Aptoptotic and histopathological defects enhanced by titanium dioxide nanoparticles in male mice after short-term exposure

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ABSTRACT
Titanium dioxide nanoparticles (TiO\textsubscript{2}NPs) are commercially utilized in diverse fields. Therefore, the current study investigated the apoptotic and histopathological defects that were caused in male mice following intraperitoneal (i.p) injection of TiO\textsubscript{2}NPs for 28 days. Doses: 2.5, 5.0, 10.0 and 20.0 mg/kg body weight were applied (10 mice for each group). Results revealed that, lactate dehydrogenase (LDH) activity was significantly increased in homogenates of liver, spleen, kidney, lung, heart, and muscles of treated animals, respect to their controls. Also, significant alterations in acid and alkaline phosphatase (ACP and ALP) activities were reported. The dose 5.0 mg/kg exhibited a significant decline in cell viability of blood samples (74.9 %) (\(P_{0.05} = 0.0177\)), followed by 2.5 mg/kg (80.8 %), and finally the 10.0 mg/kg (81.8 %) with respect to control (96.3 %). Additionally, significant increases of expressed proteins of caspases-3 and-7 were noticed in cells of the treated animals. Ultrastructural investigations in sections of liver, kidney, lung and spleen of the treated animals showed significant defects, especially in the nucleus, mitochondria and rough endoplasmic reticulum (RER), compared to normal patterns of the control. Also, significant induction of nanoparticle (NPs)-phagolysosomes was visualized in sections of the treated animals. The present findings might provide evidence for the risk pattern of TiO\textsubscript{2}NPs in mammals after short-term exposure. So, TiO\textsubscript{2}NPs-based commercial products have now increased in the markets, and it is prudent to investigate their mammalian toxicology.

1. Introduction
Titanium dioxide nanoparticles (TiO\textsubscript{2}NPs) are one of the mainly produced NPs globally attributing to unique characteristics such as biocompatibility, resistance to corrosion, low cost, whitening, photocatalysis and easy to prepare [1-3]. Thousands of tons of TiO\textsubscript{2}NPs are utilized annually in different commercial application fields such as plastic, paints, and cement. Moreover, they are the most nanomaterials (NMs) used in therapy, drug delivery, engineering, agriculture, personal care products, cosmetics, sunscreens, electronics, wastewater treatment, filtration of gases, imaging agents, foodstuffs, and many other industrial applications [4-7].

Additionally, TiO\textsubscript{2}NPs have gained much importance for their prospective applications in biology and medicine [8]. However, several studies demonstrated that TiO\textsubscript{2}NPs are capable to be bioaccumulated in human organs, e.g. kidney, liver, lung, spleen, heart, and central nervous system causing oxidative stress and cell damage. These nanoparticles can reach and distribute into different vital organs through oral gavage, inhalation, and dermal pathways [9,10]. As previously reported, lung, liver, kidney, and heart were the primary sites of NPs [11]. Thus, the kidney is susceptible to the impact of TiO\textsubscript{2}NPs as a result of their filtration through urinary tubules [12]. Its function can be altered by many factors e.g. environmental contaminants, chemicals and drugs [13]. Han et al. [14] showed that delayed clearance of titanium (Ti) and low particle translocation might account for a portion of the increase in Ti in lung tissues. Titanium (Ti) accumulation in lung tissues negatively impacted the respiratory system constantly. In contrast, its concentrations in liver tissues rose, lead to a decline in the liver’s detoxication capacity. Its build-up in the kidney damages and inhibits the excretion process. Moreover, TiO\textsubscript{2}NPs induced hepatocyte apoptosis and
increased the inflammatory reaction [15]. Also, Li et al. [16] stated that TiO₂NPs increased gene expression levels related to the reactive oxygen species (ROS) and cytochrome-P450 (CYP1A). Numerous investigations have demonstrated that TiO₂NPs might potentially produce severe inflammations and pathological alterations in the lungs and the extrapulmonary organs [17]. Also, TiO₂NPs might induce liver and kidney function damage in mice, which is closely related to the increase of ROS and the decline of antioxidant capacity [18]. Associated with mentioned above, the present study was performed to confirm such findings through evaluation of histopathological and apoptotic effects of TiO₂NPs in some organs which are mostly rich blood such as liver, lung, spleen and kidney of male mice after intraperitoneal (i.p) injection for a short-term period.

2. Materials and methods

2.1. Chemical and reagents

Titanium dioxide nanoparticles (TiO₂NPs) were obtained for trustworthy characterization procedures from Nano-Tech Lab., Dream Land, 6th October City, Egypt. The other chemicals were obtained from Sigma Chem. Co., P.O. Box 14508 St. Louis, Missouri 63178, United States of America.

2.2. Characterization of TiO₂NPs

At an accelerating voltage of 80 kev, an aliquot of produced TiO₂NPs was studied using the Scanning Electron Microscopy (SEM) (JOEL, JSM 5300, Japan) with high resolution. A copper grid was used to cover the sample, then scanned to determine its size and form. X-ray Electron Dispersive Analysis (EDA) was performed using an X-ray Oxford detection unit (model 6647, England) connected to a scanning electron microscope (JOEL, JSM 5300, Japan). Stability of NPs were studied using dynamic light scattering (DLS) (DTS Nano v 5.2; Malvern Instruments, UK). Suspension of NPs was sonicated for 20 min at 40 W using a sonicator bath at ambient conditions.

