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# Histopathological Evaluation of the Toxic Effects of Zinc Oxide (ZnO) Nanoparticles on Testicular Tissue of NMRI Adult Mice

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**Abstract**

In this study, the histopathological effect of zinc oxide nanoparticles (ZnOn) on testicular tissue of mature NMRI mice was investigated. Zinc oxide nanoparticles have various applications in industry and medicine because of their small size and high surface area. The animals were divided into five groups randomly=6. The first group (group1) was treated with ZnOn with a dose of 250 mg/kg/day dissolved in 1ml of distilled water. All injections were intraperitoneal (IP) for one day. The experimental 2 group (group 2) was treated with ZnOn with a dose of 500 mg/kg/day. The experimental group 3 (group 3) was treated with ZnOn with a dose of 700 mg/kg/day. The sham group (group 4) received only the same volume of distilled water (1ml) by IP injection during the experimental period. The control group (group 5) did not receive any solution. The results revealed significant changes in cell types of testis tissue that were treated with ZnO nanoparticle. These changes were observed as reduction and loss of cells in seminiferous tubules in testicular tissue of experimental groups. Thus according to the findings of this study, ZnO nanoparticles have destructive effects on testis tissue and effects on spermatogenesis.

**Keywords:** Zinc Oxide Nanoparticles; Histopathology; Mice; Testicular tissue

## Introduction

A nanoparticle (NPs) are materials with a size range of approximately 1-

100 nm [1].The small size of the particles provides an increased surface area and induces unique and specific physicochemical characteristics such as chemical reactivity, durability and high conductivity compared to bulk materials [2]. Thus, NPs, because of their small size, can penetrate into the cell membrane and interfere in important cell functions. Studies have demonstrated that the size of NPs also affects their internalization in terms of efficiency [3, 4].Several reports have revealed that nanoparticles (NPs) have a relatively greater toxicity compared to large size materials, therefore materials at the nano size are highly reactive. There is report suggest that nanoparticles are toxic to vascular endothelial cells. Also according to previous studies, exposures to Zinc Oxide NPs have toxicity properties on liver enzymes in male rat [5, 6]. Additionally, NPs can pass the blood-brain and blood-testes barrier [7, 8], also affect many organs such as the heart and brain through the bloodstream [9]. Based on previous studies, some nanoparticles such as zinc oxide (ZnO) and titanium dioxide ($TiO_2$) are used in products such as tiles and auto cleaning glasses[10].Also, zinc oxide nanoparticles have widespread application such as food additives, biosensors, resin production and electronic equipment [11].Furthermore, due to the extensive application of ZnOn in different industries, it is necessary to evaluate the toxic effects of this nanoparticle in biological systems, specially its effects on the male reproductive organ and productivity potential. However, research on their toxicological impact and possible hazards for human health is still incomplete. Thus, our aim for the present study was to investigate the histopathological effects of intraperitoneal (IP) injection of ZnO nanoparticles at different doses (250, 500 and 700 mg/kg/day) on the testis tissue of adult NMRI mice.

## Materials and methods

### *Physical specifications of zinc oxide (ZnO) nanoparticles*

ZnO nanoparticles used in this study were prepared by the Pars Lima Company. The properties of the ZnO nanoparticle is as follows: white powder with 20 nm diameter, nearly spherical shape, grade SSA: > 90 $m^2/g$ and purity 99 + % that is very toxic to aquatic organisms.

### *Experimental animals*

In this study, two month old 30 adult male NMRI mice, and weighed 28-32g were used. They were obtained from the Pasteur Institute (Tehran, Iran).They was used after one week. The animals were housed under standard conditions of temperature (23°C) and relative humidity of 45-50% under a well-regulated 12:12hour light/dark cycle. Also animals were given drinking tap water and rodent food pellet.

### *Experimental design*

In this study, there were five groups each containing six adult male mice. Animals in the experimental groups (3 groups) were administered the following

ZnO nanoparticle doses; 250, 500 and 700 mg/kg/day, by intraperitoneal (IP) single injection, respectively. A group designated as the sham group received 1ml of distilled water by IP, and the control group did not receive distilled water orZnO nanoparticle. Then, one week after the injection, the mice were anaesthetized by ether and their testes were removed and weighed.

### *Sample preparation for light microscopy and histopathological analysis*

Testes from each animal were fixed in Bouin's solution and directly dehydrated in a graded series of ethanol and embedded in paraffin. The samples were sectioned by microtome into 5μm thickness and stained with hematoxylin and eosin (H&E method) for histopathological examinations. Following the staining of sections, histopathological changes of testes were evaluated using a light microscope. Then different parameters were considered and data were recorded.

### *Statistical analysis*

The data of this study was analyzed using the SPSS software, version 21. Statistical significance between groups was computed by analysis of variance and analyzed by One-way ANOVA and Tukey's test. The differences were considered significant at $P<0.05$.

## Results

### *Macroscopic changes due to the effect of ZnOn on the testes in different groups*

Testes were removed from the body of adult mice and weighed. The results did not show significant change (fig. 1).Also, during the experimental period there are no teratogenic symptoms and signs (such as anomaly and malformation) were observed due to the effect of ZnOn on the testes of mice from different groups.

