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Effects of oral administration of titanium dioxide particles on sperm parameters and *in vitro* fertilization potential in mice: A comparison between nano- and fine-sized particles

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Abstract

Titanium dioxide particles (TiO_2) as the second most widely used materials in consumer products are composed of nano-sized (<100 nm) particles (NPs) and fine-sized (>100 nm) particles (FPs). Toxicological studies on animals have shown that TiO_2 NPs exposure can cross the blood-testis barrier and accumulate in the testis resulting in testicular tissue damage and reduction of sperm count and motility. However, there is no information on the toxic effects of TiO_2 FPs on male reproductive fertility. Twenty-four adult male mice were randomly divided into three groups including control, TiO_2 NPs, and TiO_2 FPs (150 mg kg^{-1} per day). After intragastric administration for 35 days, testicular tissue alterations (seminiferous tubule diameter and germinal epithelial height), sperm parameters (count, motility, viability, morphology, and DNA quality), *in vitro* fertilization potential, oxidative stress assays such as malondialdehyde (MDA) content, level of glutathione (GSH) and activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in testicular tissue were investigated. The results showed that both sizes of TiO_2 caused pathologic changes in the testis and significantly increased MDA level and decreased GSH levels and activities of SOD and GPx in testicular tissue. Moreover, the administration of both sizes of TiO_2 significantly decreased all of the sperm parameters and *in vitro* fertility (fertilization rate and pre-implantation embryos development) compared to control. Administration of TiO_2 FPs similar to TiO_2 NPs through inducing damages to testis led to a marked reduction in sperm quality, *in vitro* fertilization, and embryos development in male mice.

Introduction

The production and applications of various nano-materials and nanotechnology are growing rapidly and therefore, increase the environmental release and accumulation of these materials. Thus, environmental and occupational exposures through inhalation, oral route, and dermal penetration to nano-materials seem to be unavoidable.[1]

Titanium dioxide (TiO_2) particles as the top five particles in use are composed of nano-sized particles (NPs) lesser than 100 nm and fine-sized particles (FPs) greater than 100 nm.[2] The TiO_2 particles are widely used in many products such as sunscreens and cosmetics, white pigments, and antibacterial agents as well as authorized additive applied as a food colorant. Over 70.00% of all nanoparticles produced in the world are associated with TiO_2 NPs.[3,4] Many previous *in vivo* and *in vitro* studies have indicated that TiO_2 NPs accumulate in vital organs such as the liver, brain, lung, testis, and ovary and induce histological alterations and cell toxicity.[1,5,6] The TiO_2 NPs may induce oxidative stress in the tissue by increasing the production of reactive oxygen species (ROS) and cause DNA oxidation.[7,8] Furthermore, oxidative stress can induce inflammatory responses and mitochondrial dysfunction, which can lead to cell damage and eventually cell death.[9]

It has been reported that a 75.00 kg adult human intakes 15.00-37.50 mg per day of TiO_2 from food.[2] A large amount (64.00%) of TiO_2 particles used as a food additive

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is larger than 100 nm in size and considered as TiO_2 FPs.[10] Previous studies have indicated that TiO_2 NPs in comparison with TiO_2 FPs are more toxic.[2,11] An equal dose of TiO_2 NPs was assumed to be 40 times more potent than TiO_2 FPs.[12] Following oral administration of different sizes of TiO_2 particles in mice, it was indicated that TiO_2 FPs in comparison with TiO_2 NPs could not affect plasma glucose and ROS levels and the histopathological findings also showed that TiO_2 FPs did not induce pathological alterations in liver and pancreas.[2] In another study, it was demonstrated that TiO_2 NPs in comparison with TiO_2 FPs can induce the significant release of ROS and interleukin-8 (IL-8) in A549 human lung epithelial cells. [11] Thus, these studies have indicated that TiO_2 FPs induce less toxic effects on the body, but they are not safe. In contrast to above-mentioned reports, the results of an *in vitro* study demonstrated that the treatment of human bronchial epithelial cells with TiO_2 FPs caused a reduction of cell viability.[13] However, due to lack of sufficient information, there is no consensus on whether TiO_2 NPs are more toxic than TiO_2 FPs. There is a considerable body of reports in laboratory animal models suggesting that treatment with TiO_2 NPs can cause toxicity in the male reproductive system and induce impairment in testicular function. For example, it has been demonstrated that TiO_2 NPs supplementation in mice leads to pathological alterations in testicular tissue and a significant increase in the number of abnormal sperms.[4] Other experimental studies have also indicated that TiO_2 NPs exposure can decrease the weight of testis, the number of Leydig cells and testosterone concentration as well as sperm quality.[5,14] But, to the best of our knowledge, there is no detailed study to establish whether oral administrations of the same dose of nano- and fine-sized TiO_2 cause similar toxicity on the male reproductive system.

