RESEARCH ARTICLE

CYPRUS
JOURNAL OF MEDICAL SCIENCES

**DOI:** 10.4274/cjms.2025.2025-81
Cyprus J Med Sci 2025;10(6):389-399

# Genotoxic and Hemato-Biochemical Effects Induced By Anatase Titanium Dioxide Nano Particles in Sprague Dawley Rats

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## Abstract

**BACKGROUND/AIMS:** This study aims to examine the adverse effects of titanium dioxide nanoparticles ($TiO_2$-NPs) on rats.

**MATERIALS AND METHODS:** Twenty-five rats, each weighing 200 to 220 grams, were acquired and later transported to the animal house for acclimatization. The rats were divided into five groups of five rats each (G1, G2, G3, G4, and G5). These groups were treated with varying amounts of $TiO_2$-NPs (mg/kg body weight): 0.00 (control), 0.9 (saline), 80, 120, and 160, every other day for 28 days. The rats' weight was examined regularly. After 28 days, blood samples were collected from the orbital sinuses into ethylenediaminetetraacetic acid -coated tubes, and serum was separated for biochemical analysis.

**RESULTS:** The data suggested that the rats' weight was substantially reduced compared with the control and saline groups. Hematological indicators, such as hemoglobin, hematocrit, red blood cells, mean corpuscular hemoglobin concentration, lymphocytes, and monocytes, significantly decreased ($p<0.05$), whereas mean corpuscular volume and white blood cells significantly increased. Biochemical indicators, including alanine transaminase, aspartate transaminase, alkaline phosphatase, urea, creatinine, lactate dehydrogenase, and total bilirubin, were markedly increased. Findings indicate increased oxidative stress and antioxidant enzyme activity in response to high-dose treatment. Genotoxic measurements demonstrated a large decrease in head length and head deoxyribonucleic acid (DNA) (%), but tail length, comet length, tail DNA (%), and tail moment increased markedly.

**CONCLUSION:** Anatase $TiO_2$-NPs induced significant genotoxic and hemato-biochemical effects in Sprague Dawley rats.

**Keywords:** Titanium dioxide nanoparticles, hemato-biochemical, genotoxicity, Sprague Dawley rats

## INTRODUCTION

Nanotechnology is an emerging scientific field that focuses on the design and development of materials at the nanoscale (nanomaterials). These materials, which range in size from 1 to 100 nm, include metals such as copper, zinc, titanium (Ti), magnesium, gold, and silver. Nanomaterials have a wide range of applications from medicine and industrial products like fuel cells to everyday items such as cosmetics.[1] They are produced in large quantities because of their unique properties, which can enhance the performance of various products and processes, contribute to the development of smart medicines, and support sustainable development.[2]

Ti is the ninth most abundant element in the Earth's crust. Titanium dioxide nanoparticles ($TiO_2$-NPs) are white, odorless, and non-combustible powders that exhibit low solubility.[3] Due to their chemical

**To cite this article:** Latif MA, Jabeen F, Iqbal Z, Khalid A. Genotoxic and hemato-biochemical effects induced by anatase titanium dioxide nano particles in Sprague Dawley rats. Cyprus J Med Sci. 2025;10(6):389-399

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**Received:** 21.07.2025
**Accepted:** 19.11.2025
**Publication Date:** 16.12.2025

stability, inertness, cost-effectiveness, and wide range of applications, $TiO_2$-NPs are extensively used in fields such as photocatalysis, catalysis, dye-sensitized solar cells, photovoltaics, and water-splitting technologies.[4] Of the two primary crystalline forms of $TiO_2$-NPs, anatase and rutile, anatase is generally considered more toxic than rutile. Both types are capable of generating reactive oxygen species (ROS), which are oxygen-containing reactive molecules naturally produced during cellular metabolism. Although ROS play vital roles in immune defense and cell signaling, their excessive buildup can result in oxidative stress. This study examines the toxicity of anatase $TiO_2$-NPs, drawing on findings from Zhang et al.[5], Wang et al.[6], and Vasantharaja et al.[7]

$TiO_2$-NPs, when present in rats, can translocate systemically and accumulate in various organs. This can lead to varying degrees of damage to organs sensitive to oxidative stress, such as the liver, lungs, kidneys, small intestine, testes, and brain. These nanoparticles induce oxidative stress, damage deoxyribonucleic acid (DNA), and alter enzymatic activity in the body.[8]

The genotoxic effects of nanoparticles have been extensively investigated, revealing a considerable increase in cancer risk associated with nanoparticle-induced toxicity in the context of modern technological advances. Studies on genotoxicity encompass various aspects such as DNA damage, gene mutations, and chromosomal alterations. According to the International Agency for Research on Cancer, $TiO_2$-NPs have been classified as possibly carcinogenic to humans Baan et al.[9], Wani et al.[10], Wen et al.[11] In this study, biochemical markers related to liver and kidney function-such as aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine transaminase (ALT), lactate dehydrogenase (LDH), urea, creatinine, and total bilirubin-were evaluated because of their significance in detecting physiological abnormalities.[12] Furthermore, the ratios of tissue weight to body weight for the brain and gonads were analyzed.[13]

