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Tissue Biochemistry, Oxidative Stress and Histopathological Alterations in Accessory Reproductive Organs Following Intratesticularly Administered Chemosterilants in Rats

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Abstract

Aim: This study assessed the effects of intratesticular administration of zinc gluconate, calcium chloride ($CaCl_2$) and cadmium chloride ($CdCl_2$) on biochemical parameters, oxidative stress and histopathological changes in the accessory reproductive organs of male albino rats over a 180-days observation period. **Methods and Materials:** Animals were allocated into control and treated groups: group A (control), group B (zinc gluconate), group C ($CaCl_2$) and group D ($CdCl_2$+EDTA). **Results:** Biochemical analysis revealed significant alterations ($p<0.01$; $p<0.001$) in cholesterol, glycogen, lactate dehydrogenase in testis, sialic acid, and neutral α-glucosidase levels in epididymis, fructose levels in seminal vesicles and acid phosphatase activity in ventral prostate in groups B and C, while group D exhibited moderate biochemical changes. Oxidative stress markers such as lipid peroxidase, glutathione-s-transferase and glutathione peroxidase were significantly elevated, particularly in groups B and C. In group D, antioxidant enzyme activity varied over time, with notable increases in superoxide dismutase and catalase. Histopathological examination of groups B and C revealed the absence of spermatozoa in the lumen, accompanied by severe degenerative changes in the epididymis, characterised by epithelial disorganisation, intertubular widening, and unfolding of lobular prostatic alveoli. Group D showed milder effects, with reduced epithelial height, fragmented spermatozoa in the lumen of the epididymis and structural disorganisation. **Conclusion:** These findings suggest that intratesticular injection of zinc gluconate and $CaCl_2$ induced significant reproductive toxicity, while $CdCl_2$ produced relatively milder effects.

Keywords: Accessory Reproductive Organs, Biochemistry, Cadmium Chloride, Calcium Chloride, Intratesticular Chemosterilization, Oxidative Stress, Zinc Gluconate

1. Introduction

Stray dog overpopulation, especially in developing countries, poses major public health and socio-economic issues. Traditional surgical sterilisation is often impractical on a large scale, making chemical sterilisation a promising, cost-effective and humane alternative[1]. After initial implementation of chemical sterilisation by Wang in 1987, numerous studies have examined various chemical agents such as hypertonic saline solution[2], zinc gluconate[3,4], calcium chloride ($CaCl_2$)[5,6], chlorhexidine digluconate[7], vinylcyclohexene dioxide (VCD)[8] and cadmium chloride ($CdCl_2$)[9,10] for inducing permanent sterility through intratesticular injections. While effective in some cases, challenges remain in standardising doses and minimising adverse effects such as swelling, inflammation, residual fertility and secondary male behavior[11,12]. Additionally, there were side effects like pain, pyrexia, severe inflammation and irreversible testicular damage[13,14].

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Recent studies using zinc gluconate with DMSO in dogs showed reduced testicular size but no significant change in paired volume, with notable ultrasonographic alterations[15]. Zinc plays a crucial role in enzyme function, DNA/RNA synthesis, genomic stability, immune modulation and antioxidant defense, but at higher concentrations it inhibits spermatogenesis and induces cellular damage[16,17]. $CaCl_2$ has been reported to cause oxidative stress, tissue necrosis[6,18,19] and hormonal disruption but without alteration in vital functions[5,20]. $CdCl_2$ has been shown to severely impair testicular function by inducing oxidative stress, damaging DNA, lipids, and proteins, and compromising on the blood-testis barrier, leading to haemorrhage, necrosis and apoptosis[21,22]. When combined with EDTA, cadmium's local bioavailability increased, and systemic toxicity decreased by potentially limiting systemic exposure. $CdCl_2$ also disrupts mitochondrial function, and Sertoli cell proliferation[23,24] reduces reproductive hormone levels and alters testicular metabolism[10]. However, studies about the broader effects of these compounds on accessory reproductive organs, biochemical pathways, and the potential to induce oxidative stress leading to DNA damage are lacking. In the present investigation, comparative effects of intratesticularly administered zinc gluconate, $CaCl_2$ and $CdCl_2$ on tissue biochemistry, ROS and antioxidant system and histopathology of accessory reproductive organs were evaluated.