The current study aimed to investigate the toxic effects of TiO_2 particles in different sizes (FPs and NPs) on testicular tissue morphology, sperm parameters, oxidative stress, *in vitro* fertilization (IVF), and *in vitro* embryo development.

Materials and Methods

Animals and treatment. In this study, 24 adult male mice (8-10 weeks) were used. The animals were housed in plastic cages (four mice in each cage) in a ventilated animal house at 20.00 ± 2.00 ˚C and a 12-hr light/dark cycle. During the study, water and standard commercial food for mice were available *ad libitum*. Before experiment initiation, the mice were adapted to this new environment for a week. The animals were randomly divided into three groups (n=8) including control group receiving vehicle, TiO_2 NPs group receivingTiO_2 NPs suspension (150 mg kg^{-1} per day), and TiO_2 FPs group receiving TiO_2 FPs suspension (150 mg kg^{-1} per day). The mice received TiO_2 particle solution through oral gavage for 35 days. This duration of administration was chosen based on the timing (35 days) of the mouse spermatogenesis process.[15] The doses of TiO_2 particles were selected according to the previous study.[14] The TiO_2 particles were obtained from US Research Nanomaterials, Inc, USA. The size of TiO2 NPs (99.00% anatase) was 10-25 nm and the size of TiO2 FPs (99.99% anatase) was 125 nm. The TiO_2 particles were suspended in double-distilled water. This solution was sonicated for 10 min every day before gavage. This study was approved by the ethical committee of Urmia University of Medical Sciences, Urmia, Iran (Ir. UMSU.1396.153).

Sperm parameters evaluation. After 35 days, the animals were euthanized with ketamine (Alfasan, Woerden, The Netherlands) overdose (100 mg kg^{-1}) and weighted using a precise scale. For sperm sampling, both cauda epididymides along with vas deferens were dissected out and transferred to the dishes containing 1.00 mL human tubal fluid (HTF; Sigma, St. Louis, USA) medium supplemented with 4.00 mg mL^{-1} bovine serum albumin (BSA; Sigma).[16] Then, the epididymides were minced using the needle of insulin syringe and incubated for 15 min at 37.00 ˚C in 5.00% CO_2 to swim-out the sperms into the medium. To assess sperm motility, 10.00 µL of sperm suspension was placed on a pre-heated Neubauer slide and covered with a cover-glass, and then using a light microscope with 400× magnification the percentage of sperm motility was calculated. The sperms with no movement at all were considered non-motile and the ones displayed some movements were considered motile.[17] To evaluate sperm count, a 1:50 dilution from sperm solution with water was prepared and then, 10.00 µL of this mixture was placed on a Neubauer slide and using a light microscope with 400× magnification sperm count was calculated.[16] To assess the percentage of sperm viability, 20.00 µL of sperm sample from each mouse was mixed with the same volume of eosin and nigrosine stain mixture, and smears were prepared. Following the drying of the smears, the percentages of red-colored dead sperms and alive ones with no color were determined under a light microscope with 400× magnification. To determine the percentage of sperms with abnormal morphology, the smears stained by aniline blue were used.[16] Assessment of sperm DNA disintegrity as a marker of infertility was done using acridine orange staining. Briefly, the sperm smear slides from each mouse were fixed 2 hr in Carnoy's solution (methanol/acetic acid; 1:3). Then, the slides were stained by acridine orange solution for 7 min and dried. Under a fluorescent microscope using a 460-nm filter with 1000× magnification, at least 200 spermatozoa per mouse were evaluated and the percentages of sperms with normal DNA (green color) and abnormal ones (yellow to red) were determined.[16] Sperm nucleus maturity was analyzed with aniline blue staining. Aniline blue stains

