Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2023.078

RESEARCH ARTICLE

# Effects of Oral Administration of Titanium Dioxide Nanoparticles on Epididymal Semen Quality, Testicular DNA Damage and Histopathology in Male Rats

Serdar Aktas*[1], Serdal Ogut[2], Mehmet Fatih Bozkurt[3] and Sanan Raza[4]

[1]Aydin Adnan Menderes University, Faculty of Veterinary Medicine, Aydın, Turkiye; [2]Aydin Adnan Menderes University, Health Sciences Faculty, Department of Nutrition and Dietetics, Aydın, Turkiye; [3]Afyon Kocatepe University, Faculty of Veterinary Medicine Department of Pathology, Aydın, Turkiye; [4]Department of Clinical Sciences (Theriogenology Section), University of Veterinary & Animal Sciences, Jhang Campus, Pakistan.
*Corresponding author: saktas@adu.edu.tr

ARTICLE HISTORY (23-219)

Received: June 3, 2023
Revised: July 25, 2023
Accepted: August 2, 2023
Published online: September 05, 2023

**Key words:**
Titanium dioxide
Sperm parameters
Oxidative stress
Nanoparticles
DNA damage
Histopathology

**ABSTRACT**

This study aimed to investigate the effects of orally administered Titanium dioxide ($TiO_2$) nanoparticles on reproductive health in rats. A total of 20 rats, aged 2 to 3 months, were divided into two groups. One group (n=10) received a dosage of 250mg/kg bw $TiO_2$ (15 nm particle size) daily for 28 days, while the other group (n=10) received only tap water as a control. At the end of the study, rats were euthanized, epididymal sperm samples were collected and testicular tissues were examined using both hematoxylin-eosin (H&E) staining and immunohistochemical techniques to assess the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) and hypoxia-inducible factor 1α (HIF-1α). Oxidative stress levels were measured by Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Malondialdehyde (MDA) levels, and Glutathione peroxidase (GSH-Px) activity. The results indicated that the oral administration of $TiO_2$ nanoparticles significantly impaired sperm quality parameters ($P<0.01$). In the $TiO_2$-treated group, seminiferous tubule diameter and epithelial height were lower compared to control group ($P<0.05$). Furthermore, the levels of 8-OHdG, indicating DNA damage, and HIF-1α, a marker of tissue hypoxia, were increased in the testicular tissue. Additionally, the levels of oxidants (TOS and MDA) were increased, while antioxidant markers (TAS and GSH-Px) were decreased in $TiO_2$-treated group. In conclusion, oral administration of $TiO_2$ nanoparticles induces DNA damage, adversely affects epididymal semen quality, and elevates oxidant levels in testicular tissue. These results underscore the detrimental effects of $TiO_2$ nanoparticles (<100nm, especially <25nm) on reproductive health and highlight the importance of further investigation into their potential adverse effects in humans.

**To Cite This Article:** Aktas S, Ogut S, Bozkurt MF and Raza S, 2023. Effects of oral administration of titanium dioxide nanoparticles on epididymal semen quality, testicular DNA damage and histopathology in male rats. Pak Vet J, 43(4): 689-694. http://dx.doi.org/10.29261/pakvetj/2023.078

## INTRODUCTION

Titanium dioxide ($TiO_2$), a compound consisting of titanium and oxygen, is abundantly found in the earth crust. It is widely used in industries due to its desirable properties, such as whitening, ultraviolet reflection and opacification (Warheit and Donner, 2015). This compound is widely used in various sectors including paint production, chewing gum and confectionery, medicine, food products and sunscreen creams (Sungur *et al.,* 2020). The toxicity of $TiO_2$ is influenced by the size of its particles. Larger particles with size >150 nm are generally considered to be less toxic, whereas smaller nanoparticles (<100nm in size) possess a higher potential for toxicity due to their relatively larger surface area and their ability to induce DNA damage upon ingestion (Ali *et al.,* 2019). A previous study has demonstrated that $TiO_2$ exhibits dose-dependent toxicity in non-mammalian organisms such as bacteria, fungi, algae and aquatic organisms, with the presence of UV light in the environment exacerbating its toxicity in bacteria (Hou *et al.,* 2019).