2.3. Animals

Healthy male mice (mean: 30.0 ± 3.0 g) were obtained from the Institute of Public Health, Alexandria University, Egypt. They were allocated to plastic cages covered with metal grids and allowed to acclimate for two weeks under laboratory conditions, before being divided into experimental groups. The mice were provided with water and food ad libitum. Damanhour University Animal Health Care (DU-AHC) committee approved the experiments ethics (DMU-2020–0027). Also, the animals were handled according to the guideline of the National Institutes of Health for the care and use of laboratory animals (NIH Publications no. 8023, revised 1987).

2.4. Chemical administration and specimens sampling

The animals were randomly assigned into four treated groups and a control group, with ten mice in each group. Suspension of TiO₂NPs was i.p. administered at the following doses: 2.5, 5.0, 10.0, and 20.0 mg/kg bw once daily for 28 d. The control group was received an injection of vehicle (citrate solution). Animals were monitored daily and no animal perished during the research. The animals were sedated with 2 % phenobarbital (60 mg/kg, i.p.) and dissected for samples on the 29th d. Blood samples were drawn from the heart using a heparinized syringe and specialized blood tube. On the other hand, liver, spleen, lung, and kidney organs from the control and the greatest dosage groups were collected for histopathological examination.

2.5. Biochemical quantifications

One g of each tissue was homogenized for 15 s in potassium phosphate buffer pH 6.5 (1/10 w/v). For ten min, the samples were centrifuged at 5000 rpm. Dilute an aliquot of hemolymph with the same buffer (1/10 v/v). The supernatant was utilized to measure acid phosphatase (ACP) and alkaline phosphatase (ALP), while the homogenate was used to measure lactate dehydrogenase (LDH).

2.5.1. Lactate dehydrogenase (LDH)

An aliquot (100 µl) of homogenized tissue was added to 1 ml of the working solution which was prepared by mixing four volumes of Tris buffer pH 7.4 (80 mM), sodium pyruvate 1.6 mM, and NaCl (200 mM) with one volume of α-nicotinamide adenine dinucleotide (NADH) (240 µM). The absorbance change was recorded every min at 340 nm for 3 min, and the enzyme activity was presented as U/L [19].

2.5.2. Acid phosphatase (ACP)

The ACP enzyme activity was measured using specific biochemical kits (Bio Diagnostic Co., Germany). The absorbance of both sample and the standard against the blank were recorded at 510 nm, and the enzyme activity was presented as U/L [20].

2.5.3. Alkaline phosphatase (ALP)

The ALP enzyme activity was estimated using phenyl-phosphate as a substrate (N.S. Bio-Tec., kits, UK). So, the complex color of p-nitrophenyl phosphate was determined at 405 nm against the blank, and ALP enzyme activity was presented as U/L [21].

2.6. Cell viability

Fifty µls of whole blood sample were mixed with equal parts of 0.4 % trypan blue dye to obtain a 1–2 dilution by pipetting up and down in a cryo-vial. The mixture was incubated for up to 3 min at room temperature, and examined on a haemocytometer counter under the light microscope. Percentages of viable cells were calculated according to the formula:

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\text{% viable cells} = 1 - \frac{\text{No of blue cells}}{\text{No of total cells}} \times 100
\]

2.7. Immunohistochemistry of caspases

Immunohistochemistry investigations were done using the peroxidase technique to identify the cells expressing caspase-3 and caspase-7 proteins [22]. Four-millimeter sections of formalin-fixed paraffin-embedded tissues were created on poly L-lysine coated glass slides. Three changes of 5 min each were used to transfer the slides to xylene. Rehydration was accomplished using graded alcohol solution ranging from 100 % to 70 %, followed by 30 min under tap water. The antigen was extracted in citrate buffer for 20 min, followed by three 10-min washes with Tris-Buffered Saline (TBS). Incubation with 0.3 % hydrogen peroxide (H₂O₂) in methanol for 20 min inhibited endogenous peroxidase activity. The sections were then washed in TBS for 5 min and treated for 1 h in normal blocking serum to inhibit non-specific binding sites, followed by overnight incubation with primary polyclonal antibodies against both caspases at a dilution of 1:150 (Thermo scientific). Specificity and sensitivity of the antigen depend on the primary antibody employed. The slides were washed in TBS for 5 min, followed by applying of the primary antibody enhancer and incubation at room temperature for 10 min. Then, it rinsed four times in buffer and treated for 15 min at room temperature with a secondary antibody conjugated to horseradish peroxidase (HRP). They were rinsed as indicated before, incubated for color development in peroxidase substrate solution (peroxidase-compatible chromogen), and washed four times with deionized water.
water. Finally, the slides were counterstained with haematoxylin and cover slipped with an aqueous mounting media to prepare them for examination under a light microscope [23]. The positive immunohistochemistry stain for caspases-3 and-7 proteins was quantified and the color intensity was estimated using the Image J software program.

