Original Article

Effects of Iron Oxide Nanoparticles on Mouse Sperm Parameters and Testicular Tissue

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ABSTRACT

Background: Iron oxide nanoparticles are commonly used for various purposes, such as biomedical, medical, and cosmetic services and research. However, there is a little information about the effects of the nanoparticles on human health. The current investigation was conducted to evaluate the adverse effects of iron oxide nanoparticles (FeNP) on the reproductive organs of mice, such as the testicular tissue and sperm cells.

Methods: Twenty-eight male NMRI mice were randomly divided in four groups (N=7). The control group received only a regular diet. The experimental groups were administered FeNP in doses of 50, 150 and 300 mg/Kg intraperitoneally (IP), over four days. Epididymal sperm parameters, such as sperm number and motility were assessed by computer-assisted sperm analysis (CASA). Stereological analysis was also conducted on the histological sections.

Results: The results demonstrated that FeNP (300 mg/Kg/day) caused a significant decrease in the sperm parameters, such as motility, spermatogonia, primary spermatocytes, spermatid, Sertoli, Leydig cells, total length of seminiferous tubules, and testicular interstitial tissue volumes.

Conclusion: In summary, FeNP affected several reproductive tissue and cellular parameters at the administered dosage. Further research is required to examine the mechanism of action of FeNP the mice reproductive system.

Keywords: Iron Nano-Particles, Mice, Sperm Parameters, Testicular Tissue.

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INTRODUCTION

The spermatogenesis is the production and release of spermatozoa from the primordial germ cells in the testis. This process may be affected by such environmental factors as nanoparticles [1]. It is known that humans are in contact with nanoparticles both directly and indirectly throughout life [2]. Among the compounds used in medical research, the adverse effects of iron oxide nanoparticles (FeNP) are have caused concern in medicine because of the superparamagnetic features. These nanoparticles are used in magnetic storage, catalysis, electronic sensors, and high-sensitivity biomolecular magnetic resonance imaging (MRI) in medical diagnosis and therapeutics [3,4]. One reason that makes nanoparticles highly reactive is their surface to mass ratio which is greater than that for other substances [5]. Nanotoxicology investigations help us ascertain the extent to which they may be harmful to human health and the ecosystems [6]. Nanoparticles can enter the human body through three routes: respiratory, digestive and tactile [7,8]. They can intensify the influence of carbon nanoparticles, causing high reactivity, including increased generation of reactive oxygen species [ROS] and oxidative stress on cells, resulting in inflammatory damage to proteins, cell membranes and DNA [9]. Most nanoparticles may exert harmful effect on spermatogenesis; however, not all of them are toxic [10]. The mechanism by which nanoparticles impair spermatogenesis and the blood supply to testis is unclear [11,12]. Previous studies have reported the passage of nanoparticles through the sperm plasma membrane of mice and the detrimental effects on the reproductive function. Few in vitro studies examined the effect of nanoparticles on the spermatogenesis and reproductive system in mice [13-15].

The effect of nanoparticles on the spermatogenesis is influenced by such factors as dosage, administration routes, size of nanoparticles, and type of animal [12]. Recent animal research has demonstrated that nanoparticles can depositin various organs throughout the body, and move from the initial absorption site, e.g., the lungs and skin to secondary organs such as ovaries and testes [16,17]. The aim of this study was to examine the adverse effect of FeNP on the testicular tissue and sperms of adult male mice.

MATERIALS AND METHODS

Experimental Animals and $FeNP_{203}$

In this study, twenty-eight 6-8 week-old male NMRI mice, weighing 28-32g were purchased from Pastor Institute, Tehran, Iran. The FeNP (Fe_2O_3) with a diameter of 20-30 nm were purchased from Nano Pars Lima Company (Tehran, Iran).

Study Design

The Ethics Committee of *Shahid Beheshti University of Medical Sciences, Tehran, Iran,* approved the study design and animal use (IR. SBMU. SM. REC.1394.31). The mice were randomly divided into 4 groups consisting of seven mice per group, one controls and 3 experimentals. The experimental micewere administered with a dose of 50, 150 or 300 mg/Kg FeNP intraperitoneally repeated over four days. The mice were housed at standard temperature (22°C), humidity (45±5%) and light (12hr light/dark cycle) conditions.