$TiO_2$ is a widely produced and used chemical, with particle sizes ranging from 30 to 410 nm can expose individuals to varying levels of exposure through food and inhalation (Sungur *et al.,* 2020). This compound is of particular concern for workers who face chronic exposure.

Animal studies have demonstrated the potential for $TiO_2$ to cause tissue and organ damage, including its accumulation in liver and kidney tissue (Fabian *et al.,* 2008). Nanoparticles of $TiO_2$ (<100 nm size) have been found to bond with DNA and cause cytotoxicity in rat liver cells (Han *et al.,* 2021). This compound has also shown cytotoxic effects on neuroglia cells (Liu *et al.*, 2013), increased apoptotic activity in testis and prostate tissue (Shahin and Mohamed, 2017) and adverse effects on blood and liver tissues after 28 days of exposure to 150mg/kg/bw by subcutaneous injection in rats (Shakeel *et al.*, 2016). Oral administration of $TiO_2$ nanoparticles to rats for 5 days (0, 1, 2 mg/kg bw) has also been found to accumulate in ovaries and spleen (Tassinari *et al.*, 2014), while long-term administration of $TiO_2$ (2.5, 5.0 and 10.0mg/kg/bw for 90 consecutive days) to male mice has resulted in altered sex hormones and down-regulation of genes in spermatozoa (Gao *et al.*, 2013).

The impact of environmental pollution on reproductive health and its potential contribution to declining fertility rates in humans and livestock is of great concern. In recent decades, there has been a decline in sperm quality and quantity, along with an increase in infertility in humans (Calsen *et al.*, 1992). While the role of reproductive toxicants in this regard is plausible, the extent of adverse effects of $TiO_2$ on fertility remains unclear. Moreover, the particle size, exposure time, and dose of $TiO_2$ can influence its toxicity and accumulation in tissues and organs. Furthermore, the toxicology mechanisms of $TiO_2$ are not fully understood. To better understand the impact of $TiO_2$ on male fertility, this study aimed to assess the effects of oral administration of 250mg/kg bw of $TiO_2$ nanoparticles (Particle size: 15nm) to male rats for 28 days, evaluating epididymal sperm quality parameters, oxidative stress, DNA damage, tissue hypoxia, and histopathological effects on testicular tissue. The results of this study will provide valuable insight into the potential impact of $TiO_2$ on reproductive health and the need for further research in this area.

## MATERIALS AND METHODS

**Animals:** The experimental study was started after obtaining the permission of the local ethics committee. In the study, 2-3-month-old Spraque Dawley rats (n=20), weighing 250-300g and obtained from the Experimental Animal Production Centre (Kobay AŞ/Ankara, Turkey) were used. These rats were randomly divided into 2 groups, control group (n=10) and $TiO_2$ group (n=10). They were housed at 22°C and 40-50% humidity and were given feed (standard rat diet) and water ad-libitum. Rats of $TiO_2$ group were given Titanium dioxide anatase (99.5% 15nm, Cat No: US3492, US Research Nanomaterials, Houston, USA) at the dose of 250mg/kg bw daily for 28 days with gavage (Harward Apparatus, 16G) application. $TiO_2$ solution was prepared by dissolving in 2 ml of tween-60 buffer solution in warm water. Drinking water was administered equally to the control group rats by gavage.

**Collection of semen and testicular samples:** After 28 days, rats were euthanized by cervical dislocation under ketamine (50mg/kg, Keta-Control, Doga Ilaç, Turkey) and xylazine (5mg/kg, VetaXYL, Vetagro, Turkey) anaesthesia. Testes (right and left together) were taken and weighed on a precision scale. Afterwards, cauda epididymal semen samples were taken in sufficient quantity with a micropipette for examination.

