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Histological and Molecular Studies on the Toxicity of Titanium Dioxide Nanoparticles in Testis and Bone Marrow of Albino Mice

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Abstract: Titanium dioxide nanoparticles (TiO2-NPs) have recently recorded a public health and scientific concern due to their global use in industrial and household applications. However, there is limited information concerning its *in vivo* cytogenotoxicity. In this work, the cytogenotoxic effects of TiO2-NPs on the somatic tissue using the mouse testicular histopathology and immunohistochemistry and bone marrow comet assay were investigated. A concentration of 1944 mg/kg b.wt. was administered intraperitoneally to mice for five consecutive days. Histopathologically, TiO2-NPs disrupted the normal cellular architecture of testicular tissues in exposed mice; as it caused severe lesions such as congestion of the interstitium, oedema, vacuolation, damaged Sertoli and Leydig cells, degenerated spermatozoa, apoptosis and necrosis. TiO2-NPs also increased the immunoreactivity of caspase-3 and DNA fragmentation in testicular and bone marrow tissue respectively and decreased the testosterone level in blood. These results suggest that the bone marrow and testicular cells may be potential targets for TiO2-NPs induced DNA damage and cytotoxicity in mice. This is of public health importance considering increasing exposure to TiO2-NPs in consumer products. But, the application of vitamin E reduced the occurrence of histopathological lesions, decreased the immunoreactivity of caspase-3 and DNA fragmentation and increased testosterone level to record some sort of protection against TiO2 toxicity.

Key words: Titanium Dioxide · Testis · Albino Mice · Caspase-3 · Testosterone · Comet Assay.

INTRODUCTION

Nanoscience is an emerging technology in this millennium has led to the development in the production of nanoparticles [1].This led to a huge potential for new strategies of rapid disease diagnosis, treatment and enhanced quality of life [2]. Nanoparticles (NPs) are defined as those that range in size from 1 nm to 100 nm in diameter [3]. They have a small size and large surface area to volume ratio with high reactivity potential; as a result of these unique properties, they have being massively produced on a large scale [4, 5]. NPs have a broad range of applications in daily life, such as electronics, commercial products, medical devices and drugs [6]. Their production enhances the probability of exposure via inhalation, oral and dermal penetration, both in the occupational and environmental settings [7-9]. NPs are able to bind, absorb and carry other compounds such as drugs, probes and proteins with their large surfaces [10]. They can enter the body through the skin, lungs, or the intestinal tract, migrate and deposit in several organs and may cause adverse biologic reactions by modifying the physiochemical properties of systems and tissues at a nano level [11]. Vitamin E (α-tocopherol) is a naturally occurring antioxidant that plays an important role by inactivating harmful free radicals produced through normal cellular activity and from various stressors thus terminating lipid peroxidation and stabilizing the molecular composition of cellular membranes, preventing the harmful effects of reactive oxygen species (ROS) [12, 13]. Therefore, human exposure to nanoparticles is in continuous rising. Due to this fact the present work was designed to investigate the effect of TiO2- NPs on the testicular and bone marrow tissue from the histological, immunohistochemical, biochemical and molecular points of view. At the same time the possible protective effect of vitamin E against TiO2- exposure was tested.

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MATERIALS AND METHODS

Chemicals: Titanium dioxide nanopowder (anatase form, CAS number: 1317-70-0, product code - 637254), Purity: 99.7%, Average Particle Size: <25 nm, Specific Surface Area: 45 m^2/g, Color: white, Morphology: powder, Bulk density: 0.04-0.06 g/mL and relative density: 3.9 g/mL] was obtained commercially from Sigma Aldrich Co. Germany. This NP was chosen because of its utilization in previous studies. During use suspend the TiO2 in double distilled water at a dose of 100- 1944 mg per kg b.w. (Oral administration or IP injection).Vitamin E and all other chemicals used in the study were of high analytical grade and products of Merck and Sigma companies.

