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Evaluation of chemical castration by intra-testicular injection of zinc-doped carbon dots in mature rats

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Abstract

Sterilization in animals serves multiple purposes, such as behavior control, performance improvement, and population management. Chemical sterilization has emerged as a promising non-surgical alternative to traditional methods. This study aimed to investigate the effects of intra-testicular injection of zinc-doped carbon dots (Zn-CDs) nanoparticles as a chemical sterilant in mature rats. Twenty-five rats were randomly divided into five groups, including a control group without injection, a sham group receiving 0.50 mL distilled water, and three treatment groups administered respectively 0.50, 2.00, and 8.00 mg kg^{-1} of Zn-CDs synthesized through a hydrothermal process. Following anesthesia with ketamine and xylazine, and aseptic preparation, intra-testicular injections were administered bilaterally. At 60th day post-injections, blood samples were collected to measure serum testosterone levels using chemiluminescence immunoassay. The rats were then surgically castrated to assess sperm parameters and testicular histopathology. Testicular oxidant/anti-oxidant status was also evaluated. The results revealed a dose-dependent reduction in sperm viability, membrane integrity, and motility, accompanied by increased sperm DNA damage. The highest Zn-CDs dose caused the most significant decrease in sperm concentration, as well as severe testicular tissue damage. In addition, anti-oxidant capacity, seminiferous tubules maturation, testosterone production, and spermatogenesis declined with increasing Zn-CDs concentrations in a dose-dependent manner. These findings indicate that intra-testicular injection of Zn-CDs effectively induces infertility in mature rats and holds potential as a chemical sterilization method. With further studies to evaluate safety and efficacy, this approach could be developed as a practical solution for large-scale *in situ* castration, offering a non-surgical alternative for over-population control programs.

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Introduction

Neutering of male animals is commonly performed for population control, management of aggressive behavior, and prevention of neoplasia in later life. Traditionally, neutering is achieved surgically under general anesthesia; however, this approach carries risks, such as bleeding, infection, inflammation, cellulitis, granuloma formation, scrotal hematoma, abscess, wound dehiscence, myiasis, and hernia.[1] Chemical castration involving intra-testicular injection of cytotoxic agents offers a non-surgical alternative by inducing testicular tissue damage and disrupting spermatogenesis and testosterone production. This method has gained attention due to its simplicity, reduced procedure time, and cost-effectiveness for large-scale sterilization programs.[2]

Various chemical agents have been experimentally used for this purpose, including ethanol, chlorhexidine, formalin, lactic acid, calcium chloride, glucose, zinc compounds, and hypertonic saline.[3] The cytotoxicity of these compounds typically causes testicular necrosis and atrophy. However, adverse effects, such as severe swelling, inflammation, scrotal dermatitis, fistula formation, and self-inflicted injuries, have also been reported, necessitating surgical removal of the affected testis and scrotum.[4] For instance, intra-testicular injection of zinc gluconate caused fistula formation and scrotal ulcers, and failed to achieve complete sterilization in some species.[5,6]

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Similarly, zinc-based compounds combined with arginine induced purulent necrosis and scrotal ulcers in dogs.[7]

Carbon dots (CDs) are fluorescent nanoparticles synthesized from natural or synthetic carbon sources, recognized for their exceptional biocompatibility, tunable optical properties, and low toxicity.[8] In biological studies, CDs have exhibited selective cytotoxicity against cancer cells through reactive oxygen species generation, and triggering mitochondrial dysfunction, caspase activation, and apoptotic pathways.[9] Reportedly, CDs derived from sucrose and glucose have demonstrated potent cytotoxicity in HepG2 liver carcinoma and MCF-7 breast cancer cells.[10] Doping CDs with metals, such as zinc, enhances their therapeutic potency *via* improving reactive oxygen species generation.[11]

Previously, zinc nanoparticles, particularly in the form of zinc gluconate or zinc oxide, have been widely utilized as agents for chemical castration through intra-testicular injection. These nanoparticles induce germ cell death, disrupt spermatogenesis, and lead to permanent infertility by triggering apoptosis and fibrosis within the testicular tissue.[12] Zinc cytotoxic effect at high concentrations inhibits germ cell division and replication, resulting in fragmentation of the nucleus and cell membrane, being essential for the sterilizing action.[13] While zinc-based agents are effective for chemical castration, their use is associated with several unwanted complications. The most significant adverse effects include tissue necrosis, inflammation, and excessive scrotal swelling at the injection site, which can lead to testicular atrophy and secondary infections in some cases. High concentrations of zinc gluconate have shown genotoxic and mutagenic potential, with risks of DNA damage and chromosomal aberrations in treated tissues. Necrosis, in particular, is concerning because it can trigger inflammatory responses and is potentially carcinogenic if not properly controlled.[14,15]

The aim of this study was to evaluate the efficacy and safety of zinc-doped carbon dots (Zn-CDs) as a novel intra-testicular chemical castration agent in a rat model. By doping CDs with zinc, the study seeks to reduce the required concentration of zinc ions, thereby minimizing the cytotoxic and inflammatory complications commonly associated with conventional zinc-based chemical sterilants.

