while preserving testicular function. These observations support the epididymis as a promising target for the development of non-hormonal male contraceptive strategies.

The localized nature of this response is further supported by the absence of detectable systemic adverse effects throughout the experimental period. No persistent alterations were observed in physiological, behavioral, biochemical, or endocrine parameters, and tissue iron concentrations had returned to control values by 14 days despite persistence of the epididymal lesion at 80 days. Together, these findings indicate that the long-term biological response remained confined to the epididymis rather than resulting from persistent nanoparticle retention or systemic toxicity, an important characteristic for the development of chemical sterilization strategies (Ribeiro et al., 2026).

Overall, the present findings support the hypotheses of this study. Intraepididymal administration of eugenol reproduced the localized epididymal lesion previously described by our group (Carvalho et al., 2026), whereas Fe_3O_4 nanoparticles alone also induced epididymal remodeling, although with a milder biological response. Functionalization of Fe_3O_4 with eugenol successfully modified the physicochemical properties of the nanoparticles, as demonstrated by FTIR analysis together with changes in hydrodynamic diameter and zeta potential, and resulted in attenuation of the biological effects observed after administration of free eugenol. This modulation was accompanied by a partial reduction in contraceptive efficacy, suggesting that changes in local bioavailability or release kinetics influenced the intensity of the epididymal response rather than its underlying mechanism. Although conjugation to Fe_3O_4 did not enhance the contraceptive effect of eugenol, modulation of the local biological response may represent an important advantage for future optimization of this strategy. The ability to balance contraceptive efficacy with controlled tissue

remodeling may be particularly relevant for the development of non-surgical sterilization protocols intended for large-scale animal population management.

Despite the 80-day follow-up, the reversibility of the epididymal lesion was not investigated. Future studies should extend the observation period, evaluate different doses, and explore drug delivery strategies that take advantage of the superparamagnetic properties of Fe_3O_4 nanoparticles (Ferraz et al., 2024; Zhao et al., 2025). Despite these limitations, the present findings contribute to the understanding of intraepididymal administration as a localized, non-hormonal approach for male contraception and provide a foundation for the future optimization of this strategy.

4.5. Conclusion

In conclusion, intraepididymal administration of eugenol produced long-term infertility through localized epididymal remodeling while preserving testicular endocrine function. Functionalization with Fe_3O_4 nanoparticles modulated, but did not abolish, these biological effects, supporting further investigation of this strategy for the development of localized, non-hormonal male contraceptives.

4.6. References

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V. Final considerations

The present thesis investigated the potential of intraepididymal administration of eugenol, alone or associated with magnetite nanoparticles, as a non-surgical approach for male contraception. Considering the limitations associated with surgical sterilization, especially in free-roaming animal populations, the search for localized and minimally invasive alternatives remains highly relevant. The studies presented here provide evidence that the epididymis is a suitable target for this purpose and contributes to the understanding of its role in fertility regulation.

The systematic review in chapter 2 showed that most studies on chemical sterilization have focused on intratesticular administration, with calcium chloride and hypertonic saline being the compounds most frequently investigated. In contrast, relatively few studies have evaluated the epididymis as a target organ, despite its essential role in sperm maturation and acquisition of fertilizing capacity. This lack of information supported the development of the experimental studies presented in this thesis.

The chapter 3 demonstrated that the biological response depends on the site of administration. While intratesticular injection of eugenol resulted in marked testicular alterations, intraepididymal administration primarily affected the epididymal duct, leading to structural damage and severe impairment of sperm quality while preserving testicular morphology. Based on these findings, the epididymis was selected as the target for the subsequent investigation.

The chapter 4 demonstrated that a single intraepididymal administration of eugenol, either alone or combined with magnetite nanoparticles, was capable of reducing fertility through localized epididymal alterations. Although coating eugenol with magnetite nanoparticles modulated the tissue response and reduced the intensity of some lesions, the contraceptive effect was maintained. In addition, the observation that Fe_3O_4 nanoparticles alone also reduced fertility indicates that these nanoparticles are not biologically inert and may directly influence epididymal physiology.

Overall, the results obtained throughout this thesis support the original hypothesis that the epididymis can be selectively targeted to induce infertility without disrupting endocrine function. Beyond demonstrating the contraceptive potential of eugenol, this work also provides new information on the interaction

between nanostructured systems and epididymal tissue, contributing to the development of localized, non-hormonal contraceptive strategies.

Further studies are still required to determine whether the reproductive alterations induced by these treatments are reversible, to optimize nanoparticle formulations and drug delivery, and to evaluate this approach in target species such as dogs and cats. A better understanding of the molecular mechanisms involved in epididymal remodeling will also be important for improving the efficacy and safety of this strategy and for supporting its future application in veterinary reproductive medicine.