### *The histopathological results of ZnOn effect on the testes of mice in different groups*

The results of microscopic surveys showed that ZnOn does not have a significant effect on tunica albuginea thickness in treated groups compared to the control group. Also, the number of degenerated seminiferous tubules were increased in ZnOn administrated groups (250 and 500 mg/kg/day doses), yet this change was not significant compared to the control group. But, diameter of seminiferous tubules and germinal epithelium were significantly reduced in groups that received 250 and 500 mg/kg/day doses of ZnOn compared to the control group ($P<0.001$), yet those who received the 700 mg/kg/day dose, did not show any change (5and6Graphs, Figure 4.).

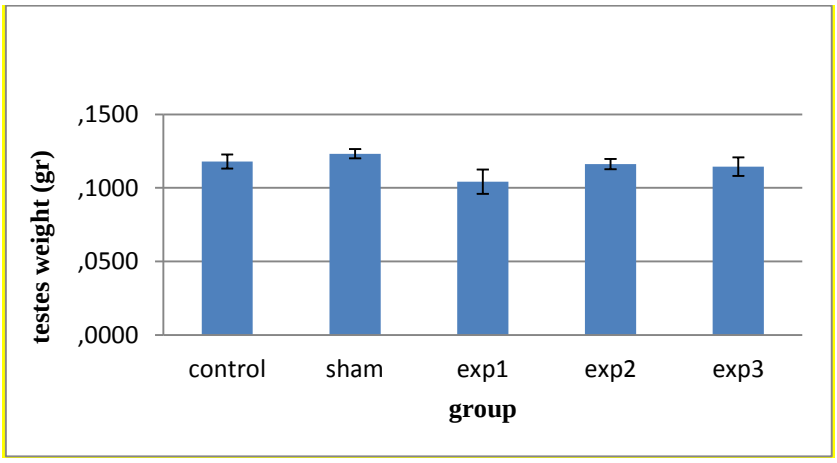



Fig.1. The Effect of Intraperitoneal Injection of Different Doses of Zinc Oxide Nanoparticles on mice testes weight. The results obtained for the effect of ZnOn on mice testes weight for experimental groups did not show significantly different from that of the control group. Test groups were indicating as exp1: 250 mg/kg/day dose, exp2: 500 mg/kg/day dose, exp3: 700 mg/kg/day dose respectively.

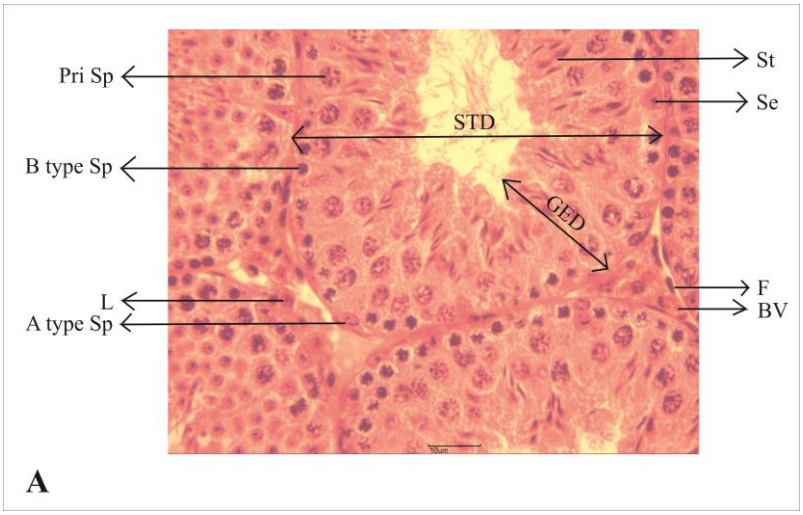
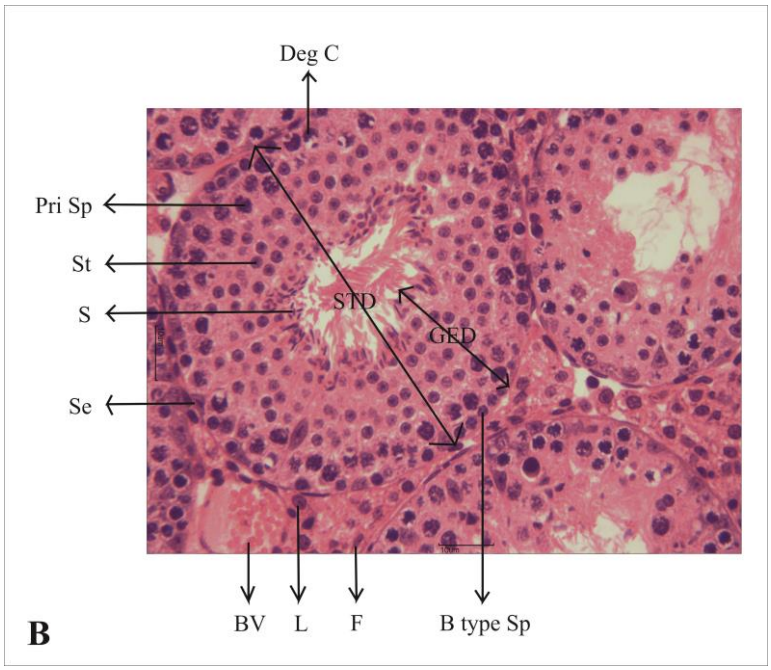