lysine-rich histones and considers as a marker of sperm chromatin evaluation. The dried sperm smear slides from each mouse were fixed for 30 min in 3.00% glutaraldehyde and then the slides were stained with 5.00% aniline blue for 7 min.[16] In this staining, the head of mature sperm appears pale, and abnormal immature sperm has a dark blue color. Under a light microscope using 1000× magnification, at least 200 spermatozoa were counted in each slide and the data were expressed as a percentage.

Oocytes collection and IVF assay. After 35 days, the male mice were prepared for IVF. Sixty adult female (8-10 weeks) mice were used for IVF assay. To induce superovulation in female mice and collect mature oocytes from oviducts, 10.00 IU pregnant mare's serum gonadotropin hormone (Intervet, Boxmeer, Netherlands) was injected, and after 48 hr, 10.00 IU human chorionic gonadotropin hormone (hCG; Intervet) was injected intraperitoneally. 12-14 hr after hCG injection (next morning), female mice were euthanized and then the oviducts were removed and placed in a drop of HTF-BSA medium previously equilibrated in an incubator (5.00% CO_2, 37.00 ˚C).[18] Then, the oviducts were dissected and the oocytes were removed and after washing, added to fertilization droplets of HTF-BSA medium which were under mineral oil. After that, 1.00 × 10^6 capacitated sperms from each male mouse were added to oocytes in the fertilization droplets. The fertilization process was evaluated after 3-5 hr by observing two pronuclei using an inverted microscope. After culturing these zygotes for 24 hr, the numbers of two-cell embryos were determined, and finally, the percentages of blastocysts and arrested embryos were evaluated 120 hr later.[18]

Histological analysis of testicular tissue. For histological assessment, the right testis was dissected out, weighed, and fixed in 10.00% formalin. After dehydration in different grades of alcohol, the tissues were embedded in paraffin, and then, 5.00 µm sections were cut and stained with Hematoxylin and Eosin (H & E). In this study, the germinal epithelium height and diameter of 10 round-shaped seminiferous tubules (SNTs) from each sample were randomly selected and measured. For this purpose, two diameters perpendicular to each other were measured in each SNT and their average was determined. To determine the average epithelial thickness of the same SNT, it was measured from the basement membrane to the luminal surface.[19] The spermiogenesis index was also calculated in the testicular tissue as the percentage of SNTs with spermatozoa in at least 20 round tubules. The gonadosomatic index or testis index was calculated by the following formula:

Testis index = testis weight (mg)/ body weight (g) × 100

Determination of oxidative stress markers in testicular tissue. To determine the MDA and GSH levels and the activity of SOD, and GPx enzymes in testicular tissue, the left testis from each mouse was homogenized in phosphate buffer (pH: 7.40) and centrifuged at 12,000 *g* at 4.00 ˚C for 20 min. Then, the supernatant was separated and stored at – 80.00 ˚C. The activities of SOD and GPx in the supernatant were determined using detection kits (ZellBio GmbH, Lonsee, Germany). The assays were performed according to the manufacturer's procedure.

To determine MDA levels, the supernatant (600 µL) was mixed with 150 µL of thiobarbituric acid (0.67% w/v), incubated in a boiling water bath (95.00 ˚C) for 30 min and extracted with n-butanol. Then, the solution was cooled and centrifuged. Absorbance was recorded at 532 nm and MDA levels were expressed as nmol mg^{-1} protein. The calibration curve of tetraethoxypropane (standard solution) was used to determine the MDA levels in testicular tissue samples.[20]