2. Materials and Methods

2.1 Animals

This study was conducted on fertile adult male Wistar albino rats (*Rattus norvegicus*), weighing 160-200 g, and aged 5-6 months. Sixty rats were housed in polypropylene cages (43x27x15 cm) in the Departmental Experimental Animal Facility under a 12-hour light and 12-hour dark cycle. The rats were provided with a pellet diet (Ashirwad Industries Limited, Chandigarh, India) and water *ad libitum*. The animals were maintained under strict veterinary supervision in compliance with the Guidelines on the Regulation of Scientific Experiments on Animals[25]. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) vide Certificate No. UDZ/IAEC/IV/01 dated December 18, 2021.

2.2 Drugs

A 0.2M zinc gluconate solution (13.3 ≥mg/mL) was prepared by dissolving 273.402 mg of zinc gluconate (>98% pure; TCI (India) Pvt. Ltd.) and 104.52 mg of L-Arginine (>99% pure; TCI (India) Pvt. Ltd.) in 3 mL of DW. For the chelated solution, 2500 µL of the zinc gluconate solution was mixed with 500 µL of L-Arginine, and the pH was adjusted to 7.0.[3,26,27]. A 20% $CaCl_2$ solution was prepared by dissolving 0.6 g of $CaCl_2$ (A.R. grade; HiMedia Laboratories Pvt. Ltd.) in 3 mL of a 0.9% saline solution, and a desired pH and chemical stability were ensured during the mixing[5]. $CdCl_2$ was prepared by dissolving 3 mg of $CdCl_2$ (≥99% pure; Rankem) in 3 mL of 1M EDTA (≥99% pure; Promega) in a glass vial, and the pH was adjusted to 7.0 [28].

2.3 Experimental Design

The rats were randomly allocated to four Groups (n=15):

Group A: Vehicle-injected control (0.9% NaCl; isotonic physiological saline)

Group B: Single intratesticular injection of zinc gluconate (13.3 mg/mL; chelated with L-Arginine) into each testis

Group C: Single intratesticular injection of calcium chloride (20% in neutralised physiological saline) into each testis

Group D: Single intratesticular injection of cadmium chloride (0.5 mg/kg b.wt.) chelated with EDTA into the left testis.

2.4 Injection Procedure

For intratesticular injections, the rats were fasted overnight and anaesthetised with sodium thiopentone (40 mg/kg b.wt., *i.p.*) (Thiosol Sodium, Neon Laboratories Ltd, Mumbai, India). Using a 28-gauge, 12-mm needle, 0.1 mL/testis of the drug was injected via linear infiltration, directed from the caudoventral to dorsocranial aspect of the testis[5].

2.5 Animal Sacrifice

Five animals from each group were euthanised with an overdose of sodium thiopentone on days 30, 90 and 180 of intratesticular injection, sacrificed and subjected to evaluation of the following:

2.5.1 Tissue Biochemistry

Upon animal sacrifice, the testis, epididymis, seminal vesicle of one side and a portion of the ventral prostate were carefully dissected out, and 50 mg tissue was homogenised in 0.5 mL deionised water to prepare 10% (w/v) homogenates. The homogenates were centrifuged at 3000 rpm for 10 minutes at 4°C, and the resultant supernatant was separated and used for the determination of the various biochemical parameters, *i.e.,* cholesterol[29], glycogen[30] and lactate dehydrogenase (LDH)[31] in testis; sialic acid[32], neutral α-Glucosidase[33] and L-Carnitine[34] in epididymis, fructose in the seminal vesicle and acid phosphatase in ventral prostate[35]. Determination of total protein of testis and epididymis[36] was also carried out.

2.5.2 Reactive Oxygen Species and Antioxidant System

The homogenates of testis and epididymis were also used for the determination of catalase (CAT)[37], lipid peroxidase (LPO)[38], glutathione peroxidase (GPx)[39], glutathione-s-transferase (GST)[40] and superoxide dismutase (SOD) activities[41].

2.5.3 Histology of Accessory Reproductive Organs

The contralateral cauda epididymis, and seminal vesicle, and the remaining portion of the ventral prostate were excised, rinsed in saline and fixed in 4% paraformaldehyde for 24 hours. The tissues were then dehydrated in graded ethanol, cleared in xylene and embedded in paraffin wax. Five-micron thick sections were cut, placed on Mayer's albumin-coated slides, deparaffinized, processed and stained with Harris' hematoxylin and eosin, mounted with DPX mountant and examined in a bright field microscope (Model: DM 1000, Leica, Wetzlar, Germany).