**Figure2**. Light microscopy of cross sections from mice testis. (H&E stained Magnification × 400). Histological studies on testicular tissue of control group mice (A) showed normal formation of testis tubules and testicular tissue cells at different stages of spermatogenesis. In thisfigure, testicular tissue histopathological parameters of control group mice were indicated by abbreviated signs as follows; sertoli (Se), A type spermatogonia (A type Sp), B type spermatogonia (B type Sp), primary spermatocyte (PriSp), spermatid (St), leydig cell (L), fibroblast cell (F), blood vesicles (BV), seminiferous tubule diameter (STD), germinal epithelium diameter (GED).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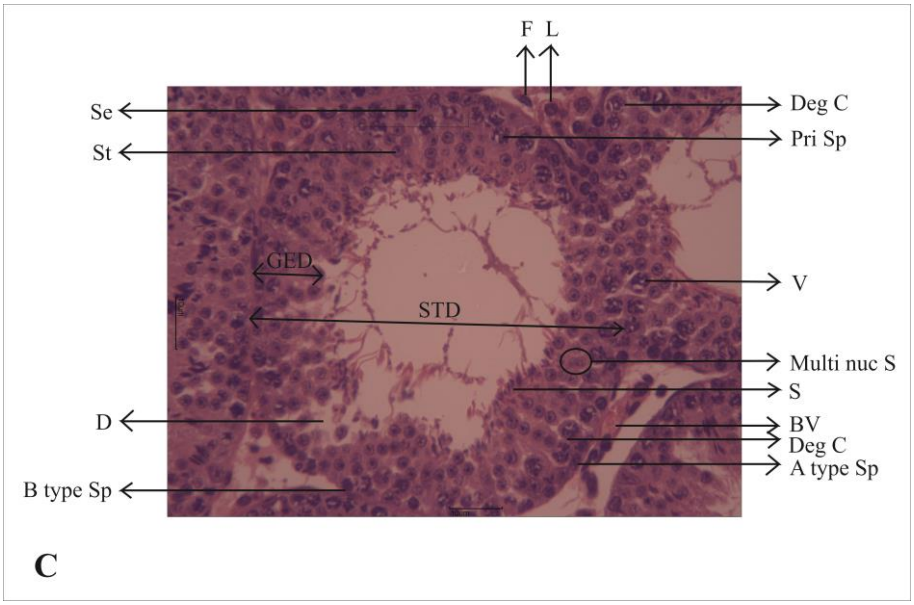
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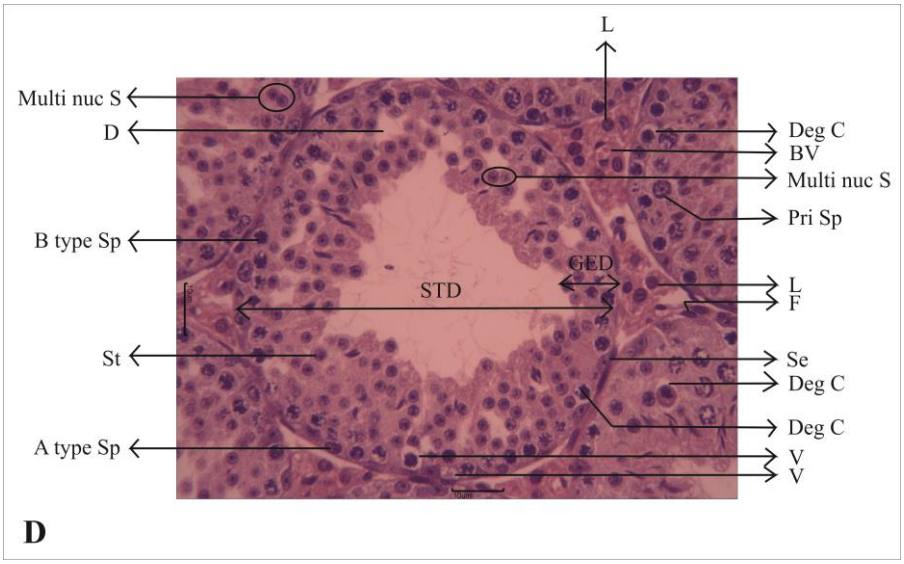