The supernatant GSH levels were measured as described previously.[21, 22] Briefly, 10.00 µL of supernatant was added to 200 µL of Tris-EDTA buffer (0.25 M Tris base and 20.00 mM, pH: 8.20 EDTA) and the absorbance of the solution was recorded at 412 nm (A_1). After adding 4.00 µL of 5, 5-dithiobis-2-nitrobenzoic acid (DTNB; 10.00 mM), the solution was incubated at 37.00 ˚C for 30 min and the absorbance was measured again (A_2) together with a DTNB blank (B). Total GSH levels were calculated as follows:

$$GSH\ concentration = (A_2 - A_1 - B) \times 1.57\ mM$$

Statistical analysis. The IVF assay data were analyzed by two proportional test using Minitab software (version 15.1; Minitab Inc., Boston, USA). Other results were examined by one–way ANOVA followed by the Tukey test using SPSS (version 16.0; SPSS Inc., Chicago, USA). All results were shown as means ± standard deviation and a p-value < 0.05 was considered as statistically significant.

Results

Testis index. The means of testis index in control, TiO_2 NPs, and TiO_2 FPs groups were 5.57 ± 0.22, 6.04 ± 0.39, and 5.61 ± 0.84, respectively. No significant difference was observed among groups.

Histological analysis of testicular tissue. There were significant histological alterations in the testicular tissue of mice received TiO_2 NPs and TiO_2 FPs (Table 1). In these groups, SNTs diameters and height of germinal epithelium were significantly reduced compared to control group (p < 0.01). The percentages of SNTs with sperm in their lumen were also significantly decreased in TiO_2 particle groups in comparison with control group (p < 0.001). Furthermore, the sloughing of germ cells into the lumen of SNTs was observed in animals exposed to TiO_2 NPs and TiO_2 FPs (Fig. 1).

Malondialdehyde content and antioxidative enzyme activities in testis. Oral administration of both sizes of TiO_2 particles (NPs and FPs) for 35 consecutive

Table 1. Seminiferous tubules (SNTs) diameter, germinal epithelium height, spermiogenesis index, and oxidative stress markers level in different groups.

Groups	SNTs diameter (µm)	Germinal epithelium height (µm)	Spermiogenesis index (%)	MDA (nmol g^{-1} tissue)	GSH (µmol mg^{-1} tissue)	GPx (U mL^{-1})	SOD (U mL^{-1})
Control	220.09 ± 12.88	72.24 ± 7.22	68.75 ± 8.34	14.11 ± 1.27	0.32 ± 0.02	271.50 ± 85.20	21.69 ± 0.92
TiO_2 NPs	190.5 ± 11.43[a]	54. 43 ± 5.20[a]	40.37 ± 6.52[b]	19.26 ± 2.02*	0.21 ± 0.03*	162.90 ± 114.50*	16.13 ± 2.23*
TiO_2 FPs	190.50 ± 7.66[a]	48.40 ± 6.68[b]	35.85 ± 7.66[b]	17.03 ± 1.35*	0.26 ± 0.01*†	165.60 ± 62.90*	17.68 ± 1.15*

NPs: Nano-sized particles; FPs: Fine-sized particles; MDA: Malondialdehyde; GSH: Glutathione; GPx: Glutathione peroxidase; SOD: Superoxide dismutase. [ab] Different letters indicate significant differences compared to control ($p < 0.01$ and $p < 0.001$). *† Different symbols indicate significant differences compared to control and TiO_2 NPs groups, respectively ($p < 0.05$).