Animals and Experimental Design: Thirty two adult healthy male albino mice (25-30 g) were obtained from the Animal house of the Faculty of Veterinary Medicine, Zagazig University. Animals were housed in clean plastic cages and maintained under standard conditions of light/dark cycle, temperature (23°C ±2) and humidity (54%). Mice were fed a standard pellet diet with free access to tap water. After 15 days of acclimatization, animals were fasted over night before treatment and divided into four groups (8 mice each):

G1: Control group (healthy animals) nourished basal diet all over the whole experimental period. **G2:** Vitamin E-orally-administered mice, 100 mg/kg/day [14]. **G3**: TiO2-NPs intraperitoneally-injected mice with 1.944 g/kg b.w./day for 5 consecutive days [15]; **G4**: TiO2-NPs- intoxicated mice with co-administration of vitamin E daily. Vitamin E in this group was orally-administered at a dose of 100 mg/kg b.w./day) for 7 consecutive days, two days before the beginning of Ti O2 application. After the last dose administration, mice were fasted overnight, euthanized, sacrificed and blood was collected. Sera were separated and kept at –20 °C for hormonal assessment. Testes were harvested and a number of them was fixed in 10% neutral buffered formalin at room temperature for histopathological and immunohistochemical examination. Bone marrow of control and treated animals was flushed from the comparable femurs using cold saline and then frozen at -20°C for the molecular study (comet assay).

Histopathological Study: Tissue of testis after formalin fixation was dehydrated in ascending grades of ethanol, cleared in xylene and embedded in melted paraffin wax at 58°C in an oven. Paraffin tissue blocks were prepared for sectioning at 3-4 micron thickness by microtome. The obtained tissue sections were collected on glass slides, dewaxed and processed for hematoxylin and eosin (H & E) staining [16].

Immunohistochemical Study: Immunohistochemical technique for caspase-3 was performed as an indicator for cell apoptosis [17].

Molecular Study

Comet Assay: The comet assay, or single cell gel electrophoresis (SCGE), is a commonly- used technic for detection, analyzing and measuring DNA damage in individual cells. The method, which involves the unwinding of DNA under alkaline conditions, was used in this study [18]. 0.5 g of crushed samples were transferred to 1 ml ice-cold PBS. This suspension was stirred for 5 min and filtered. Cell suspension (100 µl) was mixed with 600 µl of low-melting agarose (0.8% in PBS). 100 µl of this mixture was spread on pre-coated slides. Immerse coated slides in lysis buffer (0.045 M TBE, pH 8.4, containing 2.5% SDS) for 15 minutes. Slides were placed in electrophoresis chamber containing the same TBE buffer, but devoid of SDS. Conditions of electrophoresis were 2 V/cm for 2 min and 100 mA. Ethidium bromide (20µg/ml) was used for staining at 4°C. Observation was recorded with samples which still humid, the DNA fragment migration patterns of 100 cells for each treatment or dose level were evaluated with a fluorescence microscope (With excitation filter 420-490nm [issue 510nm]). The comet tail lengths were measured from the middle of the nucleus to the end of the tail with 40x. To visualize DNA damage, observations were made of EtBr-stained DNA using 40X objective of fluorescent microscope. Although any system of image analysis may be suitable for the quantitation of SCGE data, we used a comet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in cells by measuring the length of DNA migration and the percentage of migrated DNA. The percentage of fragmented DNA in the comet tail is a direct measure of DNA damage. Finally, the program calculates tail moment. The tail moment was defined by the percentage of DNA in the tail multiplied by the length between the center of the head and tail [19]. Generally, 50 to 100 randomly selected cells were analyzed per sample.

Hormonal Assay: Testosterone levels for control and experimental groups were determined For biochemical analysis, blood samples were collected from the heart puncture of control and treated animals then samples were allowed to coagulate. Sera were separated by centrifugation at 3000 rpm for 10 min, stored at -20°C and used for measuring total serum testosterone by linked fluorescent assay according to Maruyama *et al.* [20].