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Epididymal Sperm Preparation and Analysis

One week after the last FeNP injection, the mice were anesthetized by ketamine/xylazine, then the testes and epididymides were surgically removed. Immediately after dissection, caudal epididymides were minced in 5mL PBS and incubated at 37ºC for 30 minutes to allow sperms to leave the epididymal tubules. We determined the sperm concentration (sperm/mL), number of sperms with progressive motility and sperm motility parameters as follow: curvilinear velocity (V_{CL}), straight line velocity (V_{SL}) and average path velocity (V_{AP}), which were analyzed using a computer assisted program (CASA; HTM-IVOS, Version 10.8 (Hamilton Thorne Research, Beverly, MA, USA).

Tissue Preparation

For the histological examination, small pieces of testicular tissue were fixed overnight in 10% buffered neutral formalin, dehydrated and processed in paraffin wax then sectioned into 5µm sections, stained with hematoxylin and eosin, and examined by light microscopy.

Estimation of the Testis and Interstitial Tissue Volume

The total volumes of the testis and interstitial tissue were estimated stereologically by the Cavalieri method [14,15] (Fig. 1). Eight to 10 sections were selected, using a systematic uniform random sampling for stereological estimations. The image of each section was evaluated using a video-microscopy system, consisting of a light microscope (Nikon, E-200, Japan) linked to a video camera. The testis volume was estimated by the following formula: $V\ (testis) = \Sigma P \times a/p \times t$, where "ΣP" was the total points hitting the testis sections, "a/p" was the area associated with each point, and "t" was the distance among the sampled sections.

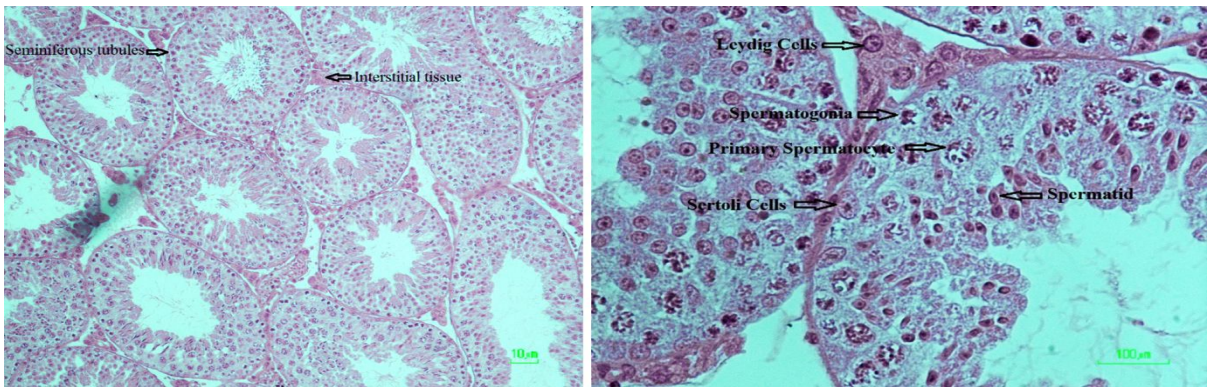

Figure. 1. Seminiferous tubules and Interstitial tissue (100x); Spermatogonia, spermatocyte, spermatid, sertoli cells and leydig cells (400x) (arrowheads).

Estimating the Length of Seminiferous Tubules

Six random fields from the sections of seminiferous tubule tissues were selected and the number of the selected tubule profiles was counted using an unbiased counting frame, i.e., an average of 130–150 seminiferous tubules per testis was estimated (Fig. 1). The length density of seminiferous tubules was also estimated using the following formula $L_v = 2 \times \Sigma Q / a/f \times \Sigma P$ [14,15], where ΣQ = the total number of the seminiferous tubules, a/f = the area per frame and ΣP = the total points superimposed on the testis tissue. The total length of the seminiferous tubules was estimated by multiplying the lengths density (Lv) by the total volume of each testis.