**Evaluation of semen quality parameters:** Rat semen samples taken from cauda epididymis were taken into 1.0 ml sterile glass petri pool containing PBS (Posphat buffer solution) for dilution, as described previously (Si *et al.,* 2006). Then, 3µl of these samples were taken and placed on a heated (37°C) bastion and examined under a phase contrast microscope for physical characteristics. Samples were also examined using a computer-assisted semen analyzer (CASA, Sperm Class Analyzer, Barcelona, Spain) by selecting 5 different microscope fields. Parameters including Curvilinear Velocity (VCL), Linearty (LIN), Straightness (STR) and Aerage Path Velocity (VAP) were recorded under CASA. After staining the preparations prepared from semen solution, 200 cells were counted under the microscope at 100X magnification.

**Determination of membrane integrity and live sperm:** For membrane integrity testing in male germ cells, the hypo-osmotic swelling test (HOST) was applied, as described earlier (Correa and Zavos, 1994). For this test, 20µl of semen sample was incubated with 180µl of fructose solution at 37°C for 15 minutes. At the end of the period, values were determined by counting at least 200 spermatozoa at 40X magnification. Sperm showing curved/swollen tail were considered as having intact plasma membranes. Eosin-Nigrosin stained slides were examined under bright field microscope for determination of live and dead sperm. Results were recorded after examination of at least 200 spermatozoa. Sperm that absorbed the eosin stain and appeared dark pink were considered as dead, while sperm that failed to absorb the stain and appeared white were taken as live.

**Measurement of oxidative stress values in testicular tissue:** Testicular tissues (0.5g) were taken and homogenized in PBS (Phosphate buffer solution, pH 7.4, 150mM) at 2000 rpm for 1 minute. Supernatants were obtained by centrifuging the homogenates at 7000 rpm for 10 minutes at 4°C and stored at -80°C for analysis. Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) values were measured with a commercial test kit, using the Erel method (Erel, 2005). Malondialdehyde (MDA) value was determined according to the procedure described earlier (Draper and Hadley, 1990). Glutathione peroxidase (GSH-Px) activity was evaluated by the method of Tietze (1969).

**Morphometric evaluation of testis:** Testicular tissue samples were taken and stored in 10% formaldehyde solution for 24 hours. Then, after necessary processing, 4µm thick sections were stained with Hematoxylin and Eosin (H&E) staining (Cardiff *et al.,* 2014) and examined under a light microscope. Seminiferous tubule diameters were recorded by measuring the thickness of the seminiferous epithelium under Olympus BX53 (Japan) microscope. Epithelial height values were obtained from images taken from tissue samples using Image J image analysis program (Image J software Version 1.47v; NIH, USA).

**Evaluation of 8-OHdG and HIF-1α:** Testicular tissue sections taken on adhesive slides were stained with the immunohistochemical Avidin-biotin complex (ABC) method, as described earlier (Vaughan *et al*., 2010; Kulasekaran *et al.,* 2020). All slides were examined under a light microscope (Zeiss Axio Lab. A1 Microscope-AxioCam ICc 5 Camera). Pictures of all rats were recorded and evaluated with the Image J program.

**Statistical analysis:** Statistical software, SPSS for Windows Version 24, was used for statistical analysis of data from different groups. Epididymal sperm quality parameters, biochemical and histopathological parameters of testes were expressed as the mean±SE for the 10 rats in each group. Sperm quality parameters, oxidative stress parameters (MDA, TAS and TOS) and histopathological data were statistically analyzed using the Independent Student's t-test. Bonferroni corrected Mann-Whitney U test (Ariagno *et al.,* 2017) was used for GSH-Px activity.