Image Analysis: The result images of the immunohistochemical reaction of caspase-3 were analysed on image pro plus 4.5.1 computer system. The system measured the relative area occupied by caspase-3 containing cells.

Statistical Analysis: The results were expressed as mean± SE. Data were analyzed by comparing values of different treatment groups with the values for controls. Significant differences among different groups were analyzed using Excel 2010 for student t-test, $p \leq 0.05$ was considered significant.

RESULTS

Histopathological Results: The light microscopic examination of testis of control mice demonstrated characteristic normal and rounded seminiferous tubules with well-organized spermatogenic cells and interstitial tissue (Fig. 1). These tubules are bounded externally by a basal lamina containing elongated myeoid cells connected with elastic fibers. Between the seminiferous tubules lie the interstitial tissue with Leydig cells and blood vessels. The wall of the seminiferous tubules contains Sertoli cells and spermatogenic cells. The cells of the spermatogenic series are spermatogonia, primary spermatocytes, short-lived secondary spermatocytes produced from meiosis, round spermatids with large spherical nuclei, elongated spermatids. The latter metamorphose to form spermatozoa. Spermatozoa aggregate at the top of Sertoli cells and are often released into the lumen of the seminiferous tubules. Sertoli cells are columnar ones and rest directly on the basal lamina. They have relatively granular cytoplasm and oval shape nucleus with dispersed chromatin as well as one or two nuclei (Fig. 2). Animals administered with vitamin E only showed the same histological structure as those of controls with well-constructed germ cells (curved arrows) and interstitial tissue (straight arrow) (Figs.3).

Animals exposed to TiO2 exhibited a distinct histological difference when compared with control ones. There were irregular & faintly-stained seminiferous tubules and vacuolated interstitial tissue, sloughing of the germinal epithelium at some points was also seen. In addition, highly-damaged spermatocytes, degenerated

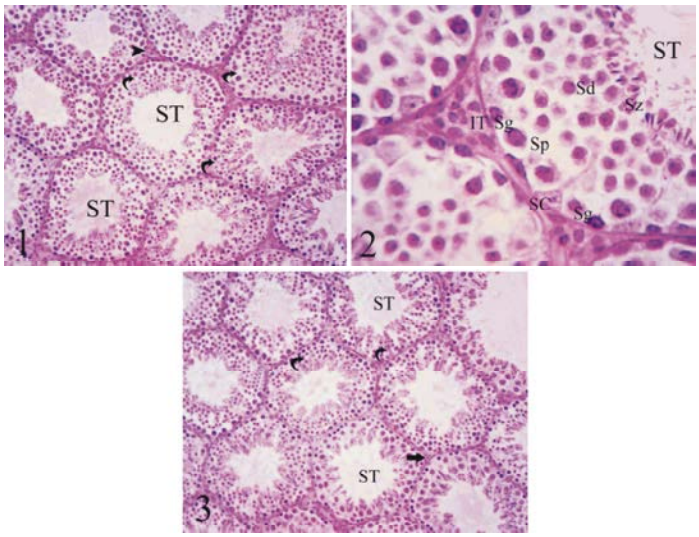

Fig. 1: Section of testis of control mice demonstrated a normal histological pattern (H&E, X400). Notice the rounded seminiferous tubules (ST), well-organized spermatogenic cells (curved arrows) and the interstitial tissue (arrow head).

Fig. 2: Section of testis of control mice with higher magnification (H&E, X1000). ST, seminiferous tubules; SC, Sertoli cells; IT, interstitial tissue; Sg, Spermatogonia; Sp, Spermatocytes; Sd, Spermatids; Sz, Spermatozoa.

Fig. 3: Section of testis of vitamin E- administered mice (H&E, X1000) showing a nearly normal histological features with well-constructed germ cells (curved arrows) and interstitial tissue (straight arrow).