Estimating the Number of Spermatogonia, Spermatocyte, Spermatid, Sertoli and Leydig Cells

We determined the number of spermatogonia, spermatocyte, spermatid, Sertoli and Leydig cells, using the optical dissector method and an unbiased counting frame (Fig. 1). The position of the microscopic fields was selected by systematic uniform random sampling, with moving the stage in equal distances in X and Y axes. The focal plane was moved downwards in Z axis. Then, a microcator (a mechanical comparator) was attached to the microscope stage (Heidenhain, Germany) to measure the z-axis traveling (depth).The number density (Nv) of different type of cells was estimated, using the following equation [18,19]:

$Nv\ (cells) = [\Sigma Q- / (h \times a/f \times \Sigma p)] \times (t / BA)$ **,** Where ΣQ = total number of counted cells, a/f = area per frame, ΣP = total number of the counting frames in all optic fields, h = height of the dissector, t = real section thickness, and BA = section thickness made by microtome. The equation product was then multiplied by the total volume of the testis to obtain the total number of cells: N (total) = NVv ×V (final).

Statistical Analysis

The statistical analysis was performed using SPSS version 22 for Windows (Chicago, IL, USA). All parameters were analyzed by one way ANOVA and the obtained data were expressed as Mean ± standard error (±SE). For the parameters with significant differences among groups, multiple comparison tests were carried out at a confidence level of $p<0.05$.

RESULTS

Sperm Motility Analysis

After for four days of tissue exposure to FeNP (300 mg/Kg/day), our computer-assisted sperm analysis (CASA) showed that the values for curvilinear velocity (V_{CL}), straight line velocity (V_{SL}) and average path

velocity (V_{AP}), progressive and immotile sperm decreased significantly (p=0.04, p=0.00, p=0.02) compared with those for the controls (Figures 2A & 2B). The percentage of sperms with rapid progressive motility was significantly (p=0.02) decreased for the highest dose (300 mg/Kg/day) of FeNP in comparison to that for the controls. Also, the percentage of immotile sperms was significantly (p=0.00) increased for the highest dose (300 mg/Kg/day) group compared to that for the control. The sperm motility parameters at doses of 150 mg/Kg/day and 50 mg/Kg/day FeNP, although decreased, were not significantly different from those observed for the controls.

Sperm Concentration

We observed a decline in sperm concentration for all experimental groups, however, the differences were not statistically significant (Fig. 2C).

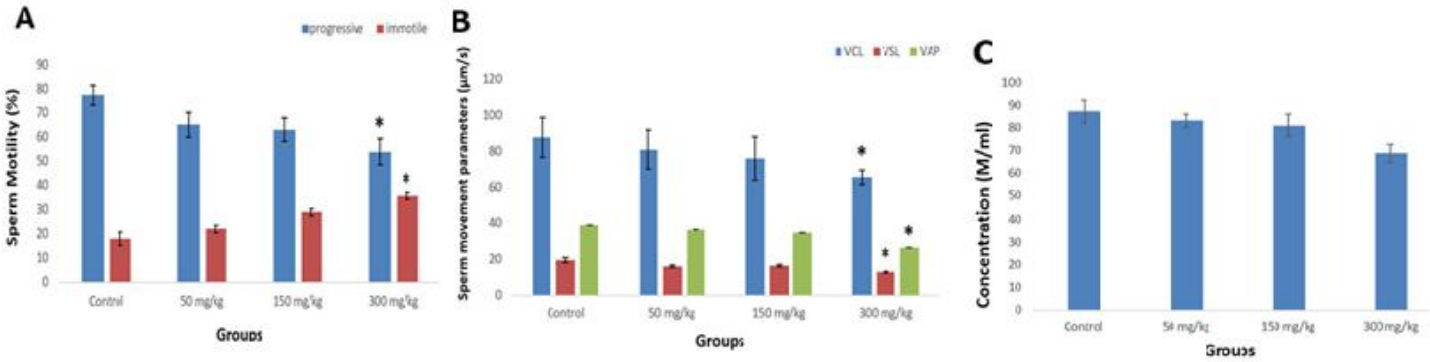

Figure 2. *(A)* The effect of Fe2O3 on Sperm progressive motility (µm/s)*; (B) Sperm movement parameters (µm/s);* and *(C)* Sperm Concentration in control and experimental groups. * Significant difference *(p=0.00 for immotile, p=0.02 for progressive,* p= 0.04 for VCL, p= 0.00 for VSL, p= 0.02 for VAP) compared with control group.

