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Histomorphometric evaluation of the testis after administration of methionine-loaded zinc oxide nanoparticles in mice

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Abstract With the remarkable progress of nanotechnology in medicine, the use of nano supplements such as methionine in low doses can be a starting point for the optimization and strengthening of the compounds that protect the testes and, subsequently, the improvement of the fertility rate of the population. Excessive amounts of methionine, an essential amino acid in mammals, have toxic effects on body organs. The beneficial effects of widely used and inexpensive zinc oxide nanoparticles (ZnO-NPs) with high antioxidant properties have been shown on the male reproductive system. This study was conducted to examine the histopathologic and histomorphometric effects of methionine-loaded zinc oxide nanoparticles on mice testes. Adult male mice (N = 50) were assigned to five groups (n = 10 in each group) randomly. The groups included M50 (50 mg/kg methionine), M200 (200 mg/kg methionine), NM50 (50 mg/kg methionine-loaded zinc oxide nanoparticles), NM200 (200 mg/kg methionine-loaded zinc oxide nanoparticles), and Control. After 42 days, the left testes of the mice were assessed for Johnson scores, seminiferous tubule epithelium height, seminiferous tubule diameter, meiotic index, rate of spermatogenesis, and histopathological features after euthanasia. The results showed that high doses of methionine (alone and attached to nano zinc oxide particles) have toxic effects on the testes. In comparison to the control group, the histopathological and histomorphometric indicators of the testis in the group receiving methionine at a dose of 50 mg/kg attached to nano zinc oxide particles had some increase (although insignificant), with better status compared to the M50 group. High doses of methionine could have undesirable effects on testes and significantly decrease the mentioned parameters. However, the results indicated that the histomorphometric indices of mice testis might benefit from zinc oxide nanoparticles loaded with methionine.

Introduction

Infertility is an increasingly concerning public health concern; it is thought that between 20-70% of fertility issues are attributed to male partners, with over 30 million men worldwide facing infertility [1]. Infertility can lead to emotional distress and social seclusion. The possibility of a connection between male infertility and toxins, alterations in diet and lifestyle, and environmental pollutants have become sources of increasing concern. Many substances in the diet have been proven to play a role in male reproductive performance, the epigenetics of reproductive cells, fetal development, and the prevention of metabolic diseases in adulthood [1-3].

Methionine is a sulfur-containing essential amino acid that has many functions, such as maintaining proper growth and development of mammals, healthy nails, skin, and hair, and synthesis of vital molecules, such as carnitine,

cysteine, etc. [4, 5]. It is also utilized for treating arthritis and is widely used in sports and food supplements. While low doses of methionine have protective effects, there have been reports of excessive methionine leading to adverse effects like growth reduction and damage to various organs, including the male reproductive system [2, 6, 7].

On the other hand, zinc is an essential element that provides high levels of protection as an antioxidant [8]. This element is present in great quantities in the testicles and peripheral gonads, playing an important role in testicular growth, function, sperm production, and maturation [8, 9]. Zinc also inhibits the oxidation of macromolecules like DNA, enhancing cellular functions such as managing oxidative stress and maintaining the structural integrity of DNA [10, 11].

Zinc oxide nanoparticles (ZnO-NPs), whose safety has recently been confirmed by the FDA (United States Food and Drug Administration), have been receiving increased attention [12]. Materials with at least one dimension smaller than 100 nm are known as nanoparticles, with an increased surface-to-volume ratio granting them unique characteristics compared to their regular form [13]. Due to the higher percentage of zinc with low toxic impact, the traditional form of zinc oxide has been used as an added ingredient for animal feeds more frequently than other salts [14, 15]. Studies have demonstrated that nanoparticles can cross the blood-testis barrier (BTB) and subsequently be stored in the testes [16–18]. It has also been indicated that ZnO-NPs have unique physical and chemical characteristics, and they can bypass some tissue barriers and exert their beneficial impacts on target cells. Although numerous studies have detailed the utility of these nanostructures in medical procedures, high levels of ZnO have been reported to be toxic to particular cell lines such as the male reproductive system [19].

The effectiveness of ZnO nanoparticles loaded with methionine on the male reproductive system has not been studied yet. Therefore, the present study aimed to evaluate the histomorphometric features of the mouse testis after exposure to methionine-loaded zinc oxide.