Antioxidants play a crucial role in neutralizing harmful free radicals and protecting cells from oxidative damage. Maintaining an appropriate balance between oxidants and antioxidants is essential for preventing chronic diseases and promoting overall health.[14] Prolonged exposure to $TiO_2$-NPs can trigger excessive oxidative stress, causing biochemical and structural damage in vital tissues. These nanoparticles disturb the oxidant-antioxidant equilibrium, leading to lipid peroxidation, protein oxidation, and DNA damage Talas et al.[15], and Gulhan et al.[16]

This study focused primarily on investigating the genotoxic and hematobiochemical effects of $TiO_2$-NPs. The wide applications of different nanoparticles, specifically $TiO_2$-NPs, and their potential toxicity in living organisms, as reviewed by previous studies, focused our attention on exploring their adverse effects in Sprague Dawley rats.

## MATERIALS AND METHODS

The details of the materials and procedures are explained in the following section.

### Biological Material and Ethical Approval

Twenty-five post-weaning male Sprague Dawley rats, weighing 200-220 grams, were purchased and kept in the animal house for acclimatization. After an acclimation period, animals were randomly assigned to five groups of five animals each. The control group received regular food and water, while the saline group (S) received a 1 mL intravenous injection of normal saline to ensure equivalence of shock. Groups 3, 4, and 5 were injected subcutaneously with $TiO_2$-NPs at doses of 80, 120, and 160 mg/kg on alternate days for 28 days.

Ethical approval was obtained on Government College University Faisalabad Ethics Review Committee (approval number: GCUF/ERC/976, date: 24.09.2019), but due to coronavirus disease-2019 (COVID-19) the study commenced in 2021. The trial was conducted from March to April 2021, after which bioassays were completed.

### Sample Collection

Blood samples were collected from all animals at the start of the experiment and after 28 days of treatment to assess their hematological and genetic health. At the end of the study, the animals were fasted overnight, anesthetized with chloroform the following day, and euthanized. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes for analysis. The brain and male gonads were carefully weighed and placed in a preservative solution for histological examination.

### Material Characterization

$TiO_2$-NPs were characterized by X-ray diffraction (XRD), fourier transform infrared spectroscopy (FT-IR), and scanning electron microscopy (SEM).

### Ti Accumulation

A 0.5-g piece of brain and gonad tissue was taken and digested in a digestion solution (5 mL nitric acid and 2.5 mL perchloric acid) in a heating digester (Velp Scientifica D-6). Initially, the fumes were yellow. The digestion process was continued until the fumes became colorless and the solution remained at 1 mL. Distilled water was added to the remaining digested solution to bring the volume to 25 mL, and the solution was filtered. The sample was analyzed using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer).[17]

### Measurement

### Body and organ weight

Body weights were recorded weekly to track changes throughout the study. Organ weights, specifically the brain and testes, were measured after euthanasia. Male gonads were measured (L*W) by vernier caliper immediately after separating.

### Blood and Serum Analysis

Blood was analyzed using the automated hematology analyzer X5-1000i, manufactured by Sysmax. Serum was analyzed using commercially available kits manufactured by Merck on the chemistry analyzer Microlab 300.

### Oxidative Stress Analysis

Oxidative stress was assessed using the following biomarkers in the brains of Sprague Dawley rats: glutathione (GSH), malondialdehyde (MDA), catalase (CAT), and lipid peroxidase (LPO).[18]

0.25 g of tissue sample (Brain) was homogenized with 2.5 mL of 0.1 M tris-hydroxymethyl aminomethane hydrochloride (Tris-HCl) buffer in bullet blinder (Pro-900) while keeping the PH 7.4 at “4 °C” and crude tissue homogenate was then centrifuged at a speed of “10,000 rpm” for

20 min in centrifuge (Sigma) at "4 °C". The superanent was stored at "-20 °C" and processed for further oxidative stress biomarker.

### Estimation of lipid peroxidation

The freshly prepared homogenate (0.1 mL) was treated with FOX reagent (0.9 mL), which was composed of butylated hydroxytoluene (88 mg), xylenol orange (7.6 mg), ammonium iron sulfate (9.8 mg) dissolved in methanol (90 mL) and sulphuric acid (10 mL). The mixture was incubated at 37 °C for 30 min. The color developed was measured at 560 nm, and LPO was expressed as mM/g of tissue.

### Estimation of malondialdehyde

A mixture of 1 mL distilled water 5 mL of n-butanol and pyridine 4 mL of sample solution (0.2 mL tissue homogenate +0.2 mL sodium dodecyl sulfate +1.5 mL acetic acid +1.5 mL thiobarbituric acid was shaken on a vortex and centrifuged at 4000 rpm for 10 min and absorbance of upper organic layer was read at 532 nm using spectrophotometer. The amount of MDA was measured as nm/g of the respective tissue.