2.8. Ultrastructural investigation

The selected organs were dissected. Small bits of were fixed as quickly as possible with 2 ml of 2.5 % glutaraldehyde diluted in 0.1 M phosphate buffer, pH 7.2, and kept at 4 °C until needed. The fixed tissues were rinsed with 0.1 M phosphate buffer pH 7.2. They were then immersed in 1 % osmium tetraoxide (OsO₄) for 1–2 h at 4 °C and washed for 2 min with buffer. For 5 min, the samples were dehydrated using an ascending sequence of acetone concentrations (25 %, 50 %, 75 %, and 100 %). Following dehydration in 100 % acetone, propylene oxide was used to enter the tissues. Epon araldite was used to embed the specimen for 48 h at 48 °C. Capsulated samples were sectioned at a thickness of 20–30 nm using an Ultratome machine. Then, the sections were collected on metal mesh (grids) and dyed with toluidine blue. After staining the grids with 4 % uranyl acetate for 5 min, they were washed in a series of four beakers of clear water. The grids were stained for 5 min with 1 % of lead acetate, washed with water, and kept in a grid box until inspected [24]. The grids were viewed using a Transmission Electron Microscope (TEM) (JOEL 1400 Plus, Japan).

2.9. Statistical analysis

The ANOVA was used to compare the significant means of treatments using the Student-Newman-Keuls test. The means were assessed for significance using the least significant difference (LSD) technique at a probability of 0.05 [25]. Additionally, images mean data were evaluated for significance using the least significant difference (LSD) approach at the 0.01 and 0.05 probability levels.

3. Results

The selected i.p doses of TiO₂NPs did not show any toxicological

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**Fig. 1.** Characterization of TiO₂NPs (a) SEM of NPs scanned at 35,000 ×, (b) EDX pattern of TiO₂ and (c) DLS pattern to obtain NPs sizes suspended with capping agent in deionized water solution.
3.1. Biochemical responses

All treatments exhibited greater activity of LDH than control (Fig. 2). At dose 10 mg/kg bw, the enzyme activity displayed the following order: spleen > heart > liver > lung > kidney > muscles with mean values: 1167.5 (P0.05 = 0.01), 908.4 (P0.05 = 0.0001), 667.9 (P0.05 = 0.0006), 651.7 (P0.05 = 0.0002), 566.4 (P0.05 = 0.0002), and 555.7 (P0.05 = 0.015) U/L, respectively, with respect to their controls: 400.8, 219.1, 251.1, 184.4, 194.1, and 172.1 (P0.05 < 0.05) U/L for liver and kidney, respectively. Other organs heart with mean values: 764.1, 737.4, 682.9, 651.9, 523.7, 325.8 U/L, respectively. Acid phosphatase (ACP) activity in all treatments significantly was greater than control (Fig. 3a). The dose 10 mg/kg bw exhibited activities in the organs of treated mice were greater than the controls. The order of enzyme activity displayed the following descending order: kidney > spleen > liver > lung > muscles > heart with mean values of 196.7 (P0.05 = 0.0004), 193.7 (P0.05 = 0.0007), 189.9 (P0.05 = 0.017), 172.1 (P0.05 = 0.011), 139.3 (P0.05 = 0.048), and 117.6 U/L (P0.05 = 0.046), respectively, with respect to their controls: 194.1, 172.9, 184.4, 129.3, 93.8, and 63.7 U/L, respectively. In contrast, the dose 20 mg/kg bw exhibited mean values lower than control in the following descending order: kidney > liver > muscles > spleen > lung > heart with mean values of 179.4, 164.2, 52.4, 36.8 and 22.2 U/L, respectively. Regarding the ALP activity, significant alterations were noticed in all treatments (Fig. 3b). In lung homogenate, significant declines were noticed in enzyme activity (291.8 and 113.6 U/L) (P0.05 = 0.0000) for doses 10 and 20 mg/kg bw, compared with control (1214.4 U/L). However, these doses exhibited increases in ALP in the liver (254.5 and 183.6 U/L) with respect to control (165.9 U/L) (P0.05 = 0.0000). The dose 20 mg/kg bw exhibited a significant increase in ALP of the spleen and kidney: (144.5 U/L) (P0.05 = 0.0000) and (1142.0 U/L) (P0.05 = 0.0000) with respect to their controls (66.4 and 844.46 U/L). Other organs heart (P0.05 = 0.0032) and muscles (P0.05 = 0.0069) displayed ALP activity lower than controls (P0.05 = 0.01).

3.2. Cell viability

Viable cells of mice blood samples of treatments showed decline profiles than the control group (untreated) (Fig. 4). Dose 5 mg/kg bw exhibited significant decline in cell viability (74.9 %), with respect to control (96.3 %), followed by 2.5 mg/kg bw (80.8 %) and 10 mg/kg bw (81.8 %). The highest dose (20 mg/kg bw) exhibited the greatest cell viability (88.6 %) (P0.05 = 0.0177).