Materials and Methods

Preparation of Zn-CDs. To prepare the Zn-CDs, 100 mL of a 1.00% acetic acid solution (Merck, Darmstadt, Germany) containing 1.00 g of zinc acetate salt (Sigma-Aldrich, St. Louis, USA) was stirred magnetically for 2 min. The mixture was then transferred into a stainless-steel hydrothermal reactor equipped with a polytetra-fluoroethylene-lined chamber and heated at 200 ± 1.00 ˚C for 6 hr. After heating, the reactor was allowed to cool to room temperature. The resulting product was centrifuged (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 15,000 rpm for 30 min to remove impurities. Finally, the supernatant containing the Zn-CDs was sterilized by filtration through a 0.45 µm sterile polytetrafluoroethylene syringe filter (Millipore Sigma, Burlington, USA) prior to injection.

Characterization of Zn-CDs. The diameter and size distribution of the Zn-CDs were measured by particle size analyzer (Scatteroscope, Qudix Inc., Seoul, South Korea). The morphology and size of the Zn-CDs were further examined by transmission electron microscopy. The transmission electron microscopy images were acquired using a Philips EM208 microscope (Philips Electron Optics, Eindhoven, The Netherlands) operated at an accelerating voltage of 90.00 kV and a magnification of 710,000×.

Animals and procedures. Twenty-five mature male rats weighing 200 - 250 g were randomly assigned into five groups. The control group received no intervention, while the sham group was aseptically administered 0.50 mL of distilled water intra-testicularly into both testes using a 26-gauge hypodermic needle. The remaining three groups received intra-testicular injections of Zn-CDs at doses of 0.50, 2.00, and 8.00 mg kg^{-1}, respectively, each in a volume of 0.50 mL, following the same procedure. The intermediate dose (2.00 mg kg^{-1}) was chosen based on promising results from our preliminary pilot study, and the lower and higher doses were included to assess the dose-dependent effects of Zn-CDs. All injections were performed under sedation with ketamine (40.00 mg kg^{-1}; Alfasan, Woerden, The Netherlands) and xylazine (5.00 mg kg^{-1}; Alfasan). The study design and all experimental protocols were reviewed and approved by the Animal Ethics Council of Urmia University, Urmia, Iran (Approval No. IR-UU-AEC-3/12, 2024). The rats were kept in groups for 60 days and then anesthetized using intra-peritoneal injection of ketamine (60.00 mg kg^{-1}) and xylazine (5.00 mg kg^{-1}). Subsequently, the epididymides and testes were surgically harvested, and the sperms were extracted from the cauda epididymis by dissecting it into a Petri dish containing 1.00 mL of human tubal fluid (Sigma-Aldrich). The testicular tissues were placed in 10.00% neutral buffered formalin for histopathological evaluations.

Clinical evaluation. All animals were examined for clinical observations, including scrotal swelling and dermatitis, fistula formation and discharge, and testicular pain on palpation, at the end of each week until the end of study.

Sperm analyses. Sperm kinematics was evaluated using computer assisted sperm analysis (Test Sperm 3.2; Videotest, St. Petersburg, Russia). The evaluated sperm kinematic parameters included total motility (%), progressive motility (%), velocity of average path (µm sec^{-1}), velocity of curved line (µm sec^{-1}), straight-line

velocity (µm sec^{-1}), linearity (%), amplitude of lateral head displacement (µm sec^{-1}), straightness (%), and beat cross frequency (Hz). The Sperm plasma membrane functionally was assessed using the hypo-osmotic swelling test. In this regard, 10.00 µL of sperms were diluted in 100 µL of a hypo-osmotic solution containing 1.35 g fructose and 0.73 g sodium citrate. Following incubation at 37.00 ˚C, sperms were examined for plasma membrane functionally using a phase-contrast microscope (BX41; Olympus, Tokyo, Japan) at 400 ×. Two hundred straight or curved sperms were counted to determine the percentage of sperms with plasma membrane functionally.[16,17] Concentrated sperm smears were exposed to a 1:3 methanol and acetic acid solution for 2 hr, air-dried and then, subjected to an Acridine Orange solution. The stained sperm samples were examined under fluorescence microscope to evaluate sperm DNA damage.[18] The morphology and viability of sperms were examined using eosin nigrosin stain in accordance with World Health Organizationguidelines.[19]

Histopathological examination. Following surgical castration, testicular tissues were fixed in 10.00% neutral buffered formalin, dehydrated through a graded ethanol series, and embedded in paraffin wax. Tissue blocks were then sectioned (5.00 µm-thick) using a rotary microtome and sections were stained with Hematoxylin and Eosin for histological examination under a light microscope (BH-2; Olympus). Spermatogenesis was assessed using Johnsen's score (100 tubules *per* slide), grading spermatogenic activity on a scale of 1-10 based on germ cell presence and seminiferous tubules organization. Johnsen scores 8, 9, and 10 indicate spermatogenesis with a few to many spermatozoa present in a section of seminiferous tubule, respectively. Scores 6 and 7 indicate seminiferous tubule with no spermatozoa, but spermatids are present. Seminiferous tubule with no spermatozoa, no spermatids, having spermatocytes is evaluated as scores 4 and 5. Seminiferous tubule with only spermatogonia as germ cells is evaluated as score 3, meanwhile tubule with no germ cells having Sertoli cells is given a score 2. Seminiferous tubule with complete absence of cells is evaluated as score 1. Scores 9 and 10 were also considered mature seminiferous tubules.[20] Thirty seminiferous tubules were randomly selected and evaluated *per* section to determine the spermiogenesis index (SPI), being calculated as the ratio of the number of seminiferous tubules containing spermatozoa to the number of tubules lacking spermatozoa.[21] Testicular damage was also classified using the Cosentino scoring system as follows: Grade 1: Normal testicular histoarchitecture and well-ordered germ cells, grade 2: Disorganized germ cells, tightly packed seminiferous tubules, and mild congestion and necrosis, grade 3: Sloughed germ cells with pyknotic nuclei, indistinct seminiferous tubules borders, and moderate congestion and necrosis, and grade 4: Severe coagulative necrosis of germ cells and congestion.[22]