2.6 Statistical Analysis

The statistical analysis was conducted using GraphPad Prism 9.5.1 software (GraphPad Software, Boston, USA). The values are represented as mean ± standard deviation (mean ± SD), and one-way analysis of variance (ANOVA) was used for statistical comparison. The $p<0.05$ is considered statistically significant.

3. Results

3.1 Tissue Biochemistry

Groups B and C showed a significant increase in testis cholesterol, glycogen, and LDH ($p<0.01$; $p<0.001$), while group D had a non-significant rise in cholesterol and glycogen. LDH increased markedly in group D by 180 days ($p<0.001$), with non-significant changes in total protein. Groups B and C had significantly lower epididymal sialic acid and neutral α-Glucosidase levels ($p<0.01$; $p<0.001$) and unaltered L-Carnitine concentration. The values of all parameters fluctuated around vehicle-treated control (group A) in group D. Total protein remained unaltered across groups. Fructose in the seminal vesicle and ACP level in the ventral prostate were significantly decreased ($p<0.01$; $p<0.001$) in all groups (Figure 1A-J).

3.2 Reactive Oxygen Species and Antioxidant System

A marked enhancement ($p<0.001$) in all ROS and antioxidant parameters was found in the testis and in the epididymis of groups B and C in comparison to group A following 180 days of intratesticular injections. The intratesticular injection in group D resulted in a significant increase ($p<0.01$) in LPO, GST and GPx levels in the testis throughout the experimental schedules. A rise in concentration of SOD in both testis and epididymis, which was found to be very significant ($p<0.01$) on day 90 of intratesticular injection. A significant increase ($p<0.01$) in CAT levels in the testis was recorded on day 30 and 90 post-injection in group D. On day 180 after intratesticular $CdCl_2$ administration in group D non-significant increase was noticed. All ROS and antioxidant parameters were found elevated in the epididymis following intratesticular administration of $CdCl_2$ in group D. The LPO at 30 and 90 days, SOD at 90 days, and GST at 30 days were found significantly ($p<0.01$) increased. The CAT level was found to be non-significantly and GPx levels significantly ($p<0.01$) increased throughout (Figure 2A-J).