3.3. Caspase’s proteins expression

Expression of caspase-3 protein was weakly noticed in cells of control samples of lung, liver, spleen and kidney (Fig. 5). Significant increases in the expressed protein were noticed in cells of the treated animals. The renal section of the treated mice showed expression of the caspase-3 protein with a mean surface area of 0.106 µm² (3.13-folds) (P0.05 < 0.01) and integrated density 6.37-folds (P0.05 < 0.01) with respect to control group (Fig. 6). In the case of hepato-section, increased values of expressed protein area (0.557 µm²; 5.74-folds) (P0.05 < 0.01) and integrated density (4.28-folds) (P0.05 < 0.01) were noticed with respect to control. An increased expression of the caspase-3 protein was conducted in the lung section of the treated mice (0.839 µm²; 20.97-folds) (P0.05 < 0.01) concerning the control which did not exceed 0.04 µm². This section exhibited integrated density (17.48-folds) (P0.05 < 0.01) of control. Spleen section of treatments showed expressed protein (0.195 µm²; 2.83-folds) (P0.05 < 0.01) and integrated density 1.99-folds (P0.05 < 0.01) of control.

Embedded section of the control group showed slightly expressed protein of caspase-7 (Fig. 7). However, significant protein expression of caspase-7 was noticed in the treated animals. The renal section exhibited a mean surface area of 0.518 µm² (13.63-folds of control) (P0.05 < 0.01) with an integrated density of 5.33-folds (P0.05 < 0.01) of control. Hepato-section of the treated mice exhibited expressed protein with a mean surface area of 0.885 µm² (23.29-folds of control) (P0.05 < 0.01) and an integrated density of 36.71-folds (P0.05 < 0.01) of control. A slight expression of the protein was conducted in the lung section (0.08 µm²; 2.16-folds) (P0.05 < 0.05) with an integrated density of 2.74-folds of control. Expressed protein pattern of spleen samples exhibited surface area of 0.099 µm² (4.13-folds of control) (P0.05 < 0.05) and an integrated density of 3.91-folds (P0.05 < 0.01) (Fig. 8).
3.4. Ultrastructural alterations

The hepato-section of control mice that was visualized at $2500 \times$ showed nucleus (N) with normal distribution of chromatin, nuclei (Nu) and regular nuclear membrane (head arrow). Numerous distributions of mitochondria (M) and smooth endoplasmic reticulum (SER) were noticed (Fig. 9a). Section of TiO$_2$NPs-treated mice (20 mg/kg bw; 28 d) at the same magnification level showed pyknotic nucleus (N) (head arrow), nuclei (Nu), migrated and condensed chromatin and irregular nuclear membrane. Numerous distributions of mitochondria (M), lipid droplets (L), some tubules (arrow) and rough endoplasmic reticulum (RER) were noticed (Fig. 9b). The magnified field of control liver tissues at $600 \times$ showed mitochondria (M) with dark dense cristae, lysosomes (Ly) as well as other organelles, which were indicated in Fig. 9a (Fig. 9c). Section of the treated mice at the same magnification level showed irregular nuclear membrane, migrated and condensed chromatin and other defects as indicated in Fig. 9b (Fig. 9d). The magnified field of treated liver section at $800 \times$ showed phagolysosomes in large membrane endosomes filled with NPs, rough endoplasmic reticulum (RER) with extensive tubules and formation of myelin (head arrow), and mitochondria (M) with dark dense cristae (Fig. 9e).

The lung section of control mice at $2500 \times$ magnification level indicated normal nucleus (N) with normal distribution of chromatin, extensive microvilli (MV) brush (double arrow), smooth endoplasmic reticulum (SER), normal distribution of lamellar bodies (LB) and alveolar lumen (AL), blood capillaries (B) and multivesicular transport vesicle (MvTV) (Fig. 10a). Section of the treated mice at the same magnification showed enlarged nucleus (N) with multi-nuclei (Nu) formation, dilated microvilli (MV) brush (double arrow), intracellular spaces, swollen mitochondria (M), disrupted and irregular vesicular transport vesicle (MvTV), rough endoplasmic reticulum (RER), and alveolar lumen (AL). A lack of lamellar bodies (LB) and bronchoalveolar junctions was noticed (Fig. 10b). At high magnification (6000 ×) of control section, all above organelles and mitochondria with tubulo-vesicular cristae were noticed (Fig. 10c). At the same magnification, the section of the treated mice showed the formation of fibrosis (Fib), dilated microvilli (MV) brush (double arrow), swollen mitochondria (M), vacuolation of some alveolar lumens (AL) with NPs (head arrow), and rough endoplasmic reticulum (RER). Migrated and condensed chromatin were also noticed (Fig. 10d). The result of the same field of