Testicular tissue oxidant/anti-oxidant status assessment. Testicular tissue samples were weighed and homogenized in ice-cold phosphate-buffered saline, followed by centrifugation at 13,000 *g* for 5 min at 4.00 ˚C to obtain a clear supernatant. This supernatant was then used for colorimetric assays to measure total anti-oxidant capacity (TAC) using anti-oxidant assay kit (Zenbio, Durham, USA) and total oxidant status (TOS) using a commercially available assay kit (Elabscience, Houston, USA). Results for both TAC and TOS were expressed as nmol *per* mg protein.

Testosterone level determination. On day 60, blood samples were collected from all rats and then, the animals were sacrificed by anesthetic over-dose. The blood samples were centrifuged at 2,000 rpm at 4.00 ˚C for 15 min. The sera were stored in a freezer at – 20.00 ˚C until the test. The testosterone level was measured using ADVIA Centaur (TSTII; Siemens Healthcare Diagnostics, Tarrytown, USA) based on chemiluminescence immuno-assay method.[22]

Statistical analysis. In this study, the obtained data were analyzed using SPSS Software (version 26.0; IBM Corp., Armonk, USA). Comparison of data means was done using one-way analysis of variance, and Kruskal-Wallis and Mann-Whitney U tests. The significance level of the means was considered to be $p < 0.05$.

Results

Characterization of Zn-CDs. Carbonization yields products with blue-to-yellow luminescence (dominantly blue/green). As shown in Figure 1, the synthesized solution appears brown under daylight but emits intense green fluorescence under ultra-violet excitation, confirming successful CDs synthesis. This green luminescence can be attributed to the oxygen-functionalized carbon groups and quantum confinement effects in the nanoscale carbon structures. The intensity distribution graph and corresponding table revealed a narrow size distribution, with the majority of particles exhibiting diameters in the range of approximately 2.00 - 30.00 nm. The peak intensity was centered on 14.60 nm. The transmission electron microscopy imaging confirmed the nanoscale size and morphology of the Zn-CDs. As shown in the micrograph, the particles are well-dispersed and predominantly spherical, with diameters consistent with the dynamic light scattering measurements. The scale bar of 30.00 nm and high magnification provide clear visualization of individual dots, supporting the conclusion that the majority of particles are within the sub-10 nm range (Fig. 1).

Clinical observation. No complications, such as fistula formation, inflammation or discharge, were observed following intra-testicular injections in all groups. The mild testicular swelling was observed as a result of the intra-testicular injections being subsided during the first week.

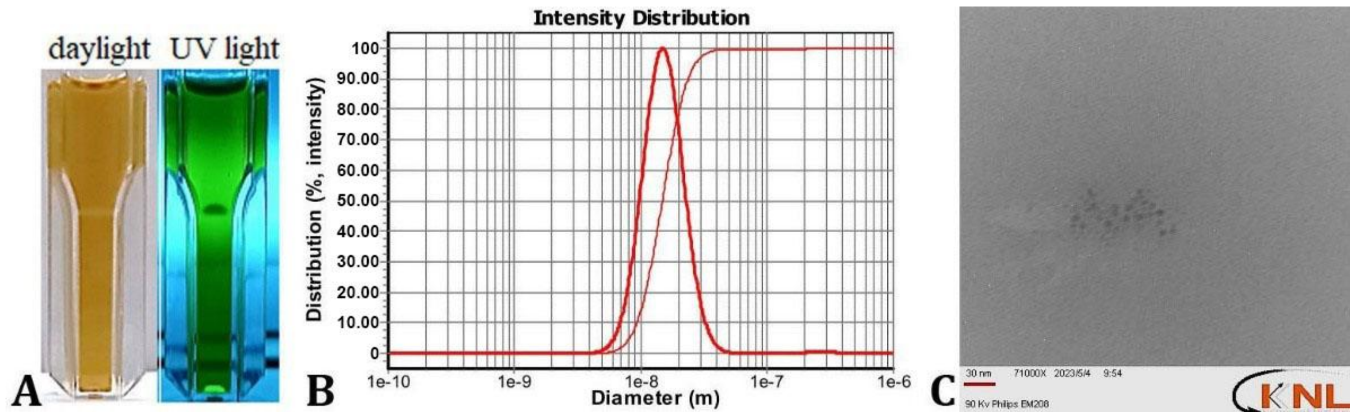

Fig. 1. A) Digital photographs of carbon dots solution under ambient light and the ultra-violet (UV) lamp; **B)** Intensity distribution graph showing the diameter of the particles, with a narrow peak centered around 14.60 nm, indicating a uniform size distribution; **C)** Transmission electron microscopy image of zinc-doped carbon dots. The zinc-doped carbon dots appear as well-dispersed and nearly spherical nanoparticles, confirming their nanoscale size and uniform morphology (bar = 30.00 nm).