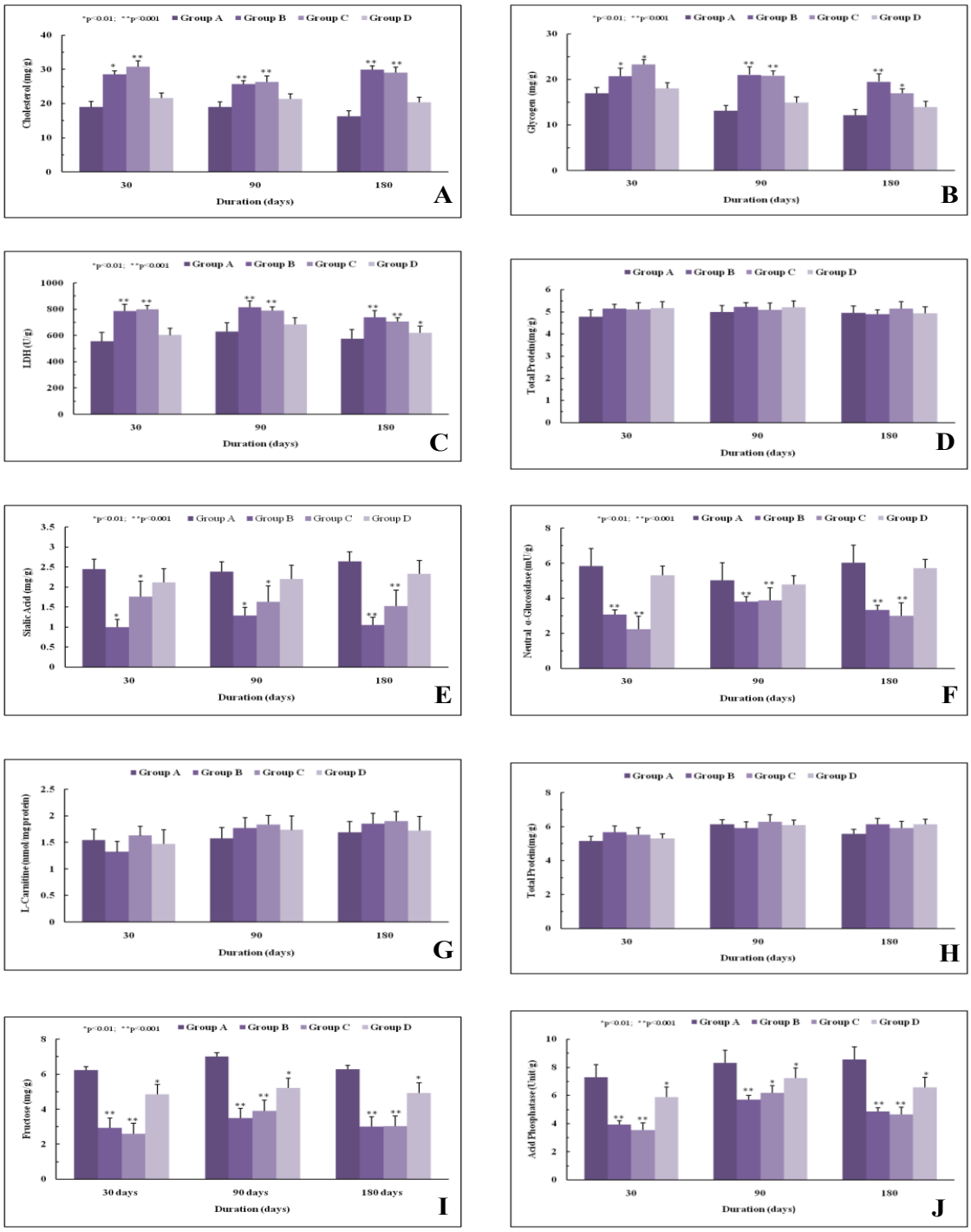


Figure 1. Tissue biochemistry on days 30, 90 and 180 of intratesticular administration of zinc gluconate, $CaCl_2$ and $CdCl_2$ in male Wistar albino rats. **A-D Testis:** (A) Cholesterol (mg/g), (B) Glycogen (mg/g), (C) LDH (U/g), (D) Total protein (mg/g). **E-H Epididymis:** (E) Sialic Acid (mg/g), (F) Neutral α-Glucosidase (mU/mL), (G) L-Carnitine (nmol/mg protein), (H) Total protein (mg/g). **I Seminal vesicle:** Fructose (mg/g). **J Ventral prostate:** ACP (Unit/g). Significant alterations were recorded in most of the parameters of groups B, C and D. Each column and vertical bar represent the mean ± S.D. [n=5] (*p<0.01; **p<0.001)