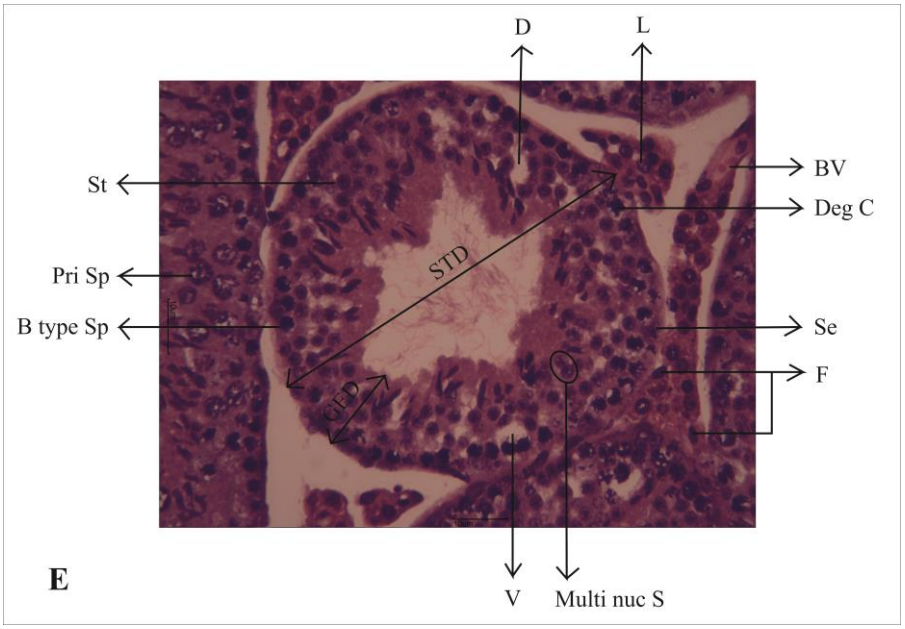
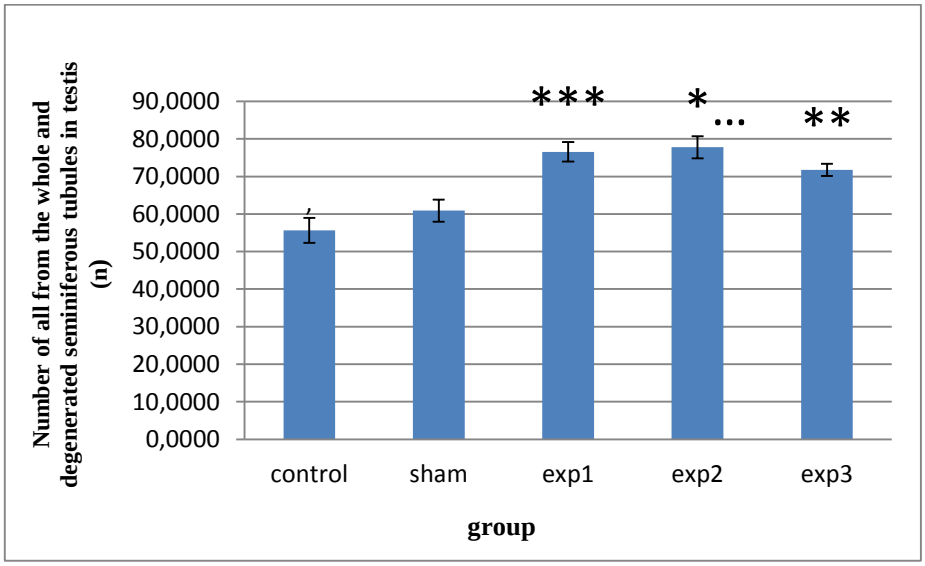



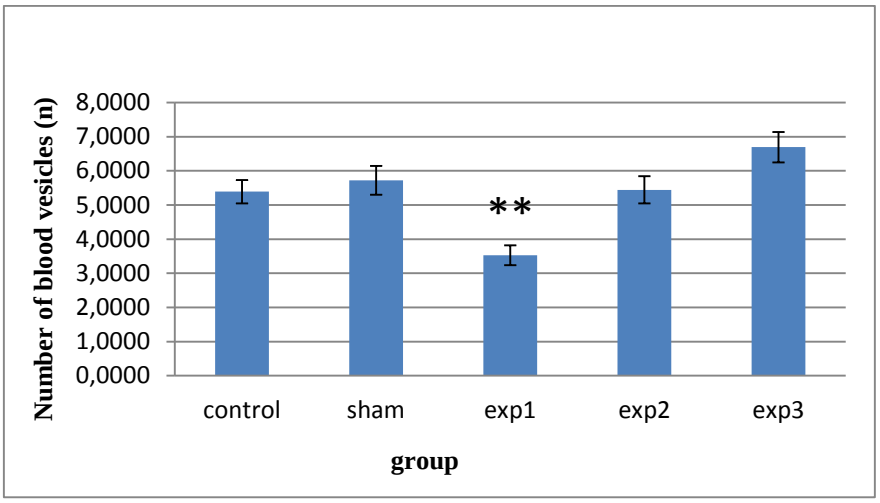

**Figure**3. Light microscopy of cross sections from mice testis tissue in sham group (B)and ZnOn treated groups in 250,500 and 700 mg/kg/day doses; C, D and E figures respectively(H&E stained, Magnification × 400). The histopathological changes of ZnOn effectin250 and 700 mg/kg/day doses of ZnOn(C and E groups) are observed as vacuolization from cells (V) and detachment of germinal epithelium (D) in tubules. This changes were markedly increased in testis tissue tubules of ZnOn treated mice in 500 mg/kg/day dose (D group).Also in the present study, testes tissue histopathological parameters of sham group and ZnOn treated groups in B - E figures were indicated by abbreviated signs as follows; sertoli (Se), A type spermatogonia (A type Sp), B type spermatogonia (B type Sp), primary spermatocyte (PriSp), spermatid (St), sperm (S), multinuclear spermatid (Multi nuc S), degenerated cell (Deg C), leydig cell (L), fibroblast cell (F), blood vesicles (BV), seminiferous tubule diameter (STD), germinal epithelium diameter (GED).