Materials and Methods

Chemicals

L-methionine was obtained from Scharlau Co., Spain. The zinc chloride (≥ 99.995%) and sodium hydroxide (≥ 98%) required for synthesis of ZnO-NPs were purchased from Sigma-Aldrich Co., USA.

ZnO-NP Synthesis

The co-precipitation method was used to prepare the necessary ZnO-NPs. 0.3 mmol/L sodium hydroxide was dissolved in distilled water. Then 0.15 mmol/L of zinc chloride was added to the above solution and stirred at 60 °C for 110 minutes to form white zinc oxide nanoparticles. After the product was centrifuged and washed with distilled water, it was dried at 50 °C under vacuum conditions.

Drug loading

First, 200 mg of ZnO-NPs were dissolved in water followed by the addition of 40 mmol/L methionine to the solution. The solution was stirred for 24 hours at 50 °C to attach methionine to the zinc nanoparticles.

Animals

A total of 50 male NMRI mice (25–30 g), 1.5–2 months old, were provided from the animal house of the Kerman University of Medical Sciences, Kerman, Iran. The mice were housed in standard polypropylene cages with ad libitum access to pellet food (Javaneh Khorasan Co., Mashhad, Iran) and water in an adequately-ventilated room (21 ± 2 °C) on a 12-hour light/12-hour dark cycle. Prior to participating in the experiment, the animals were given one week to get used to the laboratory condition.

The study was approved by the Animal Ethics Committee of Shahid Bahonar University of Kerman, and all steps were conducted following the guidelines for research animal handling and use.

Experimental design

The animals were randomly divided into five groups (n = 10). Doses 50 and 200 mg/kg (0.2 ml L-methionine dilution in distilled water) were administered to the mice in the M50 and M200 groups, respectively. 0.2 mL of methionine-loaded ZnO-NPs at concentrations of 50 and 200 mg/kg, respectively, were given to the NM50 and NM200 groups. The control group animals received normal saline in the same volume. All groups received the solutions orally daily for 6 weeks using gastric gavage. All mice were

euthanized by cervical vertebral dislocation at the end of day 42nd.

Each gram of nanomedicine contain 0.75 g methionine and 0.25 g ZnO-NPs was prepared. Considering that each 30-gram mouse in the N200 group needed about 9 mg of methionine, the amount of ZnO-NPs needed to prepare the nanomedicine was 2 mg. This rate of nanoparticle is much lower than the toxic amount causing disruption in animal tissues shown in previous studies [16, 20, 21]. Therefore, there was no need to predict the control group of ZnO-NPs in the study.

Histopathological assay

After euthanasia, the right testes of the mice were removed and kept for 48 hours in 10% neutral buffered formalin 10%. A gradation of ethanol was used to dehydrate the samples. They were then embedded in paraffin after they were cleared in xylene. The samples were then cut in 5 µm thickness. After hematoxylin and eosin (H&E) staining, the slides were studied by an optical microscope.

Morphometric analysis

Seminiferous tubules diameter and epithelial height

Germinal epithelium height and seminiferous tubule diameter were measured in 50 tubular sections of round to slightly oval shape in a random manner for each animal. The mean values of these data were then calculated. In this study, all measurements and photographs were taken using the Dino-Lite digital microscope and the Dino capture 2.0 software.

Spermatogenesis and Meiotic index

To evaluate histopathological spermatogenesis, 100 spermatogenic tubes were investigated with 100x magnification in each mouse. Each tube received a score from 1 to 10 according to Johnson scoring method, which depends on how mature the germ cells are in the spermatogenic tubes. A higher Johnson score (score 10) in comparison with a lower one suggests that spermatogenesis is in better shape (Table 1). Finally, the formula: sum of all scores / total number of seminiferous tubule sections was used to calculate the Johnson score [22]. Meiotic index (MI) was evaluated through the round spermatid/primary spermatocyte total number ratio in 50 tubules [23].

Statistical analysis

All data analysis were presented as mean ± standard deviation and P-value < 0.05 was considered statistically significant. The statistical study was performed through one-way analysis of variance (ANOVA) and Tukey's HSD post hoc analysis was used with SPSS software (version 16).

Results

Characterization of ZnO-NPs

It is important to establish the shape and size of the NPs because these physical characteristics affect the stability, cell damage, and penetration abilities of the particles. The observations under the SEM indicated that the ZnO-NPs were spherical in geometry with an average diameter of 30 nm. The shape of the nanoparticles indicated that there were no sharp edges that could stab the cells or the tissues. Their size was small enough in order to the NPs could be assimilated very easily by cells in the mice.