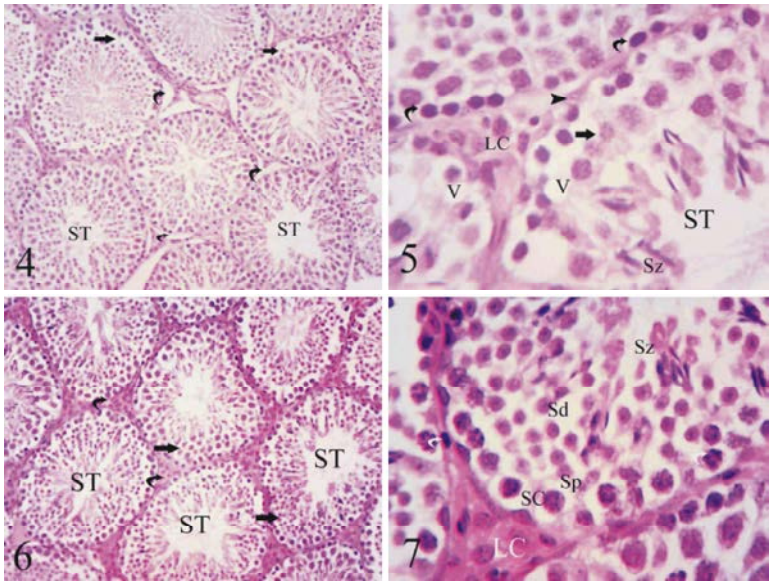

Fig. 4: Section of testis of TiO2-exposed mice (H&E, X400) showing faintly –stained seminiferous tubules (ST), vacuolated interstitial tissue (curved arrow) and sloughing of epithelia from the basal lamina (straight arrow).

Fig. 5: Section of testis of TiO2-exposed mice (H&E, X1000) showing apoptotic spermatogonia (curved arrow), degenerated Leydig cells (LC), damaged Sertoli cells (arrow head), highly-damaged spermatocytes (straight arrow), intratubular vacuolization (v) and abnormal destructed spermatozoa (Sz).

Fig. 6: Section of testis of TiO2-exposed mice treated with vitamin E (H&E, X400) showing well- constructed spermatogenic cells (straight arrows) and interstitial tissue (curved arrow) and rounded regular seminiferous tubules (ST).

Fig. 7: Section of testis of TiO2-exposed mice treated with vitamin E (H&E, X1000) showing increased-basophilia in all cells of the spermatogenic series (Sp, Sd, Sz). All cells appeared in an intact nature together with Leydig cells (LC) and Sertoli cells (SC), except some degenerated and apoptotic cells (curved arrow).

Sertoli cell and Leydig cells, abnormal destructed spermatozoa and intratubular vacuolization could be detected (Figs. 4, 5). Sections of testis of TiO2- exposed animals treated with vitamin E revealed less prominent histopathological alterations when compared with TiO2-exposed animals. Most of the seminiferous tubules were regular and compact with well- constructed germ cells (Fig. 6). The spermatogenic cells appeared with increased-basophilia together with intact SC and LC (Fig.7).

Immunohistochemical Results: ***Expression of caspase-3***

In control group, caspase-3 was expressed in cytoplasm of spermatogenic cells and few Leydig cells as brown color. The cytoplasm of germ cells was weakly- stained (Fig.8). Animals given vitamin E showed expression of casepase-3 in cytoplasm of spermatocytes nearly similar to that of control group (Fig.9). Mice exposed to TiO2-NPs showed an increase in expression of casepase-3 immunoreactivity in Leydig cells and in cytoplasm of most of germ cells (Fig.10). Testicular sections of mice exposed to TiO2 and treated with vitamin E showed a decrease in casepase-3 immuno-reactivity towards the normal status (Fig.11). The quantitative image analysis of slides confirmed the previous immunohistochemical results to record these percentage area of positivity; 16 ± 1.5%, 17.2±2.7%, 31± 2.5 %, 21± 1.4% for control, vitamin E-given, TiO2-intoxicated and TiO2+ vitamin E-treated groups(Fig. 12).