***The histopathological effect of ZnOn on the outside and inside cells of the seminiferous tubules of testicular tissue in treated mice***

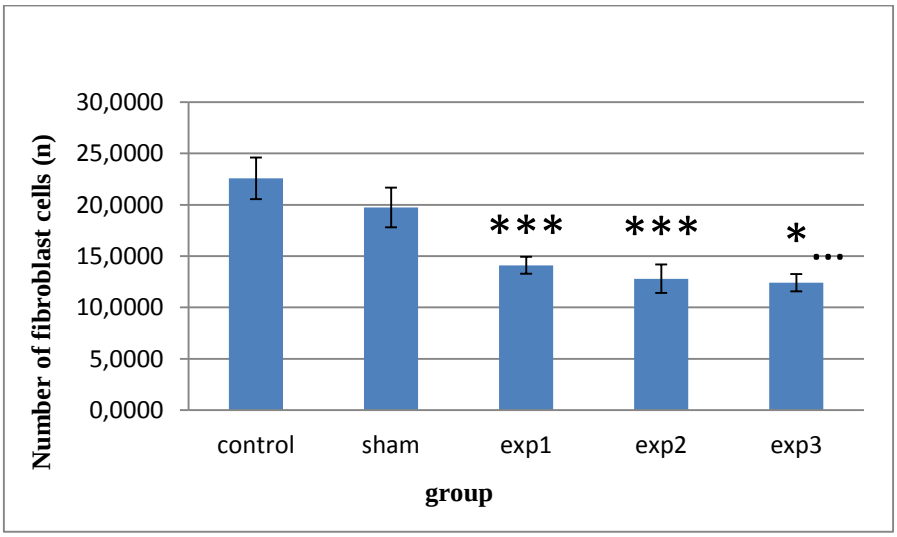
Comparison of figures (B -E) and graphs (1-10) showed that reproductive cells such as A type spermatogonia in mice treated with ZnOn (500 and 700 mg/kg/day doses) were significantly decreased compared to the control group ($P<0.01$ and $P<0.05$ respectively; Graph7, Figure 4.). The number of Primary spermatocyte cells in the ZnOn administrated group (500 mg/kg/day dose) was significantly decreased compared to the control group ($P<0.05$; Graph 8, Figure 4.).A significant reduction in fibroblast cells ($P<0.001$; Graph3, Figure 4.) and a significant increase in the number of degenerated cells ($P<0.001$; 9Graph, Figure 4.) and the number of multinuclear spermatid cells ($P<0.01$; Graph10, Figure 4.) was observed in the ZnOn treated groups (250, 500 and 700 mg/kg/day doses) compared with the control group ($P<0.01$ and $P<0.001$; 3, 9 and 10Graphsrespectively, Figure 4.). Furthermore, our results indicated that blood vesicles and leydig cell numbers were significantly decreased in the group which received the ZnOn in the 250 mg/kg/day dose compared to the control group ($P<0.01$; 2 and 4 Graphs respectively, Figure 4.).While, there were no significant changes in the number of sertoli, spermatid, sperm and B type spermatogonia cells in the ZnOn treated groups (250, 500 and 700 mg/kg/day doses) compared to the control group.



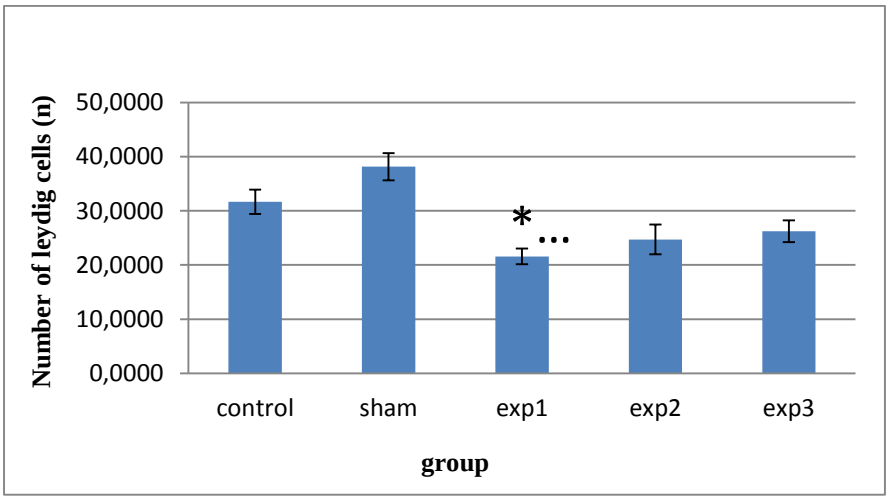

Graph1.Histogram shows number of all from the Whole and Degenerated Seminiferous Tubules in Testis of mice in control, sham and experimental groups.