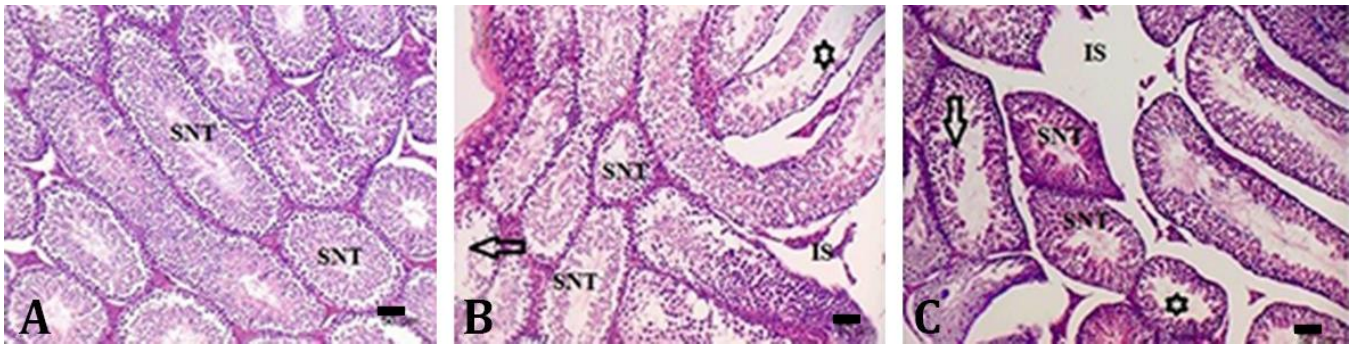

Fig. 1. Effects of TiO_2 particles exposure on testicular histology in mice. **A)** Control group, **B)** 150 mg kg^{-1} per day nano-sized TiO_2 group, and **C)** 150 mg kg^{-1} per day fine-sized TiO_2 group. Arrows are showing sloughing germ cells into the lumen of seminiferous tubules (SNTs) and stars show SNTs without sperm. Interstitial space (IS) is widened in TiO_2 particles groups, (H & E staining, Scale bars = 50 µm).

days significantly increased the levels of MDA in testis compared to control group ($p < 0.05$). Furthermore, the GSH levels and activities of SOD and GPx in TiO_2 NPs and TiO_2 FPs groups were significantly decreased in comparison with control group ($p < 0.05$). However, except for the GSH levels, no significant differences were found between TiO_2 NPs and TiO_2 FPs groups (Table 1).

Epididymal sperm parameters. Exposure of the animals to TiO_2 particles in the TiO_2 NPS and TiO_2 FPs groups significantly decreased all sperm parameters (count, motility, viability, and morphology) compared to control group ($p < 0.001$). No significant differences were observed between TiO_2 NPs and TiO_2 FPs groups. The results of sperm parameters are shown in Table 2.

Table 2 also shows the results of sperm DNA damage and the immaturity of the sperm nucleus in different groups. Supplementation with TiO_2 in both groups significantly increased the percentages of sperm DNA damage and the amount of sperm with an immature nucleus ($p < 0.001$). However, there were no significant differences between the TiO_2 NPs and TiO_2 FPs groups.

***In vitro* fertilization rate and development of embryos.** Findings of *in vitro* fertility and embryonic development in experimental groups are shown in Table 3. The percentages of fertilization and two-cell embryos in the TiO_2 NPs and TiO_2 FPs groups were significantly lower than control group ($p < 0.001$). While no significant differences were observed between TiO_2 NPs and TiO_2 FPs groups. In the TiO_2 NPs and TiO_2 FPs groups, the percentages of blastocysts and arrested embryos were also reduced in comparison with control group ($p < 0.001$). However, TiO_2 FPs-treated group showed a significant decrease compared to TiO_2 NPs-treated group regarding these parameters ($p < 0.01$; Fig. 2).

Table 2. Sperm parameters in different groups.

Groups	Sperm count ($\times 10^6$)	Sperm motility (%)	Sperm viability (%)	Abnormal morphology (%)	DNA damage (%)	Immature sperm (%)
Control	36.20 ± 2.51	68.00 ± 3.53	72.00 ± 4.35	8.60 ± 4.03	1.80 ± 0.83	1.60 ± 0.89
TiO_2 NPS	25.30 ± 2.97*	47.20 ± 5.16*	51.20 ± 5.26*	26.00 ± 3.80*	12.60 ± 3.28*	14. 40 ± 2.50*
TiO_2 FPs	23.50 ± 3.25*	44.40 ± 3.36*	49.00 ± 3.39*	28.00 ± 3.39*	13.60 ± 1.94*	11.20 ± 1.92*

NPs: Nano-sized particles; FPs: Fine-sized particles. * Asterisk indicates a significant difference compared to control ($p < 0.01$).

Table 3. The effects of oral administration of nano-sized TiO_2 and fine-sized TiO_2 on *in vitro* fertility and embryonic development in different groups.