## RESULTS

Results of this study showed that semen quality parameters decreased significantly ($p<0.01$) in the $TiO_2$ group compared to control group. Sperm concentration decreased (Table 1, Fig. 1) in the $TiO_2$ group compared to control group. Motility of sperm cells in the $TiO_2$ group (48.25±4.76%) was significantly lower than the control group (87.75±4.08%). Sımilarly, the percentage of live spermatozoa decreased from 84.25±1.94 to 63.12±3.73. Decreased forward movements of sperm were also observed (Fig. 1). Likewise, membrane integrity of spermatozoa was significantly reduced from 43.37±2.70 to 33.50±1.75%. However, there were statistically non-significant differences in VCL, LIN and STR (Table 1). Average Path Velocity values were found to be significantly lower in $TiO_2$ group compared to control group. In testicular tissue, oxidant parameters (TOS and MDA) were significantly increased, while antioxidant parameters (TAS and GSH-Px) were decreased ($p<0.01$) in $TiO_2$ group compared to control group (Table 2).

Results of hematoxylin-eosin staining showed seminiferous tubule diameter and height decreased significantly ($p<0.01$) in the $TiO_2$ group than the control group (Fig. 2, Table 3). Based on color density measurements, the values for Hif-1α did not differ significantly between the two groups (Table 4). However, for 8-OHdG color density levels were significantly increased in the $TiO_2$ group ($P<0.01$). Thus, in the $TiO_2$ group, an increased DNA damage with a tendency of hypoxia to increase were detected in the testicular tissue (Fig. 3, Table 4).

## DISCUSSION

The toxicity of Titanium dioxide ($TiO_2$) remains a topic of debate, with factors such as particle size, mode of exposure and dosage significantly influencing the outcomes. Nano-sized $TiO_2$ particles (<100nm) have been shown to induce harmful effects, including organ accumulation, such as in the liver, and potential DNA damage, leading to its classification as a carcinogenic agent (Sungur *et al.*, 2020). While the reproductive toxicity of $TiO_2$ is still not well understood, limited experimental studies have provided some insights into this issue. For instance, intravenous administration of $TiO_2$ nanoparticles to pregnant mice resulted in fetal anomalies (Ahmad *et al.*, 2022), while oral administration of 21nm $TiO_2$ to newborn rats did not show any apparent toxicity (Lee *et al.*, 2019). Another study in mice showed that exposure to 300mg/kg/bw $TiO_2$ for 42 days resulted in oxidative stress, decreased sperm motility, increased levels of abnormal sperm, and that Quercetin could potentially mitigate these negative effects (Khorsandi *et al.*, 2017).

In our study, the oral administration of 250mg/kg/bw $TiO_2$ for 28 days showed significant reductions in epididymal semen quality parameters in male mice. These results are consistent with those of previous studies, where $TiO_2$ exposure led to testicular damage and negatively affected sperm quality. For example, after 90 days of administration of 2.5, 5.0 and 10.0mg/kg BW of $TiO_2$ in mice, sex hormones were affected and 70 genes were down-regulated in spermatozoa (Gao *et al.*, 2013). The ability of $TiO_2$ nanoparticles to penetrate the blood-testis barrier and cause harm to testicular tissue and spermatozoa is thought to be the primary mechanism for these effects. Previous studies have also demonstrated that $TiO_2$ can accumulate in various tissues and organs, such as the ovaries and spleen, even after short-term exposure (Tassinari *et al.*, 2014). Chronic exposure to low doses, such as 2.5 and 5.0mg/kg, over 9 months has been reported to cause fertility problems and immunological dysfunction in testicular tissue in mice (Hong *et al.*, 2016).

Nano-sized $TiO_2$ has been shown to induce damage to spermatids and seminiferous tubules in male mice, potentially by being absorbed by Sertoli and Leydig cells (Takeda *et al.*, 2009). In this study, it was observed that tissue destruction was relatively higher in the $TiO_2$ group compared to the control group, as the diameter and thickness of the seminiferous tubules decreased in $TiO_2$ group compared to control rats. In the 8-OHdG staining, which is one of the tissue DNA damage markers, it was observed that DNA damage occurred at significantly higher rate in the $TiO_2$ group compared to the control group ($p<0.01$). HIF-1 α values, which is a marker of tissue hypoxia, were increased non-significantly in the $TiO_2$ group when compared with control group. These results suggest that $TiO_2$ administration can cause hypoxia in the testicular tissue, but application for a short time did not cause the damage to reach a significant level. It suggests that longer exposure times (>30 days) may increase HIF-1α levels significantly in treated rats.