Testicular tissue oxidant/anti-oxidant balance. The control, sham, and the lowest dose (0.50 mg kg^{-1} Zn-CDs) groups showed statistically similar baseline testicular tissue oxidant/anti-oxidant status ($p > 0.05$). However, the 2.00 mg kg^{-1} Zn-CDs produced a statistically significant increase in TOS, representing roughly a 2-fold elevation compared to the control levels and indicating that this mid-dose induced the greatest oxidative stress in testicular tissue ($p < 0.05$). The highest dose (8.00 mg kg^{-1}) showed an intermediate but still significantly elevated oxidative status, being statistically different from the control, sham, and 2.00 mg kg^{-1} groups, suggesting a biphasic response pattern where cellular adaptive mechanisms or altered bioavailability may reduce oxidative stress at the highest concentration despite still maintaining significant elevation above baseline levels ($p < 0.05$; Fig. 2A). The control, sham, and 0.50 mg kg^{-1} Zn-CDs groups exhibited similar TAC levels, with no significant difference among these groups ($p > 0.05$). Injection of 2.00 mg kg^{-1} Zn-CDs resulted in a statistically significant decrease in TAC, indicating a substantial reduction of anti-oxidant defenses compared to the lower dose, control, and sham groups. The highest dose (8.00 mg kg^{-1}) led to a TAC level that was significantly lower than the control but higher than the 2.00 mg kg^{-1} group, demonstrating a biphasic response. These findings suggest that Zn-CDs can reduce anti-oxidant capacity in testicular tissue in a dose-dependent manner, with the greatest effect observed at the intermediate dose (Fig. 2B).

Histopathological findings. Histological examination of testicular tissue sections stained with Hematoxylin and Eosin revealed dose-dependent morphological changes following intra-testicular administration of Zn-CDs (Fig. 3). The control group exhibited normal testicular histo-architecture, with intact seminiferous tubules having organized spermatogenic layers. Similarly, the sham group, which received distilled water, showed no observable histostructural alterations and seminiferous tubules appeared comparable to the control.

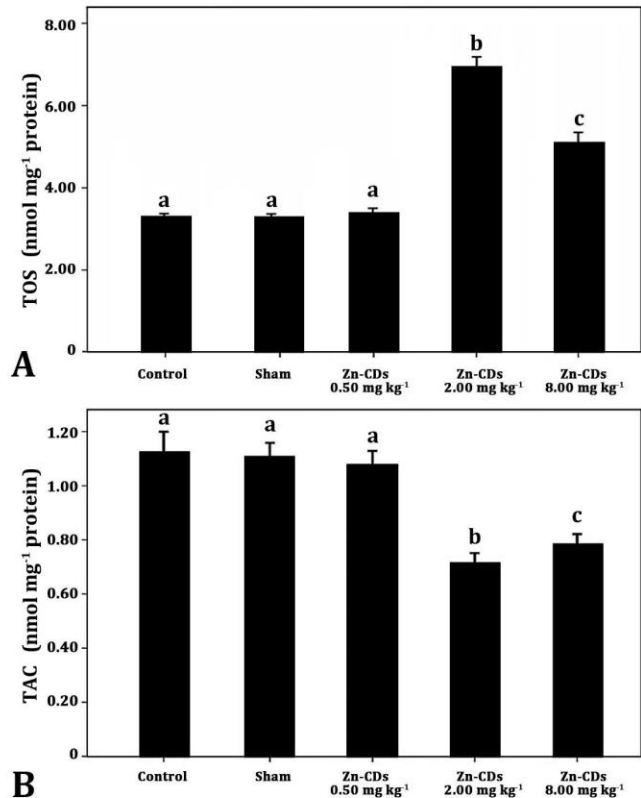

Fig. 2. A) Effect of zinc-doped carbon dots (Zn-CDs) administration on testicular total oxidant status (TOS) and **B)** testicular total anti-oxidant capacity (TAC) in all experimental groups. Bars represent mean TOS and TAC levels ± standard errors for control, sham, and Zn-CDs-treated groups (0.50, 2.00, and 8.00 mg kg^{-1}). The 2.00 mg kg^{-1} Zn-CDs group exhibited the highest TOS, indicating a dose-dependent increase in oxidative stress with a biphasic response at the highest dose. In contrast, the 2.00 mg kg^{-1} Zn-CDs group showed the lowest TAC, indicating a dose-dependent increase in oxidative stress with a peak at the intermediate dose.

[abc] Groups not sharing the same letter are significantly different ($p < 0.05$).