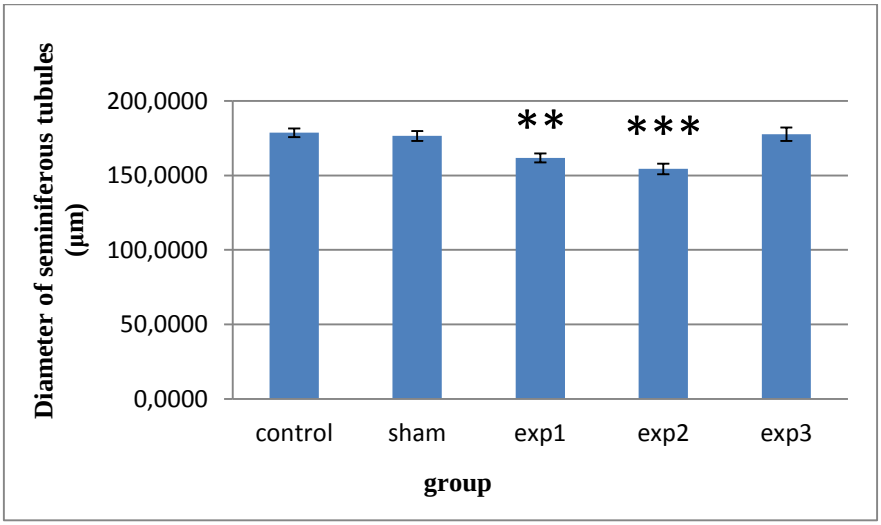

Graph2.Histogram shows number of Blood Vesicles of mice in control, sham and experimental groups.



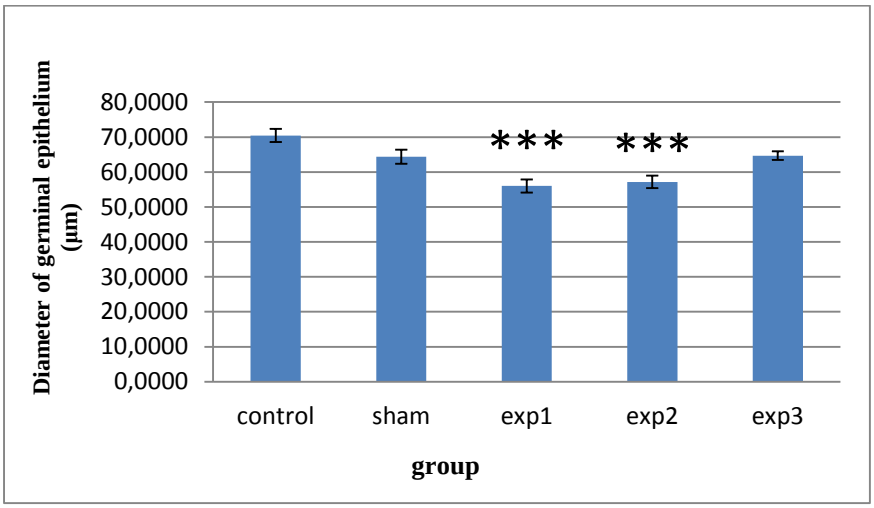

Graph3.Histogram shows number of Fibroblast Cells of mice in control, sham and experimental groups.



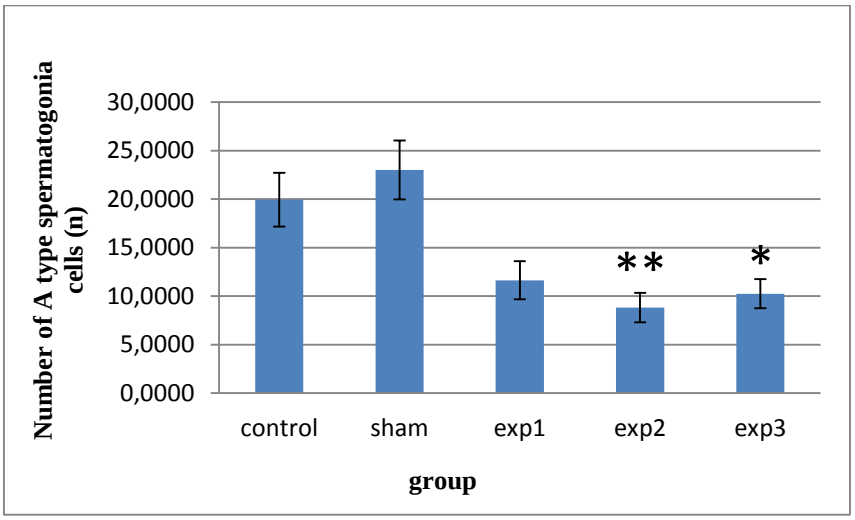

Graph4. Histogram shows number of Leydig Cells of mice in control, sham and experimental groups.



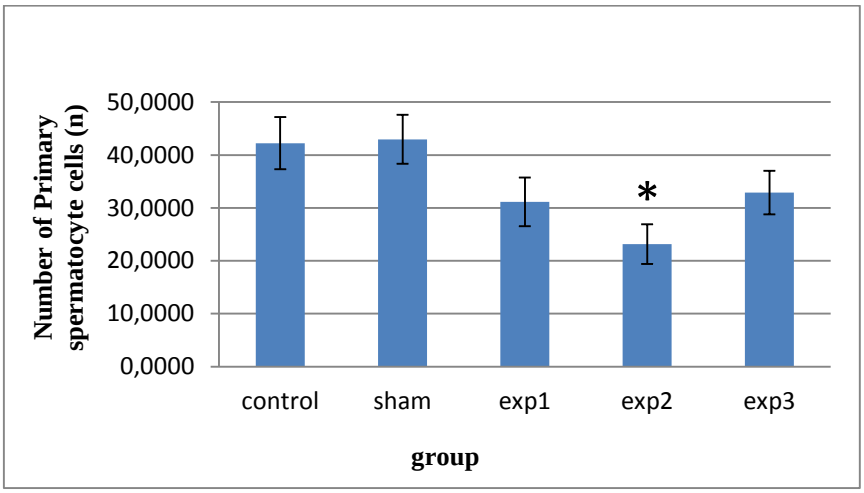

Graph5.Histogram shows Diameter of Seminiferous Tubules of mice in control, sham and experimental groups.