Oxidative tissue damage occurs due to a continuous increase in reactive oxygen species (ROS) level in the tissue with insufficient antioxidant capacity. Functional activity of the testicular tissue can be disrupted by metal ions, drugs and toxic substances, leading to an imbalance in antioxidant/oxidant equilibrium (Adedara and Farombi, 2010). The SOD and GSH-Px are reported to be the essential antioxidant systems in spermatogonia and Sertoli cells in testicular tissue (Bauché *et al.*, 1994). In a study conducted in mice, as a result of intragastric administration of 50mg/kg $TiO_2$ for 30 days, it was observed that while SOD level decreased in testicular tissue, MDA level increased (Meng *et al.,* 2022). In this study, administration of 250mg/kg BW of $TiO_2$ resulted in the increased total

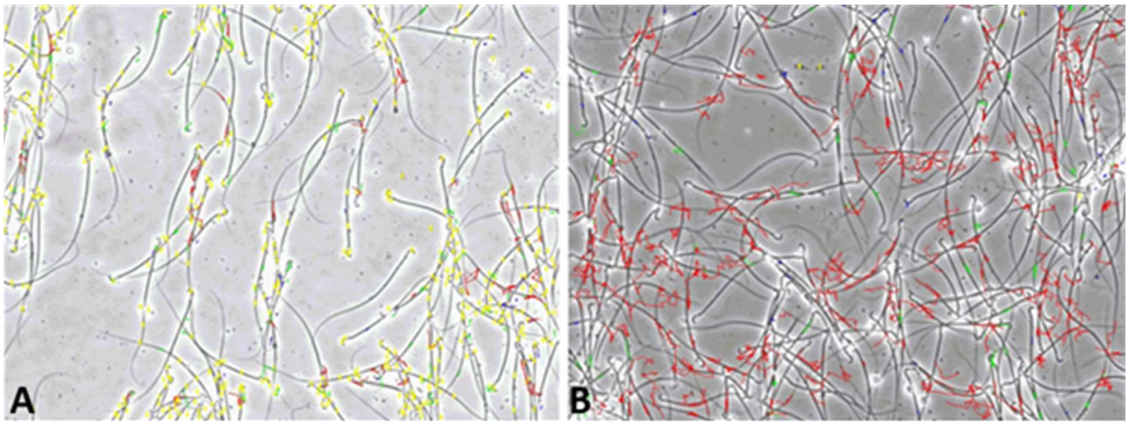


**Fig. 1:** Sperm analysis images (CASA) showing: **A)** decrease in the amount of sperm in the group treated with $TiO_2$, with decreased progressive motility, and **B)** Control group with normal epidymal semen characteristics (100X).