Groups	Number of oocytes	Fertilization rate (%)	Two-cell embryos (%)	Blastocysts (%)	Arrested embryos (%)
Control	219	207 (94.52)	188 (90.82)	107 (51.70)	100 (48.30)
TiO_2 NPS	326	259 (79.45)[a]	185 (71.43)[a]	79 (30.50)[a]	180 (69.49)[a]
TiO_2 FPs	395	313 (79.24)[a]	204 (65.18)[a]	66 (21.09)[ab]	247 (78.47)[ab]

NPs: Nano-sized particles; FPs: Fine-sized particles. [a] indicates a significant difference compared to control group at $p < 0.001$ and [b] indicates a significant difference compared to TiO_2 NPs group at $p < 0.01$.

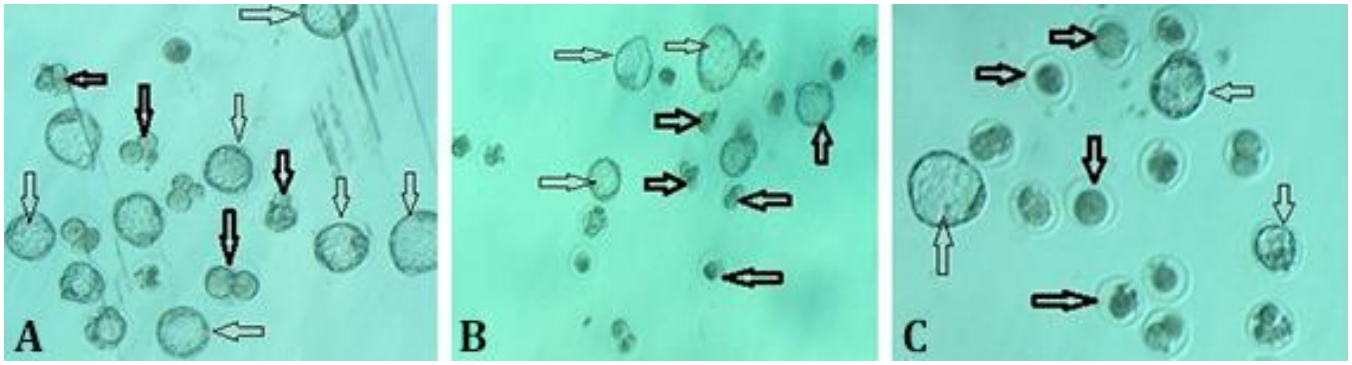

Fig. 2. Embryo culture after 120 hr. Thin arrows are showing the blastocysts and thick ones are showing the arrested embryos in different groups. **A)** Control group; **B)** Nano-sized TiO_2 group; **C)** Fine-sized TiO_2 group, (Magnification: 400×).

Discussion

This study indicated that it cannot be generalized that TiO_2 NPs are always more toxic in the male reproductive system than TiO_2 FPs, as all epididymal sperm parameters and testicular tissue alterations in groups treated with TiO_2 NPs and TiO_2 FPs were significantly decreased in comparison with control group. Furthermore, the potential of fertility and testicular oxidative stress in TiO_2 treated groups were remarkably changed. Indeed, both sizes of TiO_2 in the present study could exert toxic effects on the male reproductive system.

Various experimental studies have concluded that exposure to ultrafine particles (nanoparticles in the size range lesser than 100 nm) compared to larger size fine particles (particles greater than 100 nm) at the same doses induces greater toxicity. Surface area seems to have an important effect on the potential toxicity of nanoparticles. As the diameter of the particle reduces, the surface area increases proportionally.[23]

Several reports have dealt with the toxicological effects of TiO_2 NPs on the function and morphology of the male reproduction system, but little is known whether TiO_2 FPs could be able to affect the male reproductive system like TiO_2 NPs. In this work, the sensitivity of testicular tissue, sperm parameters, and IVF potential of mice exposed to different sizes of TiO_2 (NPs and FPs) were determined and compared with the control ones.

It should be considered that most of TiO_2 particles used as food additives are greater than 100 nm and they are not in a nanoparticle size range.[6] Thus, oral ingestion is the main exposure route of TiO_2 FPs. In the present study, we used the anatase crystalline form of TiO_2 because this form of TiO_2 is most widely used in food compounds.