Biochemical Results

Testosterone Level in Control and Treated Groups: The biochemical testing of testosterone in sera of control animals recorded a normal mean value of 0.73±0.04 (ng / ml). In the main time, those of vitamin E- treated group recorded a value (0.65 ± 0.03 ng / ml) nearly similar to that of the control group. However, animals given TiO2-NPs recorded a significant decrease in testosterone level (0.22 ± 0.02* ng / ml) as compared to the control group. Animals exposed to TiO2-NPs and treated with vitamin E recorded a significant increase (0.60 ± 0.03** ng / ml) in testosterone as compared to the TiO2-group (Table 1, Fig.13).

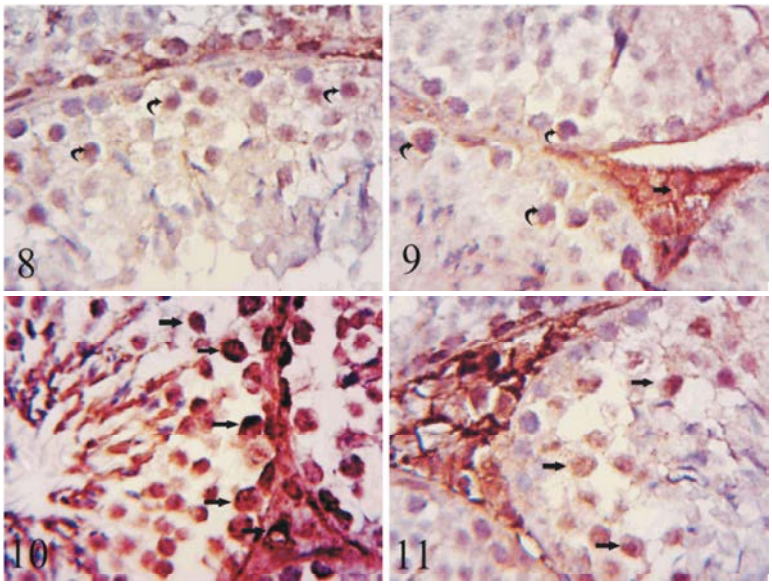

Fig. 8: Section of testis of control mice (immunostained for caspase-3, X1000) exhibiting a weak expression for caspase as brown deposits in the cytoplasm of gem cells (curved arrows).

Fig. 9: Section of testis of vitamin E-treated mice (immunostained for caspase-3, X1000) exhibiting a reaction nearly similar to that of the control (curved arrows).

Fig. 10: Section of testis of TiO2-treated mice (immunostained for caspase-3, X1000) exhibiting a strong reaction for caspase-3 in the cytoplasm of damaged spermatogenic cells and vacuolated Leydig cells (arrows).

Fig. 11: Section of testis of TiO2-exposed mice treated with vitamin E (immunostained for caspase-3, X1000) revealing a decrease in the immunoexpression of caspase-3.

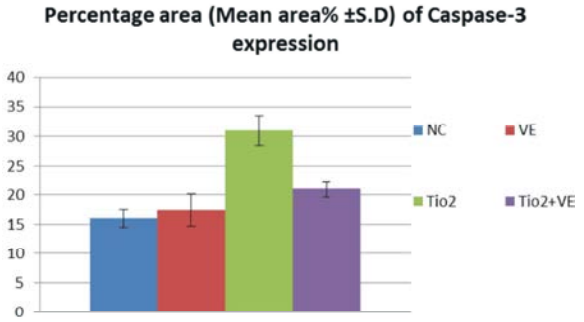

Fig. 12: Mean percentage area of caspase-3 expression in testis of control and treated mice.