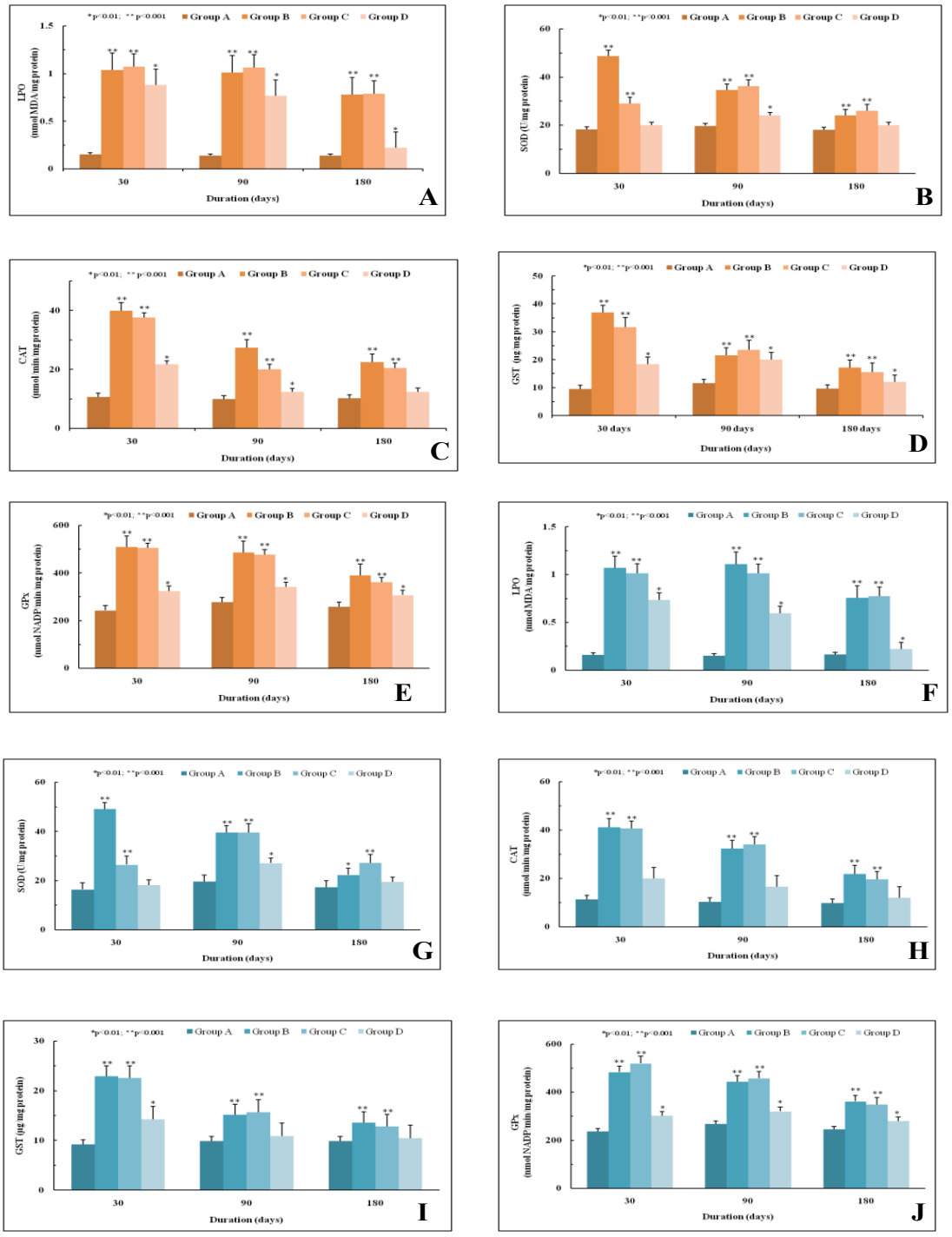


Figure 2. Reactive oxygen species and antioxidant system in testis on days 30, 90 and 180 of intratesticular administration of zinc gluconate, $CaCl_2$ and $CdCl_2$ in male Wistar albino rats. **A-E Testis:** (A) LPO (nmol MDA/mg protein), (B) SOD (U/mg Protein), (C) Catalase (µmole/min/mg protein), (D) GST (µg/mg protein) and (E) GPx (n mol NADP/ min/mg protein). **F-J Epididymis:** (F) LPO (nmol MDA/mg protein), (G) SOD (U/mg Protein), (H) Catalase (µmole/min/mg protein), (I) GST (µg/mg protein) and (J) GPx (n mol NADP/ min/mg protein). Significant enhancement was observed in all parameters. Each column and vertical bar represent the mean ± S.D. [n=5]. (*p<0.01; **p<0.001).

3.3 Histology of Accessory Reproductive Organs

3.3.1 Cauda Epididymis

The histological observation of the cauda epididymis of animals of group A on days 30, 90 and 180 showed large epididymal tubules and intertubular elements with three types of epithelial cells, *viz.,* principal (columnar with stereocilia), basal (short cuboidal), and apical (low cuboidal). The lumen was spacious and contained abundant spermatozoa (Figure 3A). The histological examination of the cauda epididymis of group B on days 30, 90 and 180 revealed complete degeneration and abundant intertubular fibrous elements with undistinguished tubules (Figure 3B-D). Following 30, 90 and 180 days of $CaCl_2$ injection in group C, the histoarchitecture of cauda epididymis exhibited disorganised tubular epithelium, widened intertubular space filled with fibrous tissue and lumen devoid of spermatozoa (Figure 3E-G). On day 30 of intratesticular injection, histoarchitecture of the cauda epididymis of group D animals revealed that the epithelium was reduced in height, with widened interstitial spaces. Fragments of spermatozoa were present in the lumen on day 30, with similar observations on days 90 and 180 (Figure 3H- J).