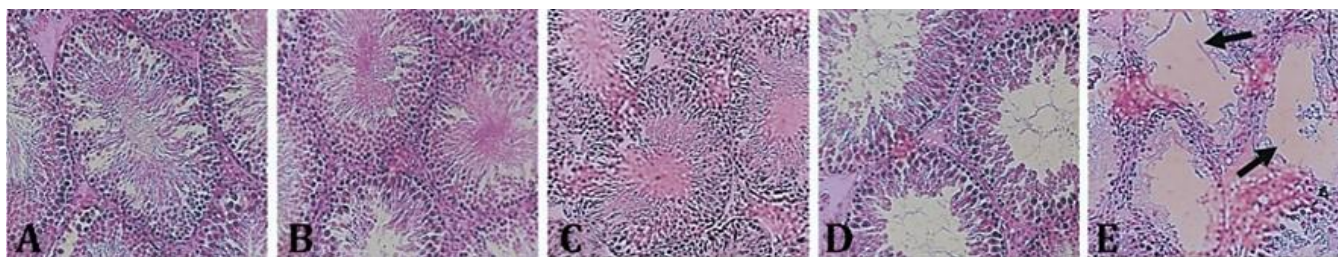

Fig. 3. Histological evaluation of testicular tissue following intra-testicular injection of zinc-doped carbon dots (Zn-CDs) at different concentrations. **A)** Control group showing normal architecture of seminiferous tubules with intact spermatogenic layers; **B)** Sham group (distilled water) exhibiting no observable histostructural alterations, similar to the control; **C)** 0.50 mg kg^{-1} Zn-CDs group showing well-preserved seminiferous tubules with minimal changes; **D)** 2.00 mg kg^{-1} Zn-CDs group displaying mild disorganization of the spermatogenic epithelium; **E)** 8.00 mg kg^{-1} Zn-CDs group demonstrating severe histopathological damage, including degeneration and disruption of the spermatogenic epithelium, as well as tubular vacuolation and atrophy (black arrows; Hematoxylin and Eosin staining, 400×).

In the group treated with 0.50 mg kg^{-1} Zn-CDs, testicular histology remained largely normal, with well-preserved seminiferous tubules and only minimal changes, indicating that this low dose did not significantly affect testicular integrity. The 2.00 mg kg^{-1} group showed mild dis-organization within the seminiferous epithelium, though overall tubular structure was retained, suggesting limited toxic effects at this concentration. Marked histo-pathological damage was observed in the 8.00 mg kg^{-1} Zn-CDs group. This group showed prominent degeneration and disruption of the spermatogenic epithelium, along with vacuolation and atrophy of the seminiferous tubules. In control, sham, and low doses (0.50 and 2.00 mg kg^{-1}) of Zn-CDs groups, the interstitial spaces appeared normal, with a dense population of Leydig cells exhibiting typical morphology and distribution. No significant signs of cellular degeneration, vacuolization, or interstitial edema were observed. In contrast, 8.00 mg kg^{-1} Zn-CDs group demonstrated marked pathological alterations in the interstitial tissue. Notably, there was an extensive vacuolation and disruption of the interstitial tissue. Large, irregular empty spaces were evident, suggestive of interstitial edema and loss of cellular components.

Cosentino score. The control group showed the lowest Cosentino score, indicating minimal tissue damage. The sham group exhibited a slightly higher score, but the difference was not statistically significant compared to the control group. The 0.50 mg kg^{-1} Zn-CDs group showed a moderate but statistically significant increase in tissue damage compared to the control group. The highest Cosentino score was related to the 8.00 mg kg^{-1} group, followed by the 2.00 mg kg^{-1} and 0.50 mg kg^{-1} groups, respectively. These results demonstrate a clear dose-dependent increase in testicular tissue damage, as measured by the Cosentino criterion, with higher doses of Zn-CDs leading to more severe histopathological changes (Fig. 4A).

Spermiogenesis index. The highest dose (8.00 mg kg^{-1}) caused a marked and statistically significant reduction in SPI compared to all the other groups (p < 0.05). Lower doses (0.50 and 2.00 mg kg^{-1}) showed intermediate effects, with the 2.00 mg kg^{-1} dose being significantly lower than control and sham groups, while the 0.50 mg kg^{-1} dose was not significantly different from the control and sham groups (p > 0.05). The control and sham groups exhibited the highest SPI, being statistically similar (p > 0.05; Fig. 4B).

Percentage of mature seminiferous tubules. Administration of Zn-CDs at doses of 2.00 mg kg^{-1} and 8.00 mg kg^{-1} resulted in a marked reduction in the percentage of mature seminiferous tubules, with mean values falling below 10.00%. In contrast, the control, sham, and 0.50 mg kg^{-1} Zn-CDs groups exhibited substantially higher percentages of mature seminiferous tubules. Statistical analysis confirmed that the reduction observed at the higher Zn-CDs doses was significant compared to the control, sham, and low-dose groups (p < 0.05; Fig. 4C).

Testicular biopsy index. Exposure to increasing doses of Zn-CDs resulted in a dose-dependent reduction in testicular biopsy scores. The control group exhibited the highest testicular biopsy score, followed by the sham group, indicating normal testicular histology in the absence of Zn-CDs exposure (Fig. 4D). Administration of Zn-CDs at 0.50 mg kg^{-1} produced a slight decrease in the biopsy score compared to the control and sham groups, though the reduction was not significant compared to the sham group (p > 0.05). Treatment with 2.00 mg kg^{-1} Zn-CDs led to a decline in testicular biopsy scores, reflecting moderate histopathological alterations. The most significant reduction was observed in the group receiving 8.00 mg kg^{-1} Zn-CDs, demonstrating markedly impaired testicular histo-structure and the lowest biopsy scores among all groups (p < 0.05).

Testosterone level. The hormone levels were significantly affected by the experimental treatments (Fig. 5). The control group exhibited the highest testosterone level, being significantly higher than all the other groups (p < 0.05) except the sham group (p > 0.05) which displayed intermediate testosterone levels, not significantly different from either the control group or the 0.50 mg kg^{-1} Zn-CDs group, but significantly higher than the 2.00 and 8.00 mg kg^{-1} Zn-CDs groups (p < 0.05).