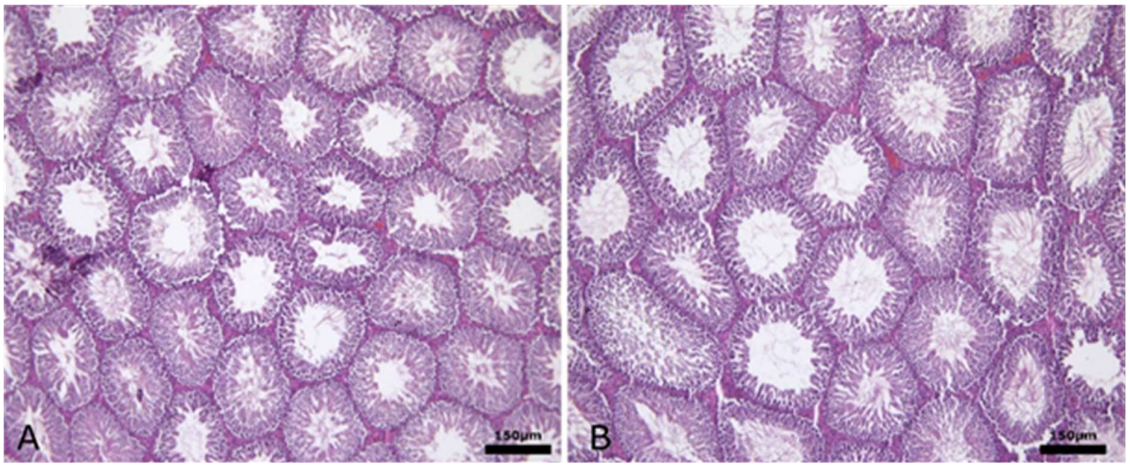


**Fig. 2:** Hematoxylin-eosin tissue staining (H&E, 100X). **A:** Titanium dioxide ($TiO_2$) treated group, showing decreased seminiferous tubule diameter and height, **B:** Control group, showing normal testicular tissue appearance (Bar length: 150µm).

**Table 1:** Mean (±SE) values of sperm concentration, sperm motility, progressive motility, live sperm, sperm integrity, Curvilinear Velocity (VCL), Linearity (LIN), Straightness (STR) and Average Path Velocity (VAP) for two groups.

| Groups | Sperm Concentration ($10^6$/ml) | Sperm motility (%) | Progressive motility (%) | Live sperm (%) | Sperm integrity (%) | VCL (µm/s) | LIN (%) | STR (%) | VAP (µm/s) |
|---|---|---|---|---|---|---|---|---|---|
| Control | 72.45±2.87[a] | 87.75±4.08[a] | 51.12±7.72[a] | 84.25±1.94[a] | 43.37±2.70[a] | 108.03±13.77[a] | 28.36±2.01[a] | 59.43±1.19[a] | 51.92±7.46[a] |
| $TiO_2$ | 44.45±1.48[b] | 48.25±4.76[b] | 19.12±2.42[b] | 63.12±3.73[b] | 33.50±1.75[b] | 82.87±6.26[a] | 24.12±1.05[a] | 58.25±0.83[a] | 33.75±2.11[b] |

Mean values with different superscripts in a column differ significantly from each other ($p<0.01$).

**Table 2:** Oxidative stress status with and without Titanium dioxide application in testicular tissue in terms of GSH-Px, MDA, TAS and TOS (mean±SE).

| Groups | GSH-Px (U/g protein) | MDA (nmol/g protein) | TAS (mmol Trolox Eq/L) | TOS (µmol $H_2O_2$ Eq/L) |
|---|---|---|---|---|
| Control | 160.16±74.01[a] | 4.03±0.08[b] | 1.59±0.03[a] | 9.51±0.29[b] |
| $TiO_2$ | 120.31±3.46[b] | 6.06±0.15[a] | 1.24±0.04[b] | 11.89±0.32[a] |

Mean values with different superscripts in a column differ significantly from each other ($p<0.01$).

**Table 3:** Measurement of seminiferous tubular diameter and epithelial height in testis samples of two groups (mean±SE).

| Groups | Seminiferous tubules diameter (µm) | Tubules epithelial height (µm) |
|---|---|---|
| Control | 311.99±6.90[a] | 73.38±1.51[a] |
| $TiO_2$ | 276.11±5.44[b] | 68.71±0.90[b] |

Mean values with different superscripts in a column differ significantly from each other ($p<0.01$).

**Table 4:** 8-OHdG and Hif-1 alpha color intensity values (pixel) in immunohistochemical evaluation of testicular tissues with Image J (mean± SE).

| Groups | 8-OHdG (Color intensity) | Hif-1 α (Color intensity) |
|---|---|---|
| Control | 83.99±4.74[b] | 66.00±1.90[a] |
| $TiO_2$ | 105.13±8.40[a] | 68.65±4.56[a] |

Mean values with different superscripts in a column differ significantly from each other ($p<0.01$).

oxidant status (MDA and TOS) in the testicular tissue, while the antioxidant level (GSH-Px and TAS) was decreased ($P<0.01$). For this reason, it is easily seen that taking $TiO_2$ at low (10mg/kg/bw) and moderate doses (250mg/kg bw) creates oxidative stress in the testicular tissue, suggesting that $TiO_2$ is a strong oxidant agent. Unfortunately, gene expressions could not be investigated in this study due to budget constraints.