![Activities of (a) acid phosphatase (ACP) and (b) alkaline phosphatase (ALP) (U/L) in organ’s homogenates of intraperitoneal TiO$_2$NPs-treated mice (10 and 20 mg/kg b. w) for 28 d. Vertical bars indicate standard errors (n = three animals each). Values with no common superscripts are significantly different (LSD multiple range test, P < 0.05).](image-url)
section when magnified at 10,000 × indicated formation of fibrosis (Fib), vacuolation of macrophages with NPs (head arrow), swollen mitochondria, loss of cristae and developed paracrystalline inclusions (M), rough endoplasmic reticulum (RER) and some of the mitochondrial inclusions (striped arrow) were noticed (Fig. 10e). The magnified field of control section when magnified at 10,000 × showed the nucleus (N) with regular nuclear membrane (head arrow), numerous distribution of mitochondria (M) around the nucleus, podocytes (P), smooth endoplasmic reticulum (SER), and regular microvilli (MV) (Fig. 11a). Section of the treated mice at the same magnification indicated rough endoplasmic reticulum (RER), increased vacuoles (V), some patterns of enlargement of mitochondria, and large-fused mitochondria. Dilated podocytes (P) and fusion of microvilli (MV) were noticed. Nucleus (N) with condensed and migrated chromatin was also noticed (Fig. 11b). Magnifying the field of control section at 6000 × showed the above organelles in Fig. 11a and the brush of microvilli (MV) (Fig. 11c). Field of the treated mice at the same magnification indicated large-fused mitochondria (M) and woolly densities in their interior. Also, it indicated migrated chromatin (Fig. 11d). At 12,000 ×, the magnified field of the treated mice illustrated precipitation of NPs in lysosomes (Ly) (head arrow). Significant defects in cytoplasm causing vacuoles (V), disrupted mitochondrial membrane and disturbed concentric cristae were noticed (Fig. 11e).

Electron micrograph of sections of the spleen of control animals at 4000 × showed normal lymphocyte (Lym) and macrophages (Mp), regular cellular membrane (arrow), numerous distribution of mitochondria (M), lysosomes (Ly) and Golgi bodies (G) (Fig. 12a). Section of the treated mice at the same magnification level showed mitochondria (M) with light dense cristae, rough endoplasmic reticulum (RER), lysosomes (Ly) (arrow), vacuoles (V), Golgi bodies (G) and destructed cellular membrane (head arrow) (Fig. 12b). The magnified field of control section at 8000 × showed lymphocyte (Lym), reticulocytes (R), reticular fibers (RF), mitochondria (M), and lysosomes (Ly) (Fig. 12c). However, the section of treated mice at the same magnification showed Golgi bodies (G), lysosomes (Ly), mitochondria (M) with light dense cristae, rough endoplasmic reticulum (RER), vacuolated cytoplasm (V), reticulocytes (R), and reticular fibers (RF) (Fig. 12d). Magnifying the section of the treatment at 12,000 × showed vacuoles (V), phagolysosomes (Ly) with NPs, blood capillaries (B), reticulocytes (R), reticular fibers (RF), and rough endoplasmic reticulum (RER) (Fig. 12e).

4. Discussion

In the current study, i.p injection of the doses of 10 and 20 mg/kg bw of TiO₂NPs induced biochemical changes in the male mice through increased ALP, ACP and LDH activities in the organ’s homogenates indicating damage of their functions. Also, defects were observed in the examined organ’s sections for immunohistochemistry and ultrastructure profiles. In this study, injection by i.p route was chosen, because the chemical absorption is excellent and following quickly i.p injection, owing to the peritoneum’s dense blood and lymph capillaries and wide surface area, which allow it to enter the circulation rapidly. Additionally, such this method avoids the frequent gastrointestinal complications in case of oral treatment [26]. Similarly, Liu et al. [27] observed marked distribution of liver function caused by i.p injection of the doses: 50, 100, and 150 mg/kg bw of TiO₂NPs (5 nm) for 14 d. Also, Alarifi et al. [28] reported a significant increase in aspartate aminotransferase (AST) and ALP activities with manifestations of apoptosis in liver cells in the form of small, condensed nucleus and chromatolysis with granular clumps of heterochromatin with irregular nuclear membrane, following i.p injection of TiO₂NPs.

In fact, the biological activity and biokinetics of NPs are dependent on variety of criteria, including their size, shape, chemistry, crystallinity, surface features (area, porosity, charge, surface modification, and coating), aggregation state, biopersistence, and dosage. Such these factors explain that biological reactions (translocation) through epithelia and other organs, activate oxidative stress, binding to proteins and receptors, and localization in the cellular organelles such as mitochondria and lysosome. The changes in the surface state might result in irreversible aggregation, which has a major influence on dispersion and distribution [29]. This concept was indicated in the present findings, where the highest dose (20.0 mg/kg bw) might be aggregated after i.p injection. This pattern resulted in low apoptotic effects on blood cells causing cell viability (88.8 %), against control (> 96 %).