Stereological Assessment of the Spermatogonia Cells

The results showed that the number of spermatogonia cells was significantly reduced in the experimental mice treated with FeNP (300 mg/Kg/day) for four days in comparison to those documented for the control group (P=0.03). The groups that received 150 mg/Kg/day and 50 mg/Kg/day FeNP showed no significant difference in the above parameter compared to those for the controls (Fig. 3A).

Number of Primary Spermatocytes

There was a significant reduction in total number of the spermatocytes in mice treated with 300 mg/Kg/day of FeNP compared to that observed for the control group (P=0.04). However, no significant differences were detected for the other experimental groups compared to that of the controls (Fig. 3B).

Number of the Spermatids

There was a significant reduction in the total number of the spermatids in the group receiving the highest dose of FENP (300 mg/Kg/day) for four days compared to those noted for the control group (P=0.04). However, no significant reduction in spermatid cells was recorded in groups receiving 150 and 5o mg/Kg/day FeNP compared to that in the control group (Fig. 3C).

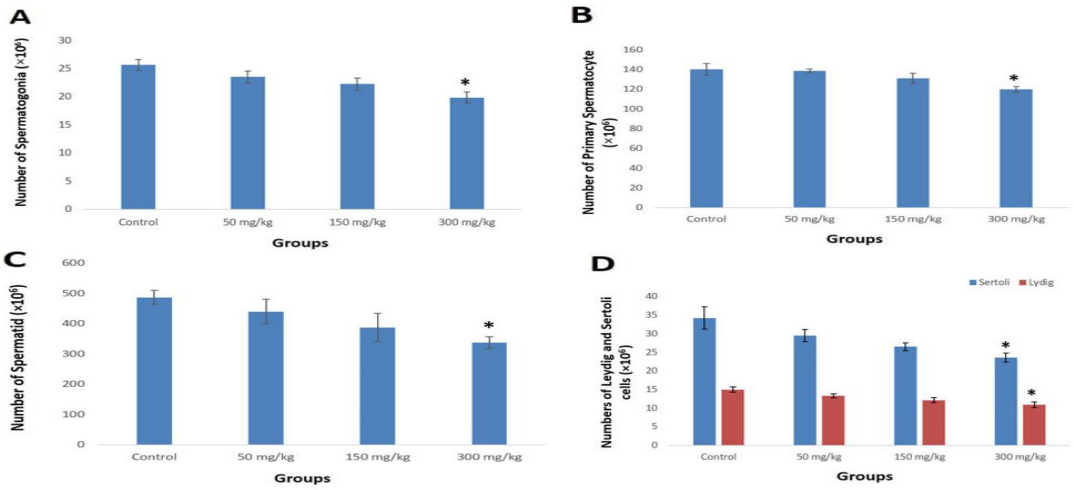

Figure 3. ***(A)*** The effect of Fe2O3 on the number of spermatogonia cells*; (B)* the number of Primary Spermatocyte Cells*; (C)* the number of Spermatid Cells*; and (D)* the number of Sertoli Cells and Leydig Cells in control and experimental groups. **Significant difference (p=0.03 for* spermatogonia*, p=0.04 for* Primary Spermatocyte, *p=0.04 for* Spermatid, *p=0.00 for* Sertoli Cells, *p=0.00 for* Leydig Cells*) compared with control group.*

Number of the Sertoli and Leydig Cells

The total number of sertoli cells was significantly reduced in the mice treated with 300 mg/Kg/day FeNP compared to that for the control group (P=0.00). While the other two experiemental showed no significant differences in the total cell numbers compared to that noted for the control group. Similarly, the results demonstrated that the total number of leydig cells was significantly lower in the mice treated with 300 mg/Kg/day FeNPcompared to that found for the control group (P=0.00). The other two experiemental groups (150 & 50 mg/Kg/day FeNP) showed no significant differences in the number of Leydig cells compared to that for the control group (Fig. 3D).