Histopathological evaluation

In the normal control group, the seminiferous tubules were covered by several layers of sex spermatogenic cells and accumulations of sperm were seen in their lumens. There was little connective tissue between the tubules. The epithelium of seminiferous tubules were formed from spermatogonia, primary spermatocytes, spermatids, and spermatozoids. Sertoli cells were located on the smooth and regular basement membrane. Leydig cells were also present in the interstitial space and the wall of each tubule was covered by a layer of myoid cells (Figure 1).

The results of the NM50 group were similar to the normal control group. In the NM200 and M200 groups, the spermatogenic tubules were wrinkled and contracted and the height of their epithelium was reduced. In some tubules, large interstitial spaces, irregular basement membrane, and lumen containing degenerated germ cells were observed. Sperm accumulation was present in a small percentage of the tubules. Also, germ cells and Leydig cells in the interstitial space had dark nuclei (Figure 2).

In general, compared to the NM200 and M200 groups, the M50 group showed much less severe destruction.

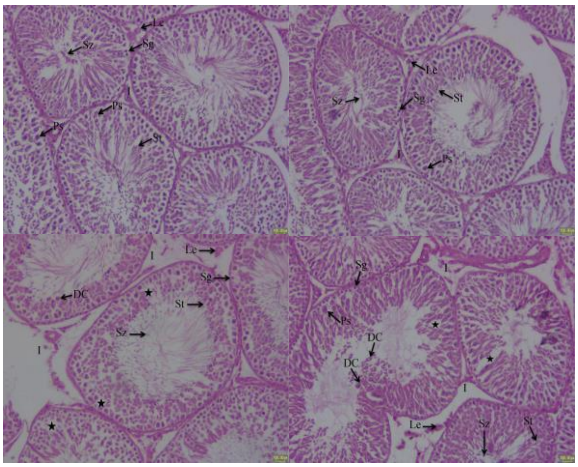

Fig 1. The photograph of the testis in the normal control group (A), MN50 (B), M50 (C), MN200 (D) and M200 (E). Sg: spermatogonial cell, Ps: primary spermatocytes, St: spermatids, Le: Leydig cells, Sz: spermatozoa, I: interstitial space, * :irregular space between spermatogenic cells, DC: degenerate cells, **: absence of spermatozoa (HE staining, Bar = 100 µm).

Morphometric analysis

Tables (1 and 2) and Figures (2 and 3) show the results of spermatogenesis percentage, meiotic index, Johnson's scores, spermatogenic tubule diameter, and seminiferous tubule epithelium height for all groups.

Spermatogenesis and meiotic index

The percentage of spermatogenesis in the rats of the M200 and NM200 groups was significantly lower than the control group ($P < 0.05$) while it was close to the normal control group in the M50 group. This percentage was higher in the NM50 group.

The mice in the M200, M50, and NM200 groups had significantly lower meiotic index ($P < 0.05$) in compared to the control group. However, this index in the NM50 group was reported in the same level similar to the control group.

The M200, M50, and NM200 groups showed significantly lower mean Johnsen's scores compared to the control normal group. The NM50 group outperformed the control group in terms of score despite the fact that this difference was not statistically significant ($P > 0.05$).

Table 1. Johnson scoring based on histopathological findings of testicular tissue

Score	Histopathological findings
1	No cells, atrophic tubules
2	No germ cells, only Sertoli cells
3	No primary spermatocytes, only spermatogonia
4	Very few primary spermatocytes
5	No spermatozoa and round spermatids, and many primary spermatocytes
6	Few round spermatids
7	No spermatozoa, and many round spermatids
8	Few spermatozoa
9	A little impaired spermatogenesis, many sperm, disorganized epithelium (marked sloughing or obliteration of the lumen)
10	Healthy spermatogenic tubules, complete spermatogenesis

Seminiferous tubule diameter and epithelial height

Compared with the control normal group, the M200 group showed lower seminiferous tubule diameters, and the difference was statistically significant ($P < 0.05$). Nevertheless, the difference between the value in other groups and the control group was either none or insignificant ($P > 0.05$). None of the studied groups showed a significant difference in seminiferous tubule epithelial height compared to the control group ($P > 0.05$) (Figure 3).