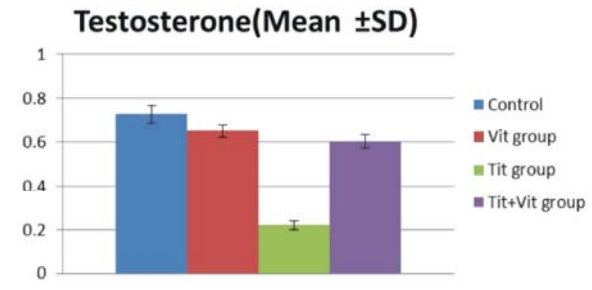

Fig. 13: Mean testosterone concentration in sera of control, treated mice

Table 1: Values of testosterone (ng/ml) in control and experimental groups

Animal groups	Mean±SD
Control group	0.73± 0.04
VE group	0.65±0.03
TiO2- group	0.22±0.02*
TiO2+VE group	0.60±0.03**

(*): significant decrease at P < 0.05 in comparison with control group
(**): significant increase at P < 0.05 in comparison with TiO2 group

Molecular Results

Comet assay for DNA Fragmentation: The application of Comet assay technology on bone marrow tissue of control mice revealed the normal DNA fragmentation with a tail DNA 1.131% and a tail moment of 1.504 (Fig.14-1, Table 2). Mice administered with vitamin E exhibited a tail DNA and tail moment nearly similar to that of control (1.063% and 1.328, respectively) (Fig.14-2, Table 2).The impact of TiO2- NPs administration on bone marrow DNA of mice is illustrated in table 2 and Fig.14-3, respectively. A pronounced increase in the tail DNA (2.695%) and tail-DNA moment (8.542) was shown in bone marrow of mice intoxicated with TiO2- NPs. Co-administration of vitamin E to TiO2-intoxicated rats protected their bone marrow from DNA damage as indicated by a

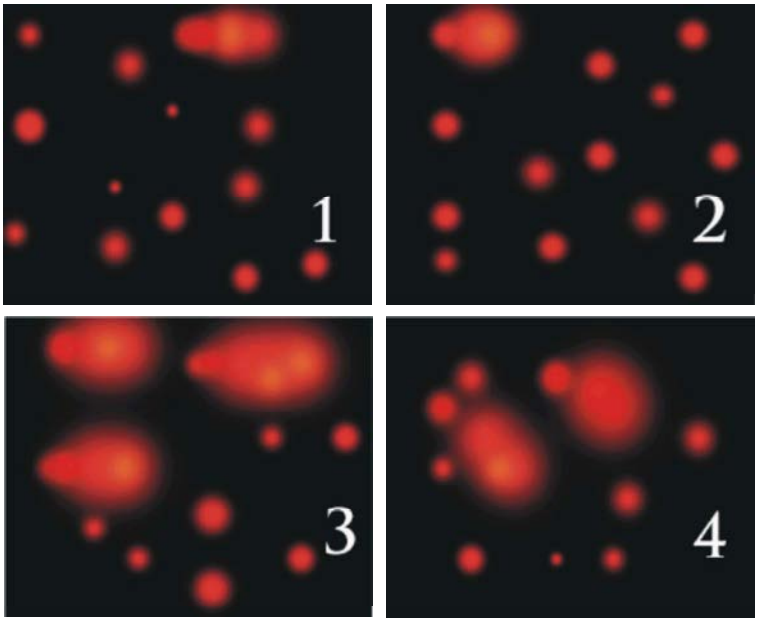

Fig. 14: COMET assay showing the extent of DNA damage in bone marrow tissue in TiO2-NPs- intoxicated mice and the effect of vitamin E treatment on the level of DNA damage. (1) normal control group, (2) group treated with VE (3) group intoxicated with TiO2-NPs (4) group intoxicated with TiO2-NPs and co-administered with vitamin E.

Table 2: Values of Comet parameters in control and treated groups of mice

Group	Tailed	Untailed	Tail Length	Tail DNA	Tail moment
	%	%	µm	%	Units
NC	4	96	1.33	1.131	1.504
VE	3	97	1.25	1.063	1.328
TiO2	14	86	3.17	2.695	8.542
TiO2+VE	10	90	2.87	2.440	7.001

significant decrease in tail DNA (2.440%) and tail -DNA moment (7.001) as compared with of TiO2 only (Fig.14-4, Table 2).