3.3.2 Seminal Vesicle

The histology of the seminal vesicle on days 30, 90 and 180 of group A showed tubular, cryptic, and highly folded normal glandular and secretory epithelium with granular columnar cells and eosinophilic material in the lumen. The histological examination of the seminal vesicle of groups B and C on 30, 90 and 180 days of intratesticular injection revealed tubular shrinkage, low epithelium, narrowed epithelial cells, less secretory material in the lumen, with thickened and vacuolated musculature. The histoarchitecture of the seminal vesicle of group D following 30, 90, and 180 days of intratesticular injection indicated low and narrow glandular epithelium, few tubular folds and less secretion of eosinophilic material in the lumen (Figures not shown).

3.3.3 Ventral Prostate

The histoarchitecture of the ventral prostate of group A on days 30, 90 and 180 showed a typical lobular structure

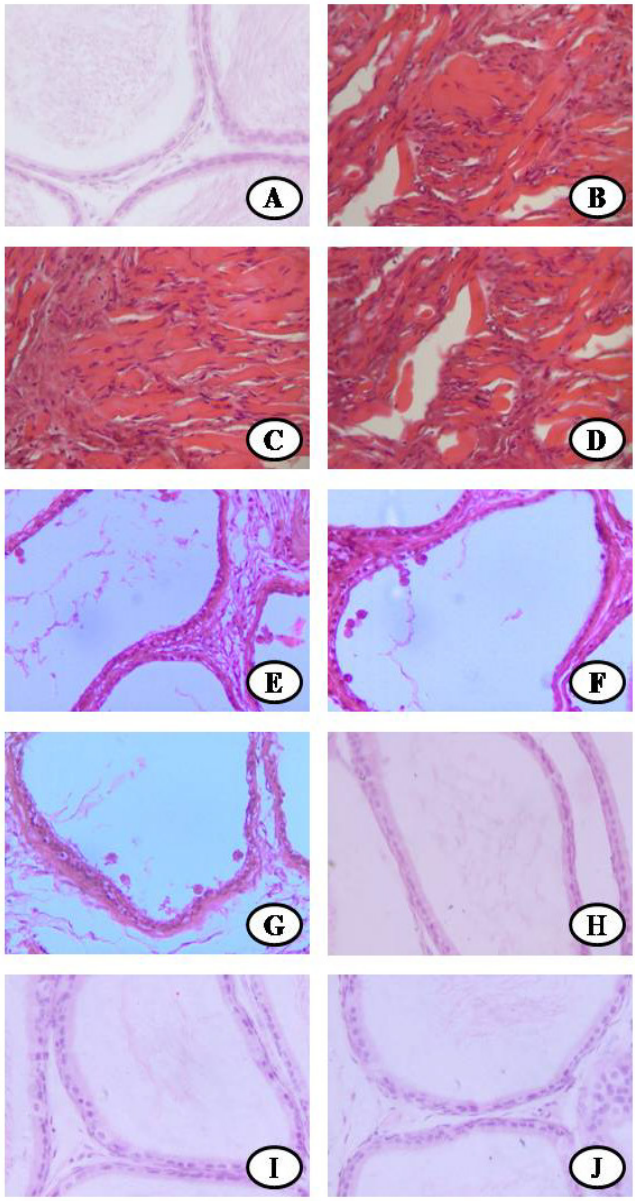


Figure 3. Histoarchitecture of cauda epididymis on days 30, 90 and 180 of intratesticular administration of zinc gluconate, $CaCl_2$ and $CdCl_2$ in male Wistar albino rats. **A**: Note large epididymal tubule with different types of epithelial cells and abundant spermatozoa in the lumen in group A. H&E x 200; **B** (30 days), **C** (90 days) & **D** (180 days): Note complete degeneration and abundant intertubular fibrous elements with undistinguished tubules in group B. H&E x 200; **E** (30 days), **F** (90 days) & **G** (180 days): Note disorganized epithelium, widened intertubular space and no spermatozoa in lumen in group C. H&E x 200; **H** (30 days), **I** (90 days) & **J** (180 days): Note low epithelium and lumen with fragments of spermatozoa in group D. H&E x 200.

with prostatic alveoli lined by low cuboidal columnar epithelium. Eosinophilic granules were present in the cytoplasm, and the lumen contained eosinophilic secretory material. In group B, the histology of the ventral prostate showed a disorganised alveolar region with nucleated columnar epithelial cells. Cellular structures appeared dispersed and indistinguishable lumen area with no eosinophilic secretory material. In group C, the histological examination of the ventral prostate revealed few lobular prostatic alveolar folding, disorganisation of the epithelial cells, and widening of the interalveolar spaces with eosinophilic secretory material in the lumen. The histological observations of the ventral prostate of group D on days 30, 90 and 180 of intratesticular injection showed normal lobular structure with intact epithelium and eosinophilic secretory material present in the lumen (Figures not shown).