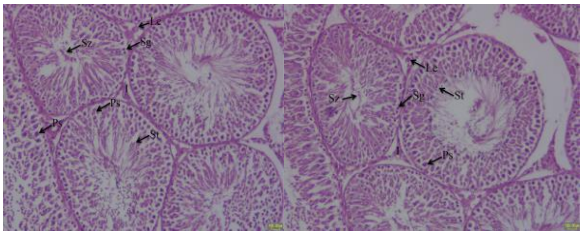

Fig 2. Seminiferous tubules of testicular tissue in normal control group (A) and MN50 (B). Spermatogonial cells (Sg), primary spermatocytes (Ps), spermatids (St), Leydig cells (Le) and spermatozoa (Sz), dense tubules, low interstitial space, and high epithelium height were observed (HE staining, Bar = 100 µm).

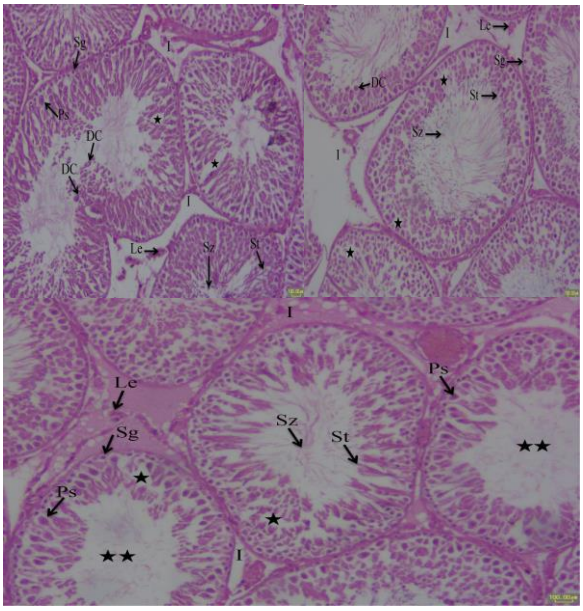

Fig 3. Cross-sections of the seminiferous tubules of testicular tissue in the group M50 (A), NM200 (B), and M200 (C). Spermatogonial cells (Sg), primary spermatocytes (Ps), spermatids (St), Leydig cells (Le), spermatozoa (Sz), and vacuole space (*) can be seen between the cell lines inside the tubes. Inside the lumen of some tubes, degenerated cells (DC) are observed. Compared to the control and NM50 groups, the tubules are denser (I). In figure C, some tubes do not contain spermatozoa (**) (HE staining, Bar = 100 µm).

Discussion

The evaluation of genetic and epigenetic data that the sperm or even the seminal fluid contributes to the offspring has received increased attention in recent years [24]. Men account for about half of infertility cases, and testicular exposure to a toxic environment can readily impair sperm production and consequently, sperm quality [25, 26]. Methionine, an essential amino acid, has vital roles in various physiological processes, such as protein synthesis, methylation, and antioxidant defense [27]. It is widely used in sports and dietary supplements as well as the treatment of a number of diseases such as arthritis. Due to the side effects of methionine, its use should be controlled and its dose adjusted [6, 28, 29].

One well-known factor of idiopathic male infertility is the cell antioxidant defense system/reactive oxygen species (ROS) imbalance leading to oxidative stress [2]. ROS can damage DNA, proteins, motility, and fluidity of the plasma membrane. Sperms are more susceptible to oxidative damage due to the lack of cytoplasmic defense enzymes such as catalase (CAT) and glutathione S-transferase (GST), and the presence of a high concentration of PUFA (polyunsaturated fatty acids) in their

Table 2. The histomorphometric results (mean ± SE) of the five studied groups.

Groups	Diameter of seminiferous tubule	Epithelium height of seminiferous tubule	Meiotic index	Johnson's score	Spermatogenesis percentage
Control	207.68 ± 16.88^{a}	65.36 ± 6.06^{a}	3.3 ± 0.3^{a}	8.93 ± 0.79^{a}	86.67 ± 8.15^{a}
M50	210.43 ± 17.54^{a}	65.09 ± 4.95^{a}	3.02 ± 0.26^{c}	8.13 ± 0.78^{b}	83.34 ± 3.85^{a}
M200	195.9 ± 12.23^{b}	64.02 ± 4.39^{b}	2.29 ± 0.3^{b}	7.07 ± 0.69^{c}	34.67 ± 5.58^{d}
MN50	214.89 ± 19.1^{c}	68.72 ± 7.1^{c}	3.27 ± 0.19^{a}	9.13 ± 0.78^{a}	94.66 ± 2.98^{c}
MN200	220.18 ± 11.99^{d}	65.84 ± 5.5^{a}	2.95 ± 0.66^{c}	7.58 ± 0.72^{bc}	58.67 ± 2.98^{b}