Length of Seminiferous Tubules

The total length of seminiferous tubules was significantly reduced in the mice treated with 300 mg/Kg/day FeNP compared to that noted for the control group (P=0.00). Likewise, no significant differences were noted for the other two experiemental (150 & 50 mg/Kg/day FeNP) compared to that seen in the control group (Fig. 4A).

Volume of Testis

The total volume of testis (VT) was significantly lower in the mice treated with the highest FeNP dose (300 mg/Kg/day) compared with that observed for the control groups (P=0.00). Similarly, the other two experimental groups (150 & 50 mg/Kg/day FeNP) showed no significant differences for this parameter compared to that for the control group (Fig. 4B).

Volume of Interstitial Tissue

There was a significant reduction in the total volume of interstitial tissue (ViT) of mice treated with 300 mg/Kg/day FeNP, compared to that for the the control groups (P=0.00). The other two experimental groups (150 & 50 mg/Kg/day FeNP) showed no significant differences for this parameter compared to that recorded for the control group (Fig. 4C).

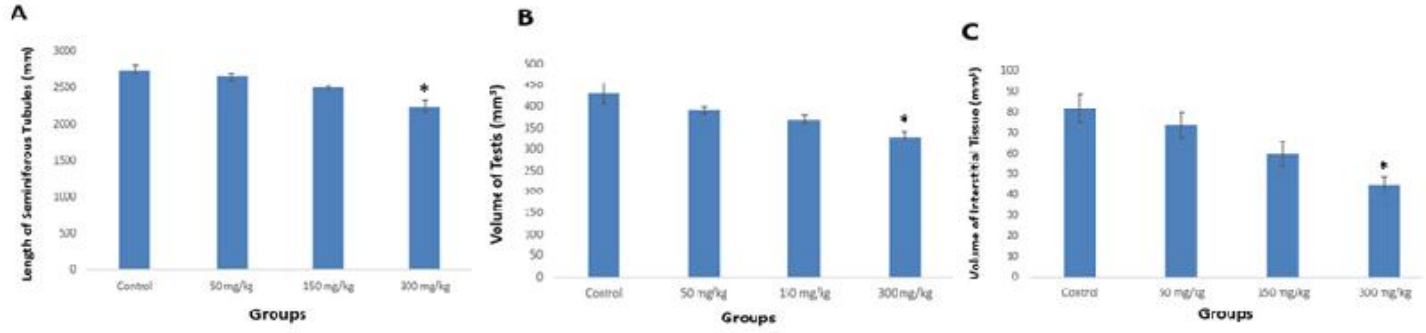

Figure 4. *(A)* The effect of Fe_2O_3 on *length of Seminiferous Tubules (mm); (B) Volume of Testis (mm3); and (C)* Volume of Interstitial Tissue (mm3) in control and experimental groups. ** Significant difference (p= 0.00 for length of Seminiferous Tubules, p= 0.00 for Volume of Testis, p= 0.00 for* Volume of Interstitial Tissue*) compared with control group.*

DISCUSSION

This study investigated that the effects of FeNP induced impairment of sperm and testicular tissue in male mice, using modern stereological methods, rendering unbiased and accurate laboratory estimations. The results demonstrated that significant reductions in the sperm motility (V_{CL}, V_{SL} & V_{AP}), the total testis volume, seminiferous tubules length, interstitial tissue volume, and the total number of leydig, sertoli, spermatocyte, spermatid and spermatogonial cells of the mice treated with 300 mg/Kg FeNP intraperitoneally compared to those noted for the control group.

Nanoparticles have been used for a variety of objectives recently and their harmful effects have been reported by previous studies [16,20]. Nanoparticles, such as FeNP have shown to induce morphological changes in testicular tissues and reproductive function [21,22]. Some studies have found that they have the ability to generate reactive oxygen species (ROS) which cause oxidative stress, cellular and DNA damage [9, 23-25].