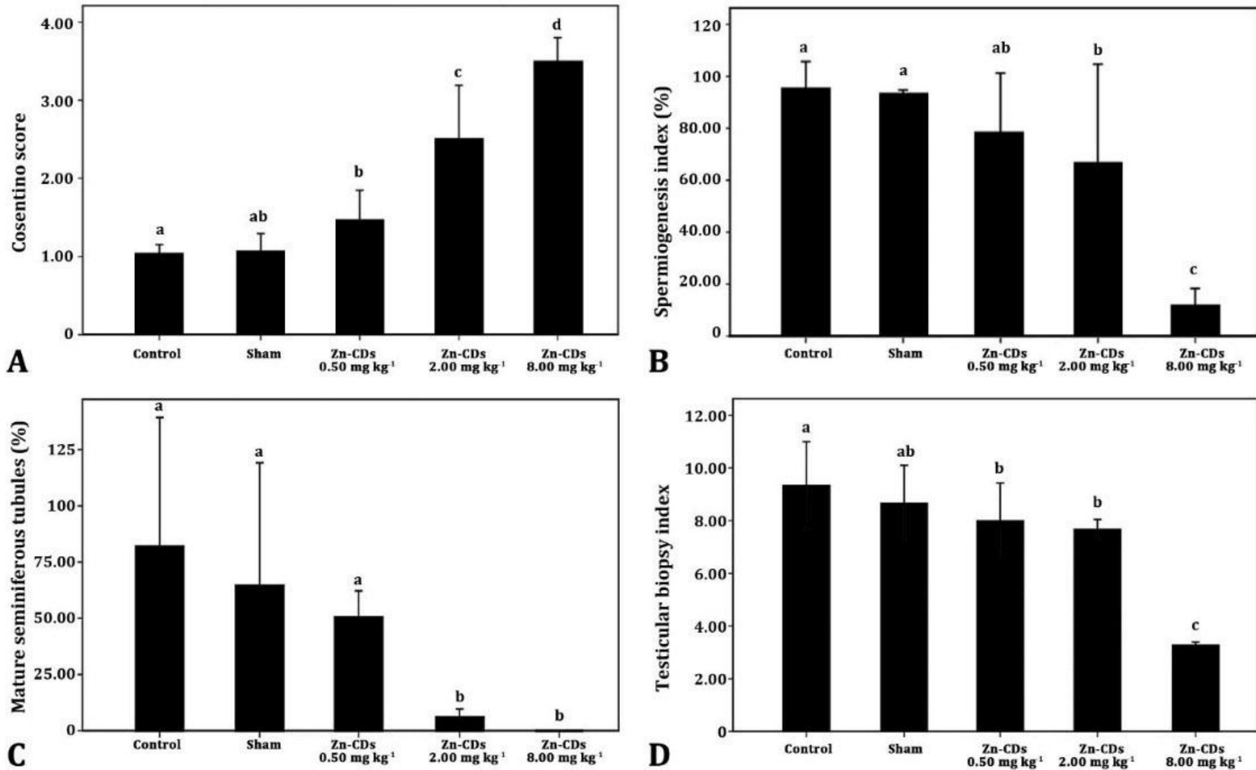

Fig. 4. Effect of zinc-doped carbon dots (Zn-CDs) administration on testicular tissue assessed by **A)** Cosentino score, **B)** Spermiogenesis index, **C)** Percentage of mature seminiferous tubules, and **D)** Testicular biopsy index in all experimental groups. Data are presented as mean ± standard error.

a-d Different letters indicate statistically significant differences between groups ($p < 0.05$).

Administration of Zn-CDs at a dose of 0.50 mg kg^{-1} led to a marked decrease in testosterone levels, being significantly lower than the control group but higher than the 2.00 and 8.00 mg kg^{-1} treatment groups ($p < 0.05$). Both the 2.00 mg kg^{-1} and 8.00 mg kg^{-1} groups showed the lowest testosterone concentrations, with no significant difference between them ($p > 0.05$), but significantly lower than all the other groups ($p < 0.05$).

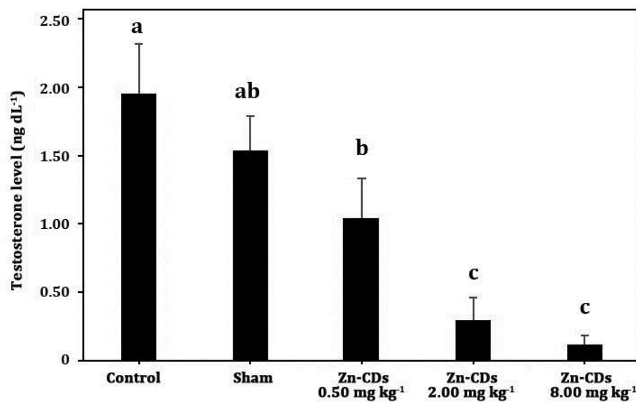

Fig. 5. Effects of zinc-doped carbon dots (Zn-CDs) treatment on serum testosterone levels. Bars represent mean ± standard error for each group.

abc Different letters indicate statistically significant differences between groups ($p < 0.05$).