The primary mechanism of TiO₂NPs toward biological toxicity in people and animals has been postulated to be an increase of ROS build-up [30,31], although this has not been proved. Hu et al. [32] demonstrated that oral treatment of TiO₂NPs resulted in not only increased ROS levels, but also endoplasmic reticulum (ER) stress in mice. The consequences of ER stress are complex, and its intermediates either activate or deactivate critical genes involved in ROS generation including the mitochondrial respiratory chain, the arachidonic acid pathway, the CYP1A-family, glucose oxidase, amino acid oxidases,
xanthine oxidase, NADPH/NADPH oxidases, and nitric monoxide (NO) synthases [31,33]. Additionally, TiO$_2$NPs can be transported into cells via phagocytosis, where they generate ROS that shift the cellular redox balance toward oxidation, resulting in abnormal function or cell death, lipid peroxidation (LPO), and altered gene expression. The TiO$_2$NPs might also bind to the mitochondrial membrane, where they increase the electron transport chain within the mitochondria, thereby activating the mitochondria-mediated apoptotic pathway [34,35]. Additionally, Ti might act through particular intracellular signaling pathways resulting in the production of certain proteins and macromolecules [36]. Thus, the histopathological changes, hepatocyte apoptosis, liver function impairment, and inflammatory cascade induced in the mouse liver following i.p injection of high doses of NPs as TiO$_2$ (5 nm) were closely related to significant changes in the mRNA and protein expression of several pro-inflammatory cytokines [37,38].

Apoptosis (programmed cell death) is a complicated biological
process that plays a critical role in controlling cell survival by eliminating damaged or diseased cells [39]. It refers to any kind of cell death mediated by an intracellular death programme, regardless of the technique used to begin it [40]. Expressed proteins (caspases-3 and-7) are well-known apoptotic indicators that may be triggered by both extrinsic and intrinsic apoptotic pathways resulting in DNA degradation [41–43]. Such this concept was indicated in the present study (Figs. 5–8) and is in accordance with a previous finding, where apoptosis in rat hepatocytes increased dose-dependently and time-dependently after i.p administration of various TiO$_2$NPs dosages. Sporadic, patchy, and well-defined necrosis were also seen in some hepatocytes, which might have been caused by oxidative stress and by glutathione deficiency in these cells [28]. According to Johnston et al. [44], the difficulties in removing these NPs in vivo might led them to be accumulated in the liver and the development of a hepatic lesion. This pattern was indicated in the present work, where treated animals showed phagolysosomes filled with NPs in the sections under TEM observation. Also, Park et al. [45] demonstrated that TiO$_2$NPs promoted oxidative stress and apoptosis in cultivated BEAS-2B cells in *in vitro* research.

The TiO$_2$NPs have been proven to produce severe inflammatory damage and pathological alterations in the lung and other organs in previous research. The present data are in accordance with different previous investigations. For instance, rats were infiltrated by macrophages and neutrophils after inhalation of TiO$_2$NPs [17]. Additionally, on 7th d after tracheal instillation of 5 nm TiO$_2$NPs, macrophage proliferation was detected, as was substantial particulate accumulation in the lung tissue gaps and the alveolar cavity [46]. Additionally, a dose-dependent substantial increase in the levels of initiator and effector caspases (caspase 9 and-3, respectively) initiates a cascade of events that terminate in cell death [47]. According to some investigators, TiO$_2$NPs increased the expression of various cytokines such as tumor necrosis factor-, interleukins (ILs), particularly IL-6 and IL-8, and chemokines, all of which are chemotactic for leukocytes and other cells involved in the inflammatory reaction and subsequent kidney damage [48]. Thus, the mononuclear cell infiltrations in this group were indicative of inflammatory cell infiltrations involved in the inflammatory response. Additionally, cerium deposition in the liver is associated with increased oxidative stress and cellular death lasting 1, 3, 14, 28, 56, or 90 d after a single inhalation of cerium oxide nanoparticles (CeO$_2$NPs). Cleavage of caspase 3 seemed to increase on 1$^{st}$ and 3$^{rd}$ d of treatment, which was consistent with the pattern seen for caspase-9 [49].

With respect to histopathological results of the present study, numerous hepatic, renal, lung and spleen alterations were detected following i.p injection of TiO$_2$NPs as presented in Figs. 9–12. These might be owing to their ease of entry into the organ’s tissues and cells, where TiO$_2$NPs interact with proteins and biological processes, generating ROS that may contribute to these modifications. For example, histological investigations of liver cells verified the toxicity induced by
TiO$_2$NPs and indicated that the central vein was packed with dilated blood vessels (angiectasis) and mononuclear cells. These lesions were regarded as indicators of hepatic damage [15,47,50]. After four weeks of tracheal perfusion with TiO$_2$NPs, pathological damage to the lung tissue and the renal fibrosis were seen [51,52]. The epithelial lining of the renal cell of rats subjected to TiO$_2$NPs (252 mg/kg for 24 h or more) showed cloudy swelling [53]. This change might imply that acute renal damage caused membrane dysfunction, resulting in water leakage and build-up owing to TiO$_2$NPs toxicity, cytoplasmic degeneration, and macromolecular crowding [54].