**Conclusions:** In conclusion, the present study highlights the potential adverse effects of nano-sized Titanium dioxide ($TiO_2$), particularly those with particle sizes smaller than 100nm, on reproductive health. Our findings revealed that the oral administration of $TiO_2$ at a dosage of

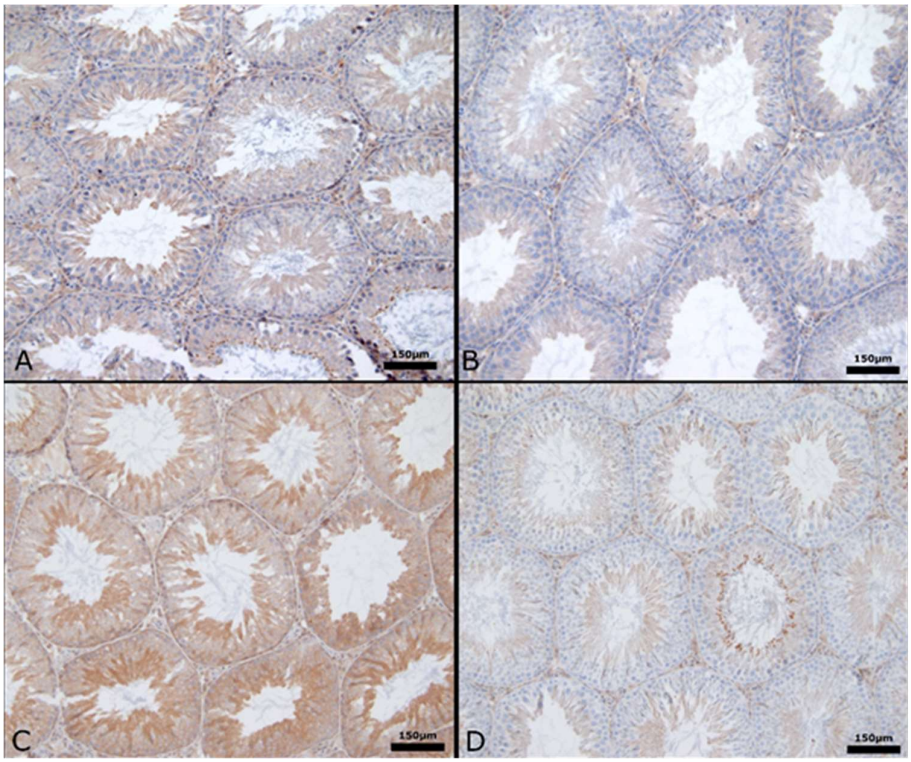


**Fig. 3:** Immunohistochemical staining (200X) of testicular tissues of the groups with 8-OHdG (**A-B**) and Hif1-α (**C-D**): Red color indicates positivity. **A:** Titanium dioxide ($TiO_2$) treated group showing higher tissue DNA damage level than control group (B). **C:** Titanium dioxide ($TiO_2$) treated group, Hif1-α staining showing intense color indicating increased hypoxia in the tissue, but difference was non-significant compared to the control group (D).

250mg/kg bw for 28 days resulted in increased tissue DNA damage and a reduction in epididymal sperm quality parameters in rats. These results raise concerns regarding the safety of larger $TiO_2$ particles found in everyday products, as these may still be source of exposure to smaller nanoparticles during the production process. Therefore, it is crucial to implement necessary precautions to minimize chances of exposure to $TiO_2$ particles smaller than 100nm in order to safeguard overall health and reproductive performance.

**Authors contribution:** In this scientific study, SO served as the project manager, SA conducted the experimental studies. SR did the sperm analysis, while MFB carried out pathological evaluations and statistics. SA contributed in the writing of the manuscript, while all authors reviewed and approved the final manuscript.

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