4. Discussion

Chemical sterilisation of stray animals using compounds such as zinc gluconate, $CaCl_2$ and $CdCl_2$ has been extensively studied. This study specifically evaluated the effects of intratesticular administration of these agents on various physiological and biochemical parameters in male albino rats. Key aspects examined included accessory reproductive organs' histology, biochemical markers, ROS and antioxidant system. The findings revealed significant alterations in different parameters, providing crucial insights into the feasibility, possible toxicology and effectiveness of these chemicals as potential non-surgical alternatives for sterilisation.

The zinc gluconate, $CaCl_2$ and $CdCl_2$ induced complete necrosis in the testes, destroying the normal architecture of seminiferous tubules, damaging Sertoli and Leydig cells and causing germ cell loss. This disruption impairs spermatogenesis and is supported by multiple studies. Zinc gluconate compromises the blood-testis barrier by altering Sertoli cell junctions, increasing permeability and triggering germ cell apoptosis via oxidative stress and zinc salt imbalance[42,43]. Calcium ions damage membrane integrity, denature proteins, impair ATP production and obstruct blood flow[44-46]. $CdCl_2$ disrupts Sertoli cell structure, causes germ cell sloughing and decreases testosterone by damaging Leydig cells; also induces oxidative stress and DNA damage[24,47].

Comprehensive oxidative, biochemical and histological assessments provided evidence of severe clinical toxicity and adverse effects associated with zinc gluconate and $CaCl_2$[27,48]. However, $CdCl_2$, when administered with EDTA, a chelating agent, produced the desired contraceptive effects with only partial toxicological consequences[23]. The effects of zinc gluconate and $CaCl_2$ were observed to be immediate, inducing 100% infertility within 30 days post-treatment. Although $CdCl_2$ produced a gradual onset of recovery, its effects persisted until the end of the study of 180 days[49].

Investigations carried out previously by us[49] revealed that the intratesticular administration of zinc gluconate, $CaCl_2$ and $CdCl_2$ led to substantial reductions in all reproductive organ weights throughout the study period of 180 days, suggesting that zinc gluconate and $CaCl_2$ induce severe testicular atrophy likely due to its ability to trigger inflammatory responses leading to fibrosis and necrosis in the testicular tissue[16,50]. Similar outcomes were observed with $CdCl_2$, though the reduction was less drastic, indicating a milder effect, suggesting less severe testicular damage[51]. Histological analysis revealed degenerative changes in the accessory reproductive organs, such as epithelial disorganisation in the epididymis, loss of glandular secretory activity of the seminal vesicle and glandular shrinkage in the ventral prostate, following treatment with zinc gluconate and $CaCl_2$. These findings suggest impaired epididymal function, diminished seminal fluid production and secretory dysfunction which are critical for sperm maturation and motility and reduced androgen-dependent accessory gland function, correlating with the observed hormonal alterations[52-54].

The administration of zinc gluconate and $CaCl_2$ significantly altered tissue biochemistry. Cholesterol levels in the testes were elevated, peaking on day 180, which indicates disrupted cholesterol metabolism and impaired steroid hormone synthesis. The LDH activity, a non-specific marker of tissue necrosis rather than metabolic stress, was significantly increased, with the highest levels recorded in the zinc gluconate group, indicating pronounced cellular injury and loss of membrane integrity[55,56]. This suggests extensive cellular necrosis and metabolic dysfunction in testicular tissues. Neutral α-Glucosidase, an essential marker for epididymal