Different letters (a, b, c, and d) in each column indicate the existence of a significant statistical difference between the results

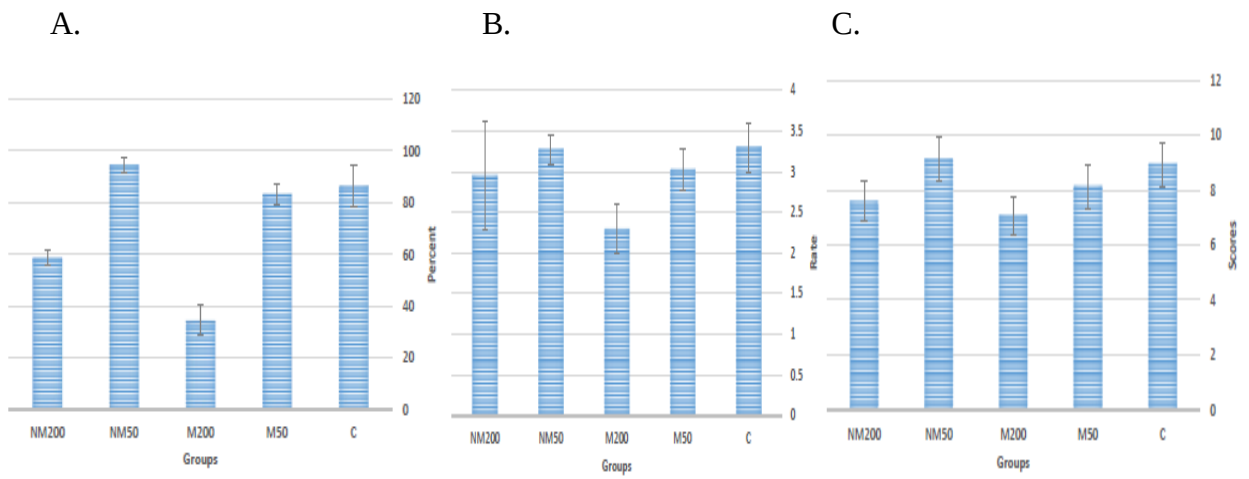

Fig 4. The effect of different treatments (control, M50, M200, NM200 and NM50) (mean ± SE) on spermatogenesis percentage (A), meiotic index (B), and Johnson's score (C).

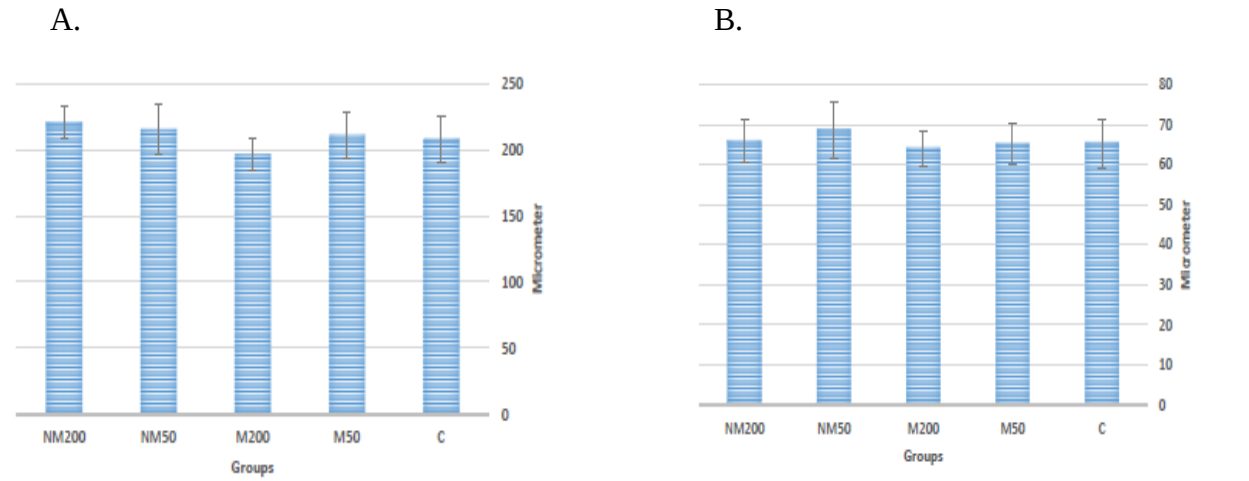

Fig 5. Mean ± SE of the diameter (µm) (A) and epithelium height (B) of the seminiferous tubules in the five groups (control, M50, M200, NM200 and NM50).