Other investigations have reported that as sperm mobility occurs in epididymis, inflammation of epididymis that is induced by nanoparticles exposure, could diminish sperm motility [1,26-29]. Reportedly, sperm motility is probably reduced due to the impact of silver nano-particles on mitochondrial functions [30]. Nasri *et al*. have also found that iron oxide nanoparticles decreased sperm mobility in male mice [31]. Further, significantly lowered sperm motility in male mice was also shown by Guo *et al*. after treating them with a high-dose (500 mg/Kg) of titanium dioxide nanoparticles (TiO_2) [32]. According to findings of Braydich-Stolle *et al*. silver and aluminum nanoparticles can pass through the cell membrane and affect mitochondrial functions [33,34]. Taken together, changes in sperm motility as seen in this study is likely to be related to the mitochondrial dysfunction as confirmed by a previous study [35]. The findings of these studies were consistent with our results regarding the adverse effect of FeNP on sperm motility. Our study showed significant histological changes occurring at dosage of 300 mg/Kg

of FeNP and to an insignificant degree in the other experimental groups (150 & 50 mg/Kg/day FeNP). The later finding has not been reported by previous studies.

Cell viability may be impaired by the disruptive effects of nanoparticles on the membrane receptors or cellular signal transduction mechanisms [36]. Braydich-Stolle *et al*. reported that cadmium nanoparticles could lead to lysosomal damages in Sertoli cells [34]. According to a previous study, zinc oxide nanoparticles caused testicular tissue damages in mice including detachment, sloughing and vacuolization of the seminiferous tubules. In this study, sertoli cells have contained vacuoles, indicative of damage resulting from exposure to FeNP [14,37]. Another study has reported that diesel-exhaust particles (DEPs) TiO_2 and carbon black (CB) nanoparticles were taken up by Leydig cells, impairing their viability and proliferation. According two studies, the viability of leydig cells were affected by iron oxide nanoparticles [32,38]. Also, silver nanoparticles have reduced the number of Leydig cells in male Wistar rats [30]. Miresmaeili *et al*. examined the effect of silver nanoparticles (AgNP) on rat spermatogenic cells but did not detect any significant reduction in the number of Sertoli cells [39]. In contrast, Ema *et al*. showed that TiO_2 nanoparticles reduced the number of Sertoli cells and disrupted the seminiferous tubules. Our previous findings demonstrated that a single intraperitoneal injection of 500 or 700 mg/Kg/day zinc oxide nanoparticle (ZnOn), the number of A type spermatogonia cells was significantly decreased [40]. Further, a significant reduction in primary spermatocyte cells was observed following exposure to ZnOn at a dosage of 500 mg/Kg/day in male mice. Sensitivity of the mammalian spermatogonial stem cells to Ag-NP has been reported by Braydich-Stolle *et al*. [41]. Some NPs are able to penetrate the blood–testis barrier and impair the spermatogenic process [12]. Previous studies have indicated that nanoparticles of gold and iron oxide (Fe_2O_3 and Fe_3O_4) could exert oxidative stress, ROS production and mitochondrial damage, which lead to the accumulation of autophagosomes and cellular autophagy [42]. We believe that these mechanisms are likely to be involved in FeNP toxicity. It has been suggested that elevated release of Fe^{2+} ions in high concentrations may enter the cells and damage intracellular structures. Consistent with our findings, Collodel *et al*. examined the damaging effect of gold and silver nanoparticles on the rat spermatocytes and spermatids [36]. The stereological findings of our study were similar to another research conducted by Ajdary *et al*. In that study Wistar adult male rats that were given Mn_2O_3 nanoparticles orally at 400 ppm for 14 days, displayed cellular damages in the testicular tissue and a reduction in the number of spermatogonial, primary spermatocyte, spermatid, and the Leydig cells [43].

CONCLUSION

The present study demonstrated that the adverse effects caused by FeNP at high dosage (300 mg/Kg/day) on sperm motility and such histological parameters as total volume of testis, total lenght of seminiferous tubules, total volume of interstitial tissue and the total number of Leydig, Sertoli, spermatocyte, spermatid and spermatogonial cells could compromise fertility capacity. Thus, the toxicity effects of these nanoparticles on human health must be given high attention, especially because of the development of nanotechnology in various industries and nations. Further investigations are required to clarify the exact mechanisms of nanoparticles effects on animal and human reproductive system.

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CONFLICT OF INTERESTS

The authors declare that there was no conflict of interest in conducting this study.

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