DISCUSSION

Nanotechnology has effectively improved a number of consumer products, through the manufacturing and use of nanoparticles. Therefore, assessment of the toxicological effects of nanoparticles on the human health and environment is inevitable because these nanoparticles through their small size, large surface area to volume ratio and other physicochemical properties are able to disrupt the biochemical and physiological functions of the cell. In the present study, the potential cytotoxic effect of TiO2-NPs was evaluated in the germ and somatic tissues (testis and bone marrow) of mice using histological, immunohistochemical, biochemical and molecular parameters. The data obtained in this study showed TiO2-NPs to be cytotoxic- and genotoxic in mice.

In the current study, the effect of TiO2 on the male reproductive system was investigated at the histopathological level in injected nano-sized TiO2 intraperitoneally at a dose of 1944 mg/kg b.w. for five days. The Tio2-exposed animals exhibited several histopathological changes in the testicular tissue which may be attributed to the oxidative stress due to Tio2 toxicity and may suggest a direct interference of these nanoparticles in the process of spermatogenesis. These alterations represented by irregular and faintly-stained seminiferous tubules, vacuolated interstitial tissue, sloughing of the germinal epithelium at some points, highly-damaged spermatocytes, degenerated Sertoli and Leydig cells, abnormal destructed spermatozoa and intratubular vacuolization. These results are in agreement with other investigators; similar observations such as oedema, reduced height of germinal epithelium, fairly numerous spermatocytes and few elongated spermatids, necrosis, vacuolation and congestion of the interstitial/testicular blood vessels and considered these

as an evidence of the toxicity of TiO2-NPs to the mouse male reproductive system were recorded [21]. Degenerative changes in the seminiferous tubules indicated that TiO2- NPs may directly interfere in the process of spermatogenesis [22]. A repeated exposure of TiO2-NPs for 90 consecutive days induced oxidative stress in testis, a depletion of reduced GSH and oxidized glutathione levels in the testis, as well as inhibition of SOD activity and a slight increase in catalase activity [23]. Reproductive organs are very sensitive to stress such as heavy metals, xenobiotics, microwaves and nanoparticles. It was recorded previously that NPs are able to cross the blood–testes and blood–brain barriers [24, 25]. Mouse Leydig cells possess a large capacity for the internalization of nanoparticles and these nanoparticles induce cytotoxicity and gene expression changes that lead to impairment of male mouse reproductive system [26, 27].

As regards the impact of TiO2 on the activity of caspase-3 in the testicular tissue, the present study recorded a marked increase in the percentage area of caspase -3 expression to provide an evidence for their apoptotic effect. These results are also in agreement with other authors; who studied the effects of intravenously injected TiO2-NPs on reproductive system. They recorded a significant decrease in antioxidant enzymes while a significant increase in lipid peroxidase was detected. Various functional and pathological disorders, such as reduced sperm count, increase in caspase-3, creatine kinase activity, DNA damage and cell apoptosis were observed in TiO2-NPs-exposed animals. Moreover, the testosterone activity was decreased significantly in a dose-dependent manner in the animals treated with TiO2-NPs as compared to control group [28].Studying the effects of beta-carotene (BC) on testicular germ cell apoptosis arising from TiO2-NPs. The authors found that expression of apoptotic related genes including Bid, FasL, caspase-3 and p38MAPK was significantly increased in TiO2-NPs treated mice. TUNEL assessments confirmed that the increase of apoptotic index of testicular germ cells in TiO2-NPs treated mice was reversed by BC. Beta-carotene pre-treatment could also effectively attenuate the expression of apoptotic related genes [29].