plasma membrane [25, 30]. It has been shown that methionine has antioxidant effects depending on the methyl cycle. The protective effect of methionine may be due to its ability to scavenge free radicals and inhibit lipid peroxidation. Methionine is a precursor for the antioxidant glutathione, which is an important intracellular antioxidant that can scavenge ROS and protect cells from oxidative damage. Also, methionine reduces the damage caused by oxidative stress by increasing GSH activity and chelating lead and removing it from tissues [31, 32]. However, high doses of methionine may have the reverse effect and a pro-oxidant effect, leading to the production of reactive oxygen species and oxidative stress [31, 33].

Although methionine has antioxidant properties, many studies have mentioned its toxic effects in high doses. The results of the present study showed that methionine gavage with a dose of 200 mg/kg had toxic effects on the testis and significantly reduced the percentage of spermatogenesis, Johnson score, meiosis index, and the diameter of the epithelium of the seminiferous tubules. Also, the spermatogenic tubules were wrinkled and contracted in various ways and the height of their epithelium had decreased. In some tubules, large interstitial spaces, irregular basement membrane, and lumen containing degenerated germ cells were observed. There was accumulation of sperm in a small percentage of tubes, and the germ cells and Leydig cells in the interstitial space had dark nuclei. However, the indices of the group receiving 50 mg/kg of methionine were at the level of the control group. In some studies, it has

been reported that the reduction of the epithelium, followed by the reduction of spermatogenesis, is due to the increase of the connective tissue between the tubes, which has led to a decrease in blood supply and the resulting hypoxia of the cells inside the lumen [34]. It has been shown that the additional consumption of methionine at a dose of 2.4% of the diet reduces feed consumption, inhibits general growth, and causes hemolytic anemia [35]. In Abu Elnaga's (2012) study, the use of high amounts of methionine (2% of the diet) showed qualitatively adverse effects (edema in the interstitial spaces and epididymal walls) on the testicular tissue, and its low doses improved spermatogenesis and structure of the epididymis [31]. Also, in the histological examination of the testis following the consumption of 2.7% (w/w) extra methionine in the diet, an increase in spermatocyte degeneration and loss of spermatids was seen but this result did not occur in the lower dose [24].

In another study in line with the present investigation, the administration of methionine (50 mg/kg) and metovitane (containing thiamine, nicotinamide, methionine, zinc salt, and α-tocopherol acetate) in anti-tuberculosis drug-treated rats reduced the amount of tubal content irregularity and increased the number of epididymal sperm. The authors noted that at least part of the protection may be through the expression and regulation of CYP isozyme activities, limiting ROS production and protecting the vital activities of testicular antioxidant enzymes, including SOD. Compared to methionine, metovitan decreased germ cell exfoliation in the spermatogenic tube lumen, and in general, the better performance of the protective effects of metovitan can be due to its additional supplements including zinc [2].

Homocysteine is a sulfur-containing amino acid that is produced as a byproduct of methionine metabolism [36]. Several studies have reported that high doses of methionine can increase homocysteine levels in blood and tissues [6, 7, 37, 38]. Accumulation of homocysteine leads to the development of a medley of diseases, including neurodegenerative and cardiovascular disease and male infertility [39, 40]. Homocysteine can have negative effects on the testicle by inducing oxidative stress and inhibiting spermatogenesis. It also causes apoptosis by breaking DNA into smaller pieces [6, 41, 42]. Des Santos et al. (2022) reported a reduction in germinal epithelium height and tubule diameter and an increase in the abnormal seminiferous tubules following hypercysteinemia, which is in accordance with the present study results in the groups receiving high-dose methionine [37]. According to these studies, homocysteine levels might rise in the 200 mg/kg methionine group compared to the control group and in the group receiving 50 mg/kg methionine post-injection for a few days. In the present study, measurement of blood homocysteine levels was not possible due to the small serum volume [7].