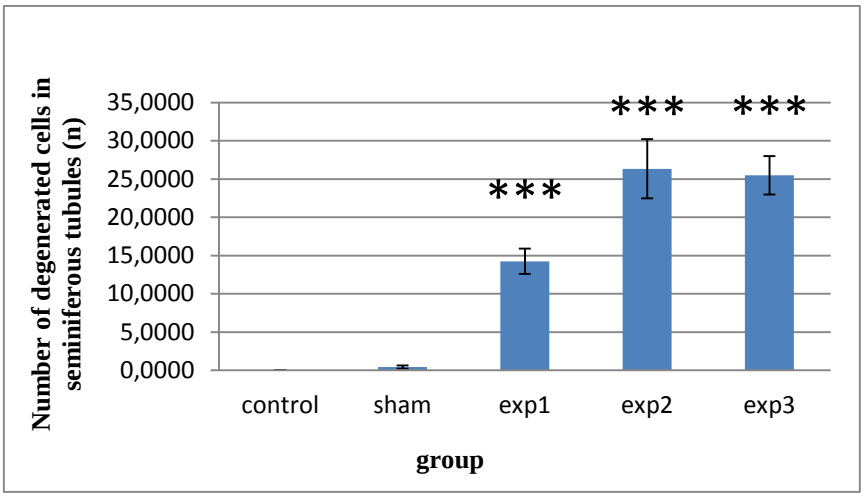

Graph6.Histogram shows Diameter of Germinal Epithelium of mice in control, sham and experimental groups.



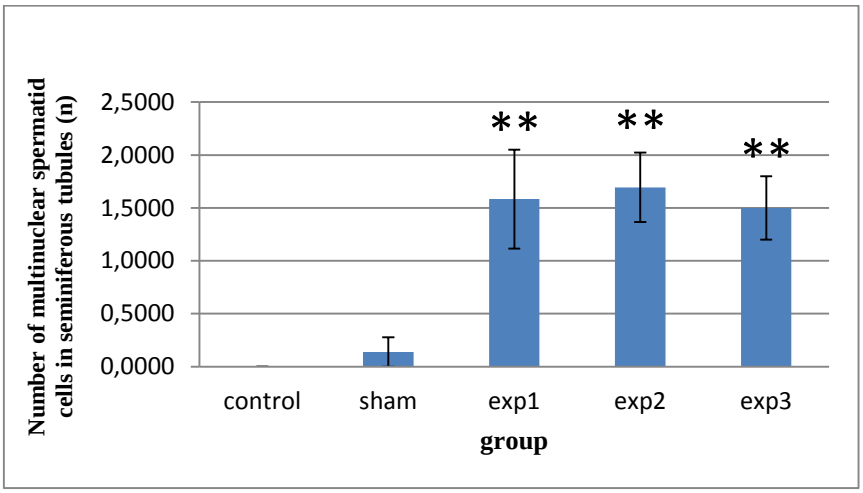

Graph7.Histogram shows number of A Type Spermatogonia Cells of mice in control, sham and experimental groups.




Graph8.Histogram shows number of Primary Spermatocyte Cells of mice in control, sham and experimental groups.




Graph9. Histogram shows number of Degenerated Cells in Seminiferous Tubules of mice in control, sham and experimental groups.




Graph10.Histogram shows number of Multinuclear Spermatid Cells in Seminiferous Tubules of mice in control, sham and experimental groups. Note: Testis histopathology assessments forcontrol, sham and experimental groups are indicated as 1-10 Graphs. Statistical analysis is also done in this study. Asterisks (*, ** and ***) indicate the level of significant differences for P<0.05, P<0.01 and P<0.001, respectively for test groups compared to the control group, exp1: 250 mg/kg/day dose, exp2:500 mg/kg/day dose, exp3: 700 mg/kg/day dose. The differences were considered significant at level P<0.05.

## Discussion

In current study, mice treated with zinc oxide nanoparticles (ZnOn) exhibited significant histopathological changes regarding to different histological parameters. It is possible that ZnOn may affect leydig cells and inhibit testosterone synthesis. Our results demonstrated that the leydig cell numbers were significantly decreased in ZnOn (250 mg/kg/day dose) treated group in comparison with the control group (p<0.01;4 Graph, Figure 4.). This was consistent with previous studies that showed ZnOn reduced the numbers of leydig cells in experimental groups that received ZnO NPs [12]. This was also supported by previous findings that have indicated the various effects of ZnOn and other nanoparticles (NPs) on the male reproductive system. It has also been shown that NPs have the capacity to penetrate the blood-testis barrier and induce toxic effects on male germ cells [13-16]. Even small amounts of silver NPs are also capable of decreasing sperm quality and inducing toxic impact on germ cells [17].There is evidence indicating that some NPs such as samar-ium/europium and gadolinium/terbium contain serious autophagy effects in human liver cells [18]. Also ZnOn is capable of inducing autophagy in normal skin cells [19].There is a study that indicated zinc oxide nanoparticles induce testicular damage and cytotoxic effects on testicular germ cells in a dose dependent manner in mice. Zinc oxide NPs might affect sertoli cell function as that causes formation of multinucleated giant cells and sloughing of immature germ cells from the seminiferous tubules and vacuolization of sertoli cells in mice [12]. There is evidence that has demonstrated direct damage to sertoli cells, (included, the presence of vacuoles in their cytoplasm) which is considered as the early morpho-