Data released from our study showed that TiO_2 FPs resemble TiO_2 NPs induced a statistically significant reduction in sperm count and motility. This is in agreement with Orazizadeh *et al*. reporting that TiO_2 NPs in mice causes pathological alterations in testis and a decrease in sperm concentration and motility.[24] In another study by Guo *et al*., it has been shown that TiO_2 NPs administration leads to a significant reduction in sperm count and motility.[25] Previous studies have documented that reductions in sperm count and motility are valid parameters to judge male infertility in animals and they are often used as markers of chemical-induced testicular toxicity.[26,27]

In our study, the decrease in sperm count was also confirmed by histological observations, as the percentage of SNTs without sperm in their lumen was significantly higher in TiO_2 particles treated groups compared to control group. This reduction in sperm count following TiO_2 treatment may be attributed to translocation of TiO_2 particles from blood circulation to testis affecting spermatogenesis process in two ways; first, inducing damage in germ cells resulting in the reduction of mature sperms number and another way is the direct effect on mature sperms.[28]

The results of the sperm morphology analysis revealed the spermatotoxic effects of TiO_2 NPs and TiO_2 FPs. This parameter has the potential in identifying chemicals inducing spermatogenesis dysfunction.[29] Sperm with abnormal morphology is associated with male infertility and sterility in most species and can directly affect fertilization and pregnancy outcome.[30] The involved mechanisms for the increase in the percentage of abnormal sperm are not fully understand, but it may occur due to abnormal chromosome, minor alteration in testicular DNA and point mutation.[31,32] In a study by Bruce and Heddle, it was revealed that the induction of abnormality in the head of sperms can be related to chromosomal aberrations and also point mutation in testicular DNA.[33] Another reason for sperm abnormality is impairments in the differentiation of sperm during spermatogenesis following a cytotoxic chemical exposure or even due to changes in testicular DNA, which in turn interfere with the differentiation process in spermatozoa.[29]

In this study, sperm viability was affected by TiO_2 NPs and TiO_2 FPs administrations. Findings revealed that TiO_2 particles significantly decreased the percentage of alive sperm in comparison with control mice. These findings may be associated with the direct effect of TiO_2 particles

on testicular tissue. A previous study has indicated that TiO_2 NPs disrupt the blood-testis barrier.[6] Therefore, probably TiO_2 particles can enter the testicular tissue through blood circulation and may affect the spermatogenesis process resulting in a decrease in sperm count, motility, and viability. There is a possibility that TiO_2 particles affect sperm cells directly. For example, TiO_2 may induce damage in the acrosome structure which is known as an important organelle in sperms involving in the fertilization process.[34] One possibility is that the TiO_2-induced alteration in the acrosome of the sperm can result in fertilization failure.

Acridine orange staining showed that DNA damage was observed in mice exposed to both sizes of TiO_2 particles. Damage in sperm DNA may transfer damaged genome into oocyte leading to a reduction in fertilization rate and embryonic development and may also affect fetal and postnatal development.[35,36] Our findings demonstrated that TiO_2 FPs same to TiO_2 NPs impair *in vitro* fertility resulting in reproduction activity reduction.

Regarding aniline blue staining which can evaluate sperm chromatin defects, it can be reported that TiO_2 particles exposure via oral gavage increased the percentage of sperm chromatin abnormality. Sperm maturation occurs in the tail of the epididymis. Thus, TiO_2 may exert its toxic effects on testis and epididymis. This alteration in epididymal sperms induced by TiO_2 may be associated with nitric oxide (NO) radicals formation.[37] It has been indicated that excessive NO production may decrease the percentage of sperm viability and motility.[38]

Data obtained from this study indicated that all sperm parameters had been impaired. A possible reason for sperm parameters impairment following TiO_2 NPs and TiO_2 FPs is that Leydig cells function may be affected by TiO_2 exposure. Orazizadeh *et al*. have shown that serum and testis testosterone levels significantly decrease in mice following TiO_2 NPs administration.[24] In a previous study, it has been demonstrated that TiO_2 NPs are taken up by mouse Leydig cells and affect the viability, proliferation, and gene expression of these cells,[39] Moreover, the findings of Gao *et al.* and Jia *et al.* have also proven the suppressive effect of TiO_2 on blood serum testosterone of treated mice.[40,41] Although we did not measure the testosterone levels, TiO_2 NPs and TiO_2 FPs may affect testosterone levels and this may explain the adverse effects of TiO_2 on sperm parameters in this study.