Zinc, a trace element with antioxidant properties, is abundant in male genitalia. It plays an important role in spermatogenesis, as it is required for DNA, RNA, and protein synthesis in the testis. Zinc's ability to bind sulphydryl groups in proteins and to occupy iron and copper binding sites in lipids, proteins, and DNA allows it to function as an oxidation inhibitor [2, 43]. It has been shown that atrophy of spermatogenic tubes and failure of spermatogenesis occurs in rats following zinc deficiency [44, 45]. Testes and epididymis of rats become more sensitive to oxidative stress because of zinc deficiency because of increased ROS production and/or impaired zinc dependent antioxidant mechanisms. In a study, zinc supplementation improved the decrease in sperm count and plasma testosterone caused by CP [46]. It is accepted that particles in their nanoform have better intestinal absorption rates, bioavailability, and catalytic activity. As a result, it is probable that converting ZnO into nanoform will boost the effectiveness of Zn by improving its absorption and bioavailability in the digestive system [47].

Zinc oxide nanoparticle (ZnO-NPs) use as a drug delivery platform has great potential to improve its therapeutic efficacy. It has been reported that these particles have antioxidant and anti-inflammatory properties and have been investigated for their potential use in the treatment of various diseases, including cancer, diabetes, and neurological disorders [48–50]. Therefore, the combination of methionine and ZnO-NPs may exert a strong antioxidant effect that can help protect against oxidative stress-induced damage to the testis. In addition, ZnO-NPs increase the bioavailability of drugs. For example, zinc oxide nanoparticles have been found to raise the bioavailability of curcumin, a polyphenol with antioxidant and anticancer properties [48, 49]. Badkoobeh et al. found ZnO-NP administration to prevent sperm damage in adult male rats treated with doxorubicin through an antioxidant mechanism [45]. It is worth mentioning that when using ZnO nanoparticles, be attention to their toxicity in high doses. In most

studies, doses above 50 mg/kg of zinc oxide nanoparticles are considered toxic, and the dose used in this study is much lower than this amount [51]. Torabi et al. (2017) showed that a dose of 5 mg/kg of ZnO-NPs did not show any toxic effect on spermatogenesis [44]. In the study of EL-Maddawy (2019) in rats treated with 3 mg/kg of zinc oxide nanoparticles five times a week for 8 weeks, testosterone, sperm count, GSH, and CAT increased and MDA decreased in the testis [52]. Additionally, ZnO-NPs have hepatoprotective effects at low dosages by scavenging free radicals or encouraging antioxidant activities, which detoxify the free radicals [53].

In our study, the results of methionine consumption at a dose of 50 mg/kg attached to nanoparticles of zinc oxide were similar to those of control animals. The increase in the investigated parameters was not significant in compared to the methionine 50 mg/kg group, which performed better. Administration of 200 mg/kg methionine-bound nanoparticles decreased the morphometric characteristics of mice testes significantly, and in terms of pathology, similar to the M200 group, they had adverse effects on the structure of the testis. The presence of irregular basement membranes, lumen containing degenerated germ cells, and dark nuclei in Leydig and germ cells indicate disruption of cellular processes and functions. The positive effects of 50 mg/kg ZnO-NPs bound to methionine on the testis can be attributed to the biocompatibility and safety of ZnO-NPs in lower doses. It can also be caused by the synergistic antioxidant effect and increased bioavailability of methionine following the simultaneous consumption of methionine and zinc oxide nanoparticles. In according to the results of the present study, it is possible that the high dose of methionine used (200 mg/kg) may have decreased the protection provided by zinc oxide nanoparticles.

Conclusion

It is worth noting that methionine in higher doses (alone and attached to zinc oxide nanoparticles) can have significantly adverse effects on testicular tissues. The implications of the study findings for the use of methionine in food supplements and animal feed and for understanding the mechanisms underlying the effects of environmental and occupational exposures to methionine can be significant. In comparison to the control group, the histopathological and histomorphometric indicators of the testis in the group receiving methionine at a dose of 50 mg/kg attached to nano zinc oxide particles had some increase (although insignificant), with better status compared to the M50 group. However, more research is needed to fully evaluate the safety and efficacy of this approach, as well as to optimize the dose for therapeutic use.

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Conflict of interest

The authors declare that there is no conflict of interests.

Ethical approval

The trial convention was affirmed by the animal welfare committee of the Faculty of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman, Iran.

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