The current data are in accordance with previous histological studies which revealed that nephrotoxic effect was associated with TiO$_2$ exposure [2,12,55]. The tubular degeneration was supported by a significant increase in caspase-3 reaction. Some studies supported this finding and showed that TiO$_2$NPs exposure could induce apoptosis in different types of cells or organs e.g. liver, spleen and kidney [2,56]. A previous study indicated that the nephrotoxic impact of TiO$_2$ is mediated by the nephrotoxic action of O$_2$ that was turn mediated by the production of oxidative stress products such as various cytokines and ROS, which simultaneously decrease cellular antioxidants. These species caused damage and inactivation of structural proteins, enzymes, and ion pumps, increased LPO, impaired cell functioning, inflammation,

Fig. 7. Photographs of paraffin-embedded sections through (a) control kidney, (b) treated kidney, (c) control liver, (d) treated liver, (e) control lung, (f) treated lung, (i) control spleen and (g) treated spleen of male mice intraperitoneal injected with TiO$_2$NPs (20 mg/kg b. w; 28 d) illustrate immuno-stained with caspase-7 antibody and counter stained with hematoxylin. The strong positive expression was observed with brown color (arrow) [40 ×].
cytolysis, interstitial fibrosis mutation, and DNA damage and death [57, 58].

Kidneys are particularly vulnerable to toxic substances due to their high blood supply. They receive about 25 % of the cardiac output [40]. They eliminate deleterious substances from the body; therefore, NPs in the systemic circulation can be filtered by renal clearance [59]. The International Program on Chemical Safety for TiO$_2$ declared that ingested TiO$_2$ is eliminated mainly through urine [60]. Thence, the kidney is viewed to be one of the vital organs susceptible to the deleterious effects of TiO$_2$. As stated by Altayeb et al. [61], i.p-treated rats with 150 mg/kg bw of TiO$_2$NPs for 28 d revealed congested dilated glomerular capillaries, mononuclear cell infiltrations and fibrosis between degenerated tubules and renal corpuscles. This finding clarified that TiO$_2$NPs after entry into cells lead to initiate inflammatory mechanisms, apoptosis and generate the oxygen free radicals which harm the nucleus and mutate DNA, and also alter the functions of cells [2,58].

Accumulation of NPs as electron-dense material and dilated RER was seen in the cytoplasmic matrix and organelles including mitochondria. Particle dispersions were observed in the Kupffer cell. Such this accumulation was more apparent in the perinuclear membrane and the inter nuclear matrix. This build-up might be a result of the smaller particle sizes that enter the cells more easily. Additionally, TiO$_2$ has a long half-life in vivo making it difficult to excrete and eliminate, implying that particle build-up in the liver must results in hepatic lesions [62]. As stated in the literature, NMs are able to make a considerable influence on the behavior and characteristics of macromolecules, cells, and body parts due to their physical and chemical properties [1]. Numerous investigations, including Zucker et al. [63] and Shukla et al. [64] substantiated these conclusions. Additionally, TiO$_2$NPs were found in mostly membranous vesicles that resembled phagosomes or endosomes, as well as many dense lysosomes of varying sizes in hepatocytes. These findings corroborate those of Mano et al. [65], Meena [66], and Schoelermann et al. [67], where they demonstrated that TiO$_2$NPs may enter cells via endocytosis and can be transported across cells via endosomes and lysosomes. Teubl et al. [68] have shown that TiO$_2$NPs are present in both vesicles and the cytoplasm in a free-floating state. According to Kettler et al. [69], macropinocytosis, receptor-mediated endocytosis, and phagocytosis are the primary processes of NPs uptake. Similarly, Gaiser et al. [70] reported that silver nanoparticles (AgNPs) were concentrated within membrane-bound vesicles implying either effective removal from the cytoplasm following membrane diffusion and incorporation into phagosomes or lysosomes, or uptake via mechanisms involving particle membrane incorporation (e.g., endocytosis). Yanglong et al. [71] established the cytotoxicity of TiO$_2$NPs by their conversion to ionic Ti in the lysosomes.

The destruction of the bile canaliculi microvilli following TiO$_2$NPs uptake and accumulation of ROS as detected in the current results, might be due to oxidative stress being the primary cause of ultrastructure changes in the hepatocytes such as swelling. Liver cells are the primary organ that excrete toxic substances perforations and disintegration of mitochondria, RER, and irregularities in the bile canaliculi. According to Shakeel et al. [3], bile is a flu nuclear membrane containing both

![Fig. 8. Estimation of images of organs of male mice sections for (a) surface area (µm$^2$) and (b) integrated density of expressed caspase-7 protein in control, and TiO$_2$NPs-treated animals (20 mg/kg b. w) for 28 d. * and ** are the significant differences at 5 % and 1 % levels of probability according Fisher test.](image-url)
condensed and fragmented chromatin. It is secreted by liver cells and assisted the body in splitting fat and processing cholesterol. Because oxidative stress increases the LPO of membranes and aids in the elimination of toxins, if the bile duct is injured, the ACP of mitochondria and RER permeability can become clogged and leak out of the liver. The mechanism by which ROS caused myelin formation and cellular membrane peroxidation has been confirmed by Long et al. [72]. They demonstrated that constituents ROS and intracellular calcium disturbance TiO$_2$NPs can bind to mitochondrial membranes causing concentration and they are related factors that confirm each other with TiO$_2$NPs collapse of the mitochondrial membrane electron transport chain and toxicity. Second, the induction of oxidative stress and the subsequent generation of reactive O$_2^-$ result in structural damage to the mitochondria, causing the permeable pore of its membrane to open and electrical, and mechanical properties of TiO$_2$NPs to be activated in comparison to apoptotic or necrotic pathways. Another proposal made by Fröhlich and Roblegg [73], where NPs have charged nuclei and attach to DNA, altering the reactive surface of the genetic information transfer, hence increasing the absorption of other undesirable chemicals and the cascade of inflammatory events.