In agreement with our observed histopathological alterations in the testicular tissue, other investigators have found that TiO_2 NPs exposure in mice/rats can result in a decrease of SNTs diameter and germinal epithelium height with germ cells sloughing in the lumen of SNTs.[24,37] Sloughing of germ cells in the lumen of SNTs may be induced by the effects of chemical toxic on microtubules and intermediate filaments of Sertoli cells.[42]

In this study, we reported clear signs of oxidative stress as our findings showed a significant reduction of antioxidant enzymes (GPx and SOD) activities and an increase of lipid peroxidation levels in testicular tissue of mice exposed to TiO_2 particles which may lead to the spermatogenesis process impairment. Oxidative stress is one of the possible mechanisms for TiO_2 NPs induced toxicity in the male reproductive system. It has been shown that TiO_2 NPs generate free radicals.[43] Also, TiO_2 NPs decrease the levels of GnRH through the formation of free radicals and thereby reduce the production of pituitary gonadotropins leading to a decrease in testosterone concentration. In the present study, TiO_2 NPs and TiO_2 FPs induced stress oxidative can cause fragmentation in double-stranded DNA of sperm resulting in a decrease in IVF rate and potential of embryo development. There is a positive correlation between sperm DNA damage and low fertilization rate following IVF and/or normal fertility.

Data from our study demonstrated that oral administration of TiO_2 FPs resemble to TiO_2 NPs caused abnormal alterations in testicular tissue and decreased quality of sperm and rate of IVF. In a recent study, it has been indicated that TiO_2 FPs cannot enter the blood circulation, remain in the digestive tract and subsequently, they are not able to induce pathological changes in organs of mice such as fatty degeneration, edema, and necrosis of liver as induced by the same dose of TiO_2 NPs.[2] This discrepancy with our study may be due to the high sensitivity of testicular tissue in comparison with other tissues making the testis more sensitive to TiO_2 FPs toxicity. In a study by Miura *et al*., it has been shown that the administration of TiO_2 NPS to the mice results in a clear testicular dysfunction, while the liver dysfunction is not observed in these animals.[44] Furthermore, in contrast to our results, in an *in vitro* study, it has been indicated that treatment of A549 human lung epithelial cells with TiO_2 NPs induces remarkable ROS and IL-8 release in comparison with TiO2 FPs, which can be reflected that TiO_2 NPs are more toxic in comparison with TiO_2 FPs.[11] In agreement with our study, in another *in vitro* study, it has been demonstrated that TiO_2 FPs exposure leads to a reduction in cell viability and DNA damage in human bronchial epithelial cells.[13]

In vitro fertility rate and embryonic development in the TiO_2-treated groups were significantly lower than control group and the percentage of arrested embryos was higher in groups received TiO_2 NPs and TiO_2 FPs. This is the first evidence on the relationship between rate of IVF and embryonic development impairments and TiO_2 particles supplementation. Thus, to compare our results with other studies, we did not find a similar study.

Our study was not without limitations. The apoptosis of germ cells and the accumulation of TiO_2 particles in testis were not evaluated. Evaluating each of them could

provide additional information about the effects of TiO_2 particles on the male reproductive system.

In conclusion, according to the data from the present study, oral administration of TiO_2 FPs similar to TiO_2 NPs has detrimental effects on all sperm parameters, rate of fertilization, and *in vitro* embryo development and also induces oxidative stress in testis. The exact mechanisms by which TiO_2 FPs induced testicular toxicity and impairments of the spermatogenesis process and *in vitro* embryonic development are not obtained from this study. Further investigations are needed to elucidate the mechanisms by which TiO_2 FPs affect the male reproductive system. However, the obtained findings from this study can provide useful information on the risk of applying TiO_2 FPs as food additives.

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Conflicts of interest

Authors have no conflict of interest to declare.

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