Spermatology. The results of sperm analyses are presented in Tables 1 and 2. The control group, receiving no treatment, maintained baseline sperm functionality, while the sham group, administered intra-testicular distilled water, exhibited no statistically significant differences ($p > 0.05$), suggesting the injection procedure alone had minimal effect. In contrast, Zn-CDs treatment induced a clear dose-dependent decline, with the 2.00 mg kg^{-1} dose causing a significant reduction in sperm concentration, overall motility, progressive motility, and kinematic parameters ($p < 0.05$) compared to the controls, and the 8.00 mg kg^{-1} dose eliciting the most severe impairment, including the lowest sperm concentration and the sharpest drops in motility and velocity metrics. The 0.50 mg kg^{-1} dose showed a moderate, non-significant decrease in some parameters relative to control and sham groups, highlighting a progressive deterioration with increasing concentration. Further analysis underscored the escalating reproductive toxicity of Zn-CDs, with the 8.00 mg kg^{-1} group displaying the most pronounced damage, including significant reductions in velocity of average path, straight-line velocity, linearity, straightness, and beat cross frequency ($p < 0.05$), while ALH remained unchanged across all groups. Progressive motility followed a similar trend, with a marked decline at higher doses, and membrane functionality and viability showed significant

Table 1. Epididymal sperm concentration kinematic characteristics in different experimental groups. Values are expressed as mean ± standard deviation.

Parameters	Control	Sham	Zn-CDs 0.50 mg kg^{-1}	Zn-CDs 2.00 mg kg^{-1}	Zn-CDs 8.00 mg kg^{-1}
Sperm concentration (10^6 mL^{-1})	66.71 ± 16.77 ^{a}	64.08 ± 20.26 ^{ab}	60.36 ± 16.54 ^{b}	46.83 ± 14.61 ^{c}	13.81 ± 3.20 ^{d}
Total motility (%)	83.94 ± 3.41 ^{a}	81.22 ± 3.84 ^{a}	76.39 ± 2.92 ^{b}	54.05 ± 2.89 ^{c}	19.33 ± 1.47 ^{d}
Progressive motility (%)	48.10 ± 2.18 ^{a}	46.95 ± 2.51 ^{a}	40.09 ± 2.27 ^{b}	23.28 ± 1.34 ^{c}	2.47 ± 0.61 ^{d}
VAP (µm sec^{-1})	32.61 ± 1.45 ^{a}	31.53 ± 1.27 ^{ab}	29.61 ± 1.39 ^{b}	24.43 ± 1.86 ^{c}	20.19 ± 1.54 ^{d}
VCL (µm sec^{-1})	92.84 ± 3.18 ^{a}	90.18 ± 3.83 ^{ab}	87.23 ± 3.61 ^{b}	83.06 ± 2.77 ^{c}	74.63 ± 3.62 ^{d}
VSL (µm sec^{-1})	18.66 ± 0.83 ^{a}	17.39 ± 0.75 ^{a}	14.80 ± 0.43 ^{b}	9.41 ± 0.61 ^{c}	3.08 ± 0.34 ^{d}
LIN (%)	17.04 ± 0.71 ^{a}	16.27 ± 0.89 ^{a}	13.34 ± 0.61 ^{b}	8.20 ± 0.83 ^{c}	4.81 ± 0.66 ^{d}
ALH (µm sec^{-1})	8.24 ± 0.78 ^{a}	8.19 ± 0.75 ^{a}	7.64 ± 0.83 ^{a}	7.21 ± 0.91 ^{a}	6.68 ± 0.63 ^{a}
STR (%)	49.43 ± 2.43 ^{a}	47.25 ± 2.10 ^{a}	44.88 ± 2.54 ^{b}	31.43 ± 0.95 ^{c}	22.59 ± 0.77 ^{d}
BCF (Hz)	13.20 ± 0.88 ^{a}	12.49 ± 0.64 ^{a}	10.02 ± 0.71 ^{b}	8.39 ± 0.62 ^{c}	5.18 ± 0.51 ^{d}

VAP: Velocity of average path; VCL: Velocity of curved line; VSL: Straight line velocity; LIN: Linearity; ALH: Amplitude of lateral head displacement; STR: Straightness; BCF: Beat-cross frequency; Zn-CDs: Zinc-doped carbon dots.
[a-d] Different superscripts within the same row demonstrate significant differences ($p < 0.05$).

Table 2. Epididymal sperm plasma membrane functionality, DNA damage, viability, and abnormal morphology in different experimental groups. Values are expressed as mean ± standard deviation.

Parameters	Control	Sham	Zn-CDs 0.50 mg kg^{-1}	Zn-CDs 2.00 mg kg^{-1}	Zn-CDs 8.00 mg kg^{-1}
Plasma membrane functionality (%)	88.29 ± 3.51 ^{a}	86.59 ± 2.67 ^{a}	78.24 ± 3.51 ^{b}	59.31 ± 2.69 ^{c}	25.07 ± 1.55 ^{d}
Viability (%)	92.43 ± 3.40 ^{a}	90.19 ± 3.19 ^{a}	81.36 ± 3.77 ^{b}	62.88 ± 2.30 ^{c}	27.19 ± 1.94 ^{d}
DNA damage (%)	9.71 ± 1.22 ^{d}	11.83 ± 1.34 ^{d}	17.89 ± 1.20 ^{c}	30.34 ± 2.08 ^{b}	71.30 ± 3.77 ^{a}
Abnormal morphology (%)	8.22 ± 0.76 ^{d}	9.41 ± 0.82 ^{d}	15.77 ± 0.66 ^{c}	27.06 ± 1.24 ^{b}	64.76 ± 3.45 ^{a}

Zn-CDs: Zinc-doped carbon dots.
[a-d] Different superscripts within the same row demonstrate significant differences ($p < 0.05$).

differences in treated groups ($p < 0.05$), unlike the control and sham groups where no significant differences were observed. The DNA damage and sperm malformation also escalated with dose, with the 8.00 mg kg^{-1} group exhibiting the highest levels, followed by 2.00 and 0.50 mg kg^{-1}, all showing statistically significant differences ($p < 0.05$) compared to the control and sham groups, which remained statistically similar. These findings suggest a concentration-dependent toxicological impact on spermatogenesis, warranting further mechanistic studies.