Concerning the effect of TiO2 exposure on the testosterone level of experimental animals, the current study recorded a significant decrease in this concentration due to NP-toxicity to reflect the impaired testicular function together with the structure. In this respect, other investigators are in agreement with our results; the intraperitoneal injection of TiO2 -NPs at doses of 50, 100 and 150 mg / kg for five days did not alter FSH hormone level and the number of spermatogonial cells, but the mean number of spermatocytes and spermatid cells decreased significantly in experimental groups. In addition at the highest dose LH and testosterone level decreased significantly [30]. Administration of 10 and 100 ppm of TiO2- NPs to mice in water for 14 days significantly reduced the amounts of FSH and LH in both male and female mice and testosterone in males [31]. In another study, intraperitoneal injection of TiO2- NPs with 30 and 50 mg/kg doses for 21 days resulted in significant increase and decrease in the level of LH and testosterone, respectively, with no change in the levels of FSH hormone and structure of testicular tissue [32]. Testosterone was produced in Leydig cells and in supported Sertoli cells. Low levels of testosterone might suppress spermatogenesis because they could lead to dysfunction of the Sertoli cells. Our results are also on line with the previous study which showed that exposure to carbon black (CB) nanoparticles induced the dysfunction of Leydig cells and consequently the fluctuation of serum testosterone levels in the CB nanoparticle-exposed group [33]. The effect of pubertal TiO2-NPs exposure on the synthesis of testosterone and spermatogenesis in Kunming male mice was studied by other investigators who reported that the percentage of spermatozoa abnormality in epididymis was markedly increased in mice exposed to TiO2-NPs; decreased layers of spermatogenic cells and vacuoles in seminiferous tubules were also observed in the TiO2-NPs-treated group [34].

Concerning the effects of TiO2 on the DNA fragmentation, the current study recorded a marked increase in the tail DNA moment of bone marrow cells to provide another evidence, at the molecular level, for the genotoxicity of these NPs in bone marrow cells. These results come on line with those of other authors; who reported that TiO2-NPs are photocatalytic and have been implicated to generate free radicals directly [35-37]. NPs are able to generate reactive oxygen species caused by secondary mechanical processes associated with inflammatory responses ultimately causing cell damage and eventually cell death [38, 39]. Likewise, the small particle size and large surface area of TiO2-NPs enables them to easily penetrate cells and cellular components thus interfering with several sub-cellular mechanisms and biomolecules causing lipid peroxidation, mitochondrial disruption, immune reactivity and protein damage [40]. The cytotoxicity and genotoxicity of TiO2-NPs at different doses and particle sizes to bone marrow cells after intravenous injection was studied and revealed that a single exposure to TiO2-NPs showed positive effect at 24 h only using micronucleus test and Comet assay to detect

DNA damage [41]. Cytotoxicity, DNA damage and apoptosis induced by TiO2-NPs (5 nm) in A549 cells was studied. The TiO2-NPs induction in DNA damage was observed by the comet assay. A significant induction of micronucleus formation determined by 4,6-diamino-2-phenylindole (DAPI) staining was also observed. Typical apoptotic morphological feature and apoptotic bodies in A549 cells induced by TiO2-NPs also observed [42]. The genotoxic potential of the TiO2 NPs by *in vitro* alkaline comet assay was investigated. DNA damage induced by the NPs was dose dependent and was significant at higher concentrations, the toxicity of the NPs is due to the generation of ROS thereby causing oxidative stress. Concerning the effect of using vitamin E in a dose of 100 mg/kg b.w., it was observed that it minimizes the frequency of histopathological lesions, decreased the immunoreactivity of caspase-3, increased the blood activity of testosterone and reduced the DNA fragmentation due to TiO2- exposure.

CONCLUSIONS

Our study has shown that TiO2-NPs have the ability to interact with mice genetic materials / machinery under the tested conditions (high dose of TiO2; 1944 mg/kg bw) and increased the DNA fragmentation. The exposure of mice to TiO2-NPs, also, affected the histological structure and function of the testicular tissue and increased the apoptotic index in these germ cells. This is of public health importance considering industrial and household applications of TiO2-NPs. Chemically induced genetic damage has been implicated in the etiology of many diseases; thus, there is an urgent need for stringent policies as regards the use of these nanoparticles in human consumable and cosmetic products as well as their disposal into the environment. So it is concluded that TiO2-NPs induce oxidative stress, which produce cytotoxic and genotoxic changes in sperms which may affect the fertilizing potential of spermatozoa and vitamin E minimizes this toxicity.

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