As mentioned in the literature, TiO$_2$NPs accumulated in the abdominal cavity of the majority of the studied animals. In another view, it was stated that NPs when lose their qualities and characteristics after reach the body become mildly toxic or non-toxic. Many NPs are converted to hunks in mammals as a result of attaching them with organic molecules in the body. As a result, several unique qualities such as size, shape, surface charge, and capacity to permeate most organs and tissues are lost [74]. Another discovery indicates that when NPs reach the blood stream, they may be eliminated by a variety of methods based on their absorption pathway and surface characteristics. The most typical method of the removal is via the kidneys. This procedure entails the filtration of blood via the glomeruli of the nephron of the kidney [75]. Xiong et al. [76] discovered a link between the cytotoxicity of TiO$_2$NPs and particle size, where tiny particles have a greater specific surface area, which may absorb more biomolecules in the environment. Biological effects may be related to particle size, dry weight, and surface...
area. Typically, TiO$_2$NPs enter the body via the skin, breathing, or digestion. For two weeks, mice were administered a TiO$_2$ suspension (5 mg/kg bw), and TiO$_2$ was shown to accumulate in the liver, spleen, kidney, and lung tissues [77]. Shinohara et al. [78] found that after 6 h intratracheal exposure of TiO$_2$NPs, 94 %, 2.0 %, 0.17 %, and 0.023 % Ti were detected in the liver, spleen, lung, and kidney, respectively. After inhalation of 2, 10, and 50 mg/m$^3$ of TiO$_2$NPs for 6 h daily for 5 d, the NPs burdens in the lung were 118.4, 544.9, and 1635 µg, respectively. Following a 16-d recovery period, NPs loads in the lung reduced to 25, 144.5, and 295 µg, respectively [17]. Oberdrster et al. [79] showed that individuals exposed to fine particles (250 nm TiO$_2$) had retention half-lives of 117 d, while those exposed to ultrafine particles had retention half-lives of 541 d (20 nm TiO$_2$). In a similar view, multiple investigations revealed that TiO$_2$NPs accumulated in a variety of organs of the experimental animals, most notably the liver, kidney, spleen, lymph node, lungs, and heart were unable to be eliminated from the liver and kidney until 15 d following treatment [62,80]. Han et al. [14] mentioned the sluggish clearance of Ti and low particle translocation may help explain why Ti levels were greater in the lung tissue than in liver and kidney.

Finally, with the growing number of applications more concerns are elevated for the potential risk of TiO$_2$NPs exposure in human health and the environment [7]. These problems must be studied to establish scientific evidence supporting the safe use of nanotechnologies. Given the rising market presence of TiO$_2$NP-based commercial products, it is crucial to study their destiny in the mammalian system.

![Fig. 10.](image-url)
5. Conclusion

The present study provides evidence for TiO$_2$NPs toxicity on mice after i.p injection during short-term exposure. Alterations in the activities of LDH, ACP and ALP might introduce patterns for organ dysfunction parallel with other defects. Also, the increased expressions of caspase’s proteins and the decline in cell viability of blood samples might be recognized as biomarkers for cell death patterns. Moreover, ultrastructural investigations of selected organs showed significant defects compared to the normal controls. Such measurements may provide suitable diagnosis for NPs toxicity and their accumulation in organs that might induce adverse effects. So, the toxicity of TiO$_2$NP-based products (food and pharmaceuticals) should be thoroughly studied to understand their fate in the mammalian system.

Fig. 11. Electron micrographs illustrate (a) renal section of control mice showing normal nucleus (N), mitochondria (M), podocytes (P), smooth endoplasmic reticulum (SER), regular microvilli (MV), (b) section of TiO$_2$NPs-treated mice (20 mg/kg b. w) for 28 d at 2500 × showing rough endoplasmic reticulum (RER), vacuoles (V), enlargement and large-fused mitochondria (M), dilated podocytes (P), and fusion of microvilli (MV), (c) magnified fields at 6000 × of control documents the above organelles in section a, (d) magnified field of the treated mice at 6000 × indicates organelles in section c as well as large-fused mitochondria (M) and woolly densities with their interior, and (e) magnified field at 12,000 × of the treated mice shows precipitation of NPs in lysosomes (head arrow), vacuoles (V), rough endoplasmic reticulum (RER) and destructed mitochondria (M). [Glutaraldehyde-OsO$_4$ fixed uranyl acetate lead citrate stained preparation].

Ethics approval and consent to participate

Applicable.

Consent for publication

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Supplementary data

No supplementary data are provided.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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