logical sign of testicular injury and is the main response of sertoli cells to many xenobiotic [20-22].NPs have many applications in the fields of biology and medicine due to their unique chemical and physical properties such as high surface area to volume ratio, shape and size [23]. Therefore, investigation of their toxic effects seems reasonable. ZnO nanoparticles have widespread applications such as use as a biosensor, pigments and food additives and prevention of sunburn and production of resin and electronic materials [11]. Thus, because of the wide use of ZnOn and also due to exposure of animals and human to this material, we in present study aimed to investigate the effects of exposure to ZnOn at different doses (250, 500, and 700 mg/kg/day) on testis tissue of adult mice. Statistical analysis of our results indicated that there are significantly reduction in the number of A type spermatogonia cells in mice treated with ZnOn(500 and 700 mg/kg/day doses) and primary spermatocyte cells in the ZnOn administrated group (500 mg/kg/day dose) compared to the control group($p<0.05$).Also the number of fibroblast cells were significantly decreased in ZnOn treated groups(250, 500 and 700 mg/kg/day doses) compared with the control group($P<0.001$).Nanoparticles have adisruptive effect on mitochondria. They also, by increasing the release of super oxidase molecules and protein oxidation, can lead to cell death and finally decreased testosterone-producing cells [24].There are evidences indicating that $TiO_2$nanoparticles (by hypodermically injection) can accumulate in spermatid, sertoli and leydig cells of rat testis [25]and also it was observed that intraperitoneal injection of $TiO_2$NPs to male rats causes an increase of sperm abnormalities, reduction in sperm count, apoptosis of germ cells and finally destruction of male rats reproductive system [26]. Nanoparticles can have adverse effects on the male reproductive organ. There is also evidence indicating that the disadvantages of other nanoparticles on male reproductive system function are particularly due to alteration of the testicular structure and damage of spermatogenesis. Therefore, exposures to different types of nanoparticles (NPs) such as carbon-based NPs, nanoparticle-rich diesel exhaust NPs and metal-based NPs (for example; $TiO_2$ NPs) cause changes in testicular tissue in laboratory animals. As carbon-based NPs (carbon black- NPs) induced vacuolization of sertoli cells and seminiferous tubules, decrease cellular adhesion of seminiferous epithelia, reduce thickness and diameter of the germinal layer and nanoparticle-rich diesel exhaust, induce desquamation of the seminiferous epithelium and loss of spermatozoa count, while metal-based NPs cause a reduction of the number of sertoli cells and disturbance of seminiferous tubules[27].Our results showed that ZnO nanoparticle in 250 and 500 mg/kg/day doses were capable of inducing damage to mouse seminiferous epithelium and reducing tubules diameter and altering spermatogenesis. These changes were serious significant at doses of 250 and 500 mg/kg/day of ZnOn when compared to the control group ($p<0.001$), yet these changes were not significant at higher doses (700mg/kg/day). In this study, we observed signs of lesions in histology examinations of testis tissue and altering spermatogenesis in mice that were treated with ZnO nanoparticle. These alterations were observed as reduction of spermatogonia (A and B types), primary spermatocyte and sperm cells. Spermatogenic arrest together with other symptoms were also observed, which included; multinuclear spermatids; the presence of giant

cells corresponding to spermatids; the appearance of immature germinal cells in the epididymis; sever disorganization of epithelium and seminiferous tubules; seminiferous epithelium vacuolation and the presence of many degenerated cells in seminiferous tubules. Also, in the current study, we observed degenerated spermatocytes and exfoliated to the lumen of seminiferous tubules, loss of sperm cells, and the presence of degenerated sperm cells and exfoliated to epididymis tubules in ZnO nanoparticle treated mice. Therefore, according to the findings obtained from this study, we suggest that ZnO nanoparticles have destructive effects on the male reproductive system in treated mice by this nanoparticle in exhibited doses. According to previous studies, it was confirmed that ZnO nanoparticles have toxicity effects on the some organs [28-30].The mechanism by which ZnO nanoparticle exerts its effects needs more investigations. Thus, further studies are required to identify the exact and precise underlying mechanisms.

## Conclusion

In summary, histopathological observations found in our study indicated that ZnO nanoparticles have cytotoxic actions on male reproductive system and induce damage to testis tissue. The ZnO nanoparticles are capable of degeneration and decrease cell types in the seminiferous tubules (such as spermatogonia, primary spermatocyte, spermatid and sperm cells), outer part of tubules (such as leydig, fibroblast cells and blood vesicles), seminiferous epithelium and tubule diameters in adult mice. Nowadays, due to the development of nanotechnology and wide uses of these nanoparticles in industries, medicine and biology, there is wide exposure of this material to humans and the environment. Therefore, this study can be an exhortation for worker's health, consumers and public health, because of the toxic effects of nanoparticles.

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