function and sperm maturation, was significantly reduced, particularly in the zinc gluconate and $CaCl_2$ groups[54]. This reduction correlates with impaired sperm maturation, and epididymal dysfunction, contributing to infertility. These biochemical disruptions align with reductions in reproductive organ weights and hormonal imbalances[57]. Furthermore, by reducing cadmium's toxic effects, EDTA helps maintain the metabolic integrity of the testis and epididymis, ultimately supporting reproductive health. Therefore, $CdCl_2$ when chelated with EDTA, not only reduces toxicity but also allows for the preservation of essential biochemical processes, resulting in improved cholesterol utilisation, sustained glycogen levels, and maintained LDH activity in the testis. Chelation of $CdCl_2$ with EDTA protects the testis and epididymis by preserving biochemical markers like cholesterol, sialic acid, neutral α-Glucosidase, and L-Carnitine levels, etc. For instance, cadmium exposure reduces sialic acid and neutral α-Glucosidase, resulting in impairment of sperm maturation, reducing energy availability for sperm, hindering sperm metabolism and motility, but EDTA prevents this decline by reducing oxidative stress[24,58-60]. Overall, compared to the other treated groups, EDTA chelation mitigates cadmium-induced reproductive toxicity, which supports sperm function and fertility.

This study observed elevated ROS levels in the testis, accompanied by increased antioxidant enzymes such as SOD, catalase and glutathione. These findings indicate oxidative stress development due to excessive ROS generation, which may impair antioxidant defense mechanisms. Testicular ROS levels were significantly higher in all three chemosterilant-treated groups compared to controls. Increased ROS production is known to cause oxidative damage to cellular macromolecules, including lipids, proteins, and DNA, potentially leading to the suppression of sperm parameters. Heavy metals like cadmium, as well as zinc and calcium salts, contribute to ROS generation through various mechanisms. LPO levels peaked at 30 and 90 days and gradually declined, suggesting acute oxidative damage followed by a compensatory response. Concurrently, antioxidant enzymes such as SOD, CAT, and GPx were markedly elevated, reflecting the body's attempt to counteract oxidative stress[61-64]. Zinc gluconate induced the highest oxidative stress response, indicating its strong cytotoxic potential. Persistent oxidative stress and elevated antioxidant activity through 180 days suggest chronic tissue damage and systemic oxidative burden, further contributing to reproductive dysfunction[57]. Over-supplementation of zinc gluconate and $CaCl_2$ can induce oxidative damage, alter enzymatic activities and negatively impact sperm maturation and motility[65]. These findings highlight the role of oxidative stress in chemically induced testicular atrophy and infertility[6].

This study provides compelling evidence that intratesticular administration of zinc gluconate, $CaCl_2$ and $CdCl_2$ significantly affects the reproductive physiology of male albino rats, alterations in the histoarchitecture of accessory reproductive organs and marked oxidative and biochemical disturbances. Among the tested chemosterilizing agents, zinc gluconate and $CaCl_2$ showed severe and rapid-onset toxic effects, while $CdCl_2$, especially when administered with EDTA, exhibited milder toxicity, making it a potentially safer alternative for chemical sterilisation. The elevated ROS levels, altered enzymatic activities and histological disruptions underline the oxidative stress-mediated mechanisms contributing to infertility in all injected groups. However informative, the rodent model has limitations of species difference, limiting the direct translational applicability to the dog. Future studies should include larger animal models, longer follow-up periods and a comprehensive toxicity assessment to validate the feasibility of these agents in field application for stray animal population control.

5. Conclusion

This study provides a comprehensive evaluation of the effects of intratesticular administration of zinc gluconate, $CaCl_2$ and $CdCl_2$ on the histology of accessory reproductive organs, tissue biochemistry and oxidative stress. The findings of this study substantiate the effectiveness of zinc gluconate and $CaCl_2$ in inducing long-term reproductive suppression by targeting not only the testes but also severely affecting the accessory reproductive organs over the study period. However, $CdCl_2$ with EDTA as a chelating agent had comparatively lesser effects on accessory organ histoarchitecture and relatively milder biochemical effects, ROS and antioxidant oxidative stress, reflecting its relatively weaker impact on overall reproductive function, positioning it as a safer alternative for sterilisation.

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7. Competing Interests

All authors declare no significant competing interests.

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Author Contributions

ASA designed the study. TC, S and RKD conducted the experiment, TC and BK analyzed the data and drafted the manuscript. ASA and NKL finally approved the final version to be published. All authors read and approved the final manuscript.