Discussion

Chemical castration offers a non-surgical alternative for population control in animals. The zinc-based compounds are the most widely studied agents due to their cytotoxic effects on testicular tissue and ability to induce permanent infertility. Traditional agents, such as zinc gluconate, have been used in various species, including dogs, cats, rats, and monkeys, primarily functioning by inducing germ cell death and spermatogenesis disruption. However, these agents are associated with several adverse effects, including tissue necrosis, inflammation, and incomplete sterilization in some cases.[23] The present study investigated the utility of Zn-CDs, hypothesizing that the incorporation of zinc into CD nanostructures may enhance anti-microbial efficacy and concurrently attenuate adverse effects commonly encountered with conventional sterilizing agents.[24]

The results of the present study revealed a pronounced dose-dependent reduction in sperm concentration, motility, viability, and membrane integrity following Zn-CDs administration. The highest dose (8.00 mg kg^{-1}) resulted in the most severe impairment of sperm function and morphology, with significant increases in DNA damage and malformation rates. It indicates that Zn-CDs exert a progressively detrimental effect on spermatogenesis, with higher doses correlating with more substantial alterations in sperm function, necessitating further investigation into the underlying toxicological mechanisms. These results align with previous studies demonstrating the cytotoxicity of zinc-based nanoparticles and their capacity to disrupt spermatogenesis through oxidative stress and germ cells apoptosis induction.[25] The observed histopathological changes, including seminiferous tubules degeneration and vacuolation, as well as testicular histo-architecture deterioration at higher Zn-CDs doses, further support the potent sterilizing effect of these nanoparticles.[26]

The SPI and percentage of mature seminiferous tubules were significantly reduced in the higher doses' groups, corroborating the detrimental impact of Zn-CDs on testicular function. These findings are consistent with reports on other chemical sterilants, such as zinc gluconate, which similarly induce testicular atrophy and impaired spermatogenesis in animal models.[27]

A key mechanism underlying the reproductive toxicity of Zn-CDs appears to be the induction of oxidative stress.[28] This study demonstrated a significant increase in TOS and a resulting decrease in TAC in testicular tissue, particularly at the intermediate and high Zn-CDs doses. Oxidative stress leads to lipid peroxidation, DNA damage, and

apoptosis in reproductive cells.[29] The biphasic response observed in this study, with the highest oxidative stress at the intermediate Zn-CDs dose and partial attenuation at the highest dose, may reflect cellular adaptive mechanisms or altered nanoparticle bioavailability at higher concentrations.[28] The vulnerability of spermatozoa to oxidative damage is well documented, given their limited anti-oxidant defenses and high content of poly-unsaturated fatty acids in the plasma membrane.[30] The current findings reinforce the role of oxidative imbalance as a central pathway in Zn-CDs-mediated testicular toxicity and infertility.

In the present study, serum testosterone levels were significantly reduced in Zn-CDs-treated rats, especially at the 2.00 and 8.00 mg kg^{-1} doses. This suppression of androgen production may be associated with the damage to Leydig cells and disruption of steroidogenic pathways, as reported in studies focusing on zinc oxide nanoparticles and other chemical castration agents.[31]

While zinc gluconate and related compounds efficacy in inducing infertility has been demonstrated previously, their use is often limited by adverse effects, such as tissue necrosis, inflammation, and incomplete sterilization.[32] The present study suggests that Zn-CDs may offer a more controlled and potentially less invasive approach, with the added advantage of nanoparticle-mediated delivery and enhanced cellular uptake.

The application of metal-doped CDs in biomedical fields is expanding, with recent studies highlighting their multi-functional properties, including photoluminescence, targeted delivery, and catalytic activity.[33] In the context of reproductive biology, the present findings contribute to a growing body of evidence supporting the potential of engineered nanoparticles as chemical sterilants. Nevertheless, concerns regarding long-term safety, systemic toxicity, and environmental impact remain. Further research is needed to elucidate the pharmacokinetics, bio-distribution, and potential off-target effects of Zn-CDs, as well as their efficacy in other animal species and under field conditions.

Despite the significant findings, several methodological limitations warrant consideration. Long-term studies are necessary to assess the permanence of infertility, potential recovery of testicular function, and any delayed adverse effects. Additionally, mechanistic investigations into the molecular pathways of Zn-CDs-induced cytotoxicity, including apoptosis and autophagy, would provide valuable insights or the development of safer and more effective sterilization strategies. Future studies could consider assessing fertility in female rats through pregnancy rates and litter size, as well as investigating the expression of apoptosis-related genes in testicular tissue to further clarify reproductive effects and underlying mechanisms.

In conclusion, this study demonstrates that intra-testicular injection of Zn-CDs induces dose-dependent reproductive toxicity, oxidative stress, and testicular damage in mature rats, effectively leading to infertility. The results highlight the promise of Zn-CDs as a novel chemical sterilant, while also emphasizing the importance of dose optimization and comprehensive safety evaluation. These findings pave the way for further research into nanoparticle-based approaches for animal population control and reproductive management.

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Conflicts of interest

None to declare.

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