### Estimation of catalase

CAT was estimated by mixing the sample with 1.90 mL of potassium phosphate buffer. 1 mL of hydrogen peroxide was added to initiate the reaction. The optical density was measured twice at 240 nm with a 30-sec interval between measurements. CAT activity was expressed as units per mL.

### Estimation of glutathione

GSH was estimated by mixing the homogenate and sulfosalicylic acid in equal volumes and incubating the mixture at 4 °C. The mixture was centrifuged at 12000 rpm for 15 min. The supernatant (0.5 mL) was taken and mixed with 2.5 mL of potassium phosphate buffer; after adding 0.4 mL 5,5'-dithiobis (2-nitrobenzoic acid), the reaction was started, and absorbance was measured at 412 nm using a spectrophotometer; GSH was expressed as uM/g of tissue.

### DNA Damage (Comet Assay)

Blood and organ samples (brain and gonads) were used to perform the comet test. The comet test was performed promptly after dissection. Frosted-end microscope slides were used for the comet assay. These slides were scorched with a blue flame to remove oil and dust particles, then soaked in methanol and cleaned with a gentle cloth. To protect them from dust, the slides were stored in a closed box and left overnight to dry. The humidity in the air was monitored because high moisture levels can prevent the slides from drying properly, which affects the electrophoresis process.

The day before starting the assay, slides were prepared by coating them with 1% normal melting point agarose. Once dry, a second layer of low-melting-point (LMP) agarose, mixed with the sample, was applied at 40 °C. In an Eppendorf tube, 100 µL of the sample and 300 µL of LMP agarose were gently mixed; 100 µL of this mixture was poured onto each slide. A coverslip was placed over each slide to evenly spread the sample, followed by cooling on an ice plate for 2-3 min. The coverslips were then gently removed.

Next, the slides were placed in a jar with a chilled lysis buffer (89 mL lysis solution, 18.62 g EDTA, 0.68 g Tris-HCl, 500 mL distilled water, 10 mL dimethyl sulfoxide, and 1 mL Triton X and kept in a dark box at 4 °C for 1.5 h. Electrophoresis was then performed using a horizontal electrophoresis unit (Wealtec) in a cold buffer (0.18 g EDTA, 6 g sodium hydroxide, and 250 mL distilled water) at 300 mA and 25 V for 20 min.

After electrophoresis, the slides were transferred to a stand and neutralized using a cold neutralizing buffer (24.22 g Tris-Base and 2.5 mL distilled water, pH 7.5). The neutralizing solution was left on the slide for 5 min before washing, which was performed under low-light conditions. Once dry, ethidium bromide (75 µL per slide) was applied and a coverslip was placed over each slide. After 20 min, the coverslips were removed, and the dried slides were examined under a fluorescence microscope (Nikon DS-Fi2) at 40× magnification. Fifty cells were analyzed on each slide. The comet assay showed comet-like structures in damaged cells, indicating varying degrees of DNA damage. Computer image analysis (using Casp software) was employed to measure parameters such as head length, tail length, comet length, head DNA, and tail DNA.

### Statistical Analysis

Statistical significance was determined using Minitab software (Minitab 15). Body weights, organ weights, Ti accumulation, hematological parameters, oxidative stress analyses, and genotoxicity analyses were compared using the Duncan test. Group comparisons were made using a one-way analysis of variance, with significance tested at $p<0.05$.

## RESULTS

The widespread industrial and medical applications of $TiO_2$-NPs-including their use in various consumer products, cosmetics, food items, and pharmaceuticals-have raised concerns about their potential adverse health effects. The extensive utilization of $TiO_2$-NPs has therefore prompted our research to focus on understanding their toxicological impacts.[19] These nanoparticles are known to target cellular mitochondria, disrupting oxidative phosphorylation and ultimately causing damage to cells and tissues.[20] The present study investigates $TiO_2$-NPs -induced toxicity in male Sprague Dawley rats. Variations in hematological, biochemical, genotoxic, cell-viability, and histopathological (brain and gonadal) parameters were evaluated. Additionally, nanoparticle characterization was performed using XRD, FT-IR, and SEM.

### Characterization of $TiO_2$-NPs

Nanoparticles of $TiO_2$ were purchased from Sigma-Aldrich and analyzed using powder XRD, FT-IR, and SEM. The Graphical results of the XRD pattern shown in Figure 1 reveal the key peaks corresponding to specific diffraction angles (2θ) and their respective intensities. The highest peaks occur at 25.5°, 38°, 48.5°, and 54.5°, with intensities of 101, 112, 200, and 211, respectively. The prominent peaks were compared with Joint Committee on Powder Diffraction Standards data, and the peaks observed in the pattern coincide well with those reported in the literature. The 2θ peak at 25.5° confirms the anatase structure of $TiO_2$. Strong diffraction peaks at 25.5° indicate $TiO_2$-NPs in the anatase phase. FT-IR analysis provides functional characterization of $TiO_2$-NPs. The results were presented as a graph of % transmittance versus wavenumber Figure 2. The various bands observed in the FT-IR spectra of $TiO_2$-NPs confirm the presence of different functional groups in the molecule. Peaks were obtained in the range of 400-4000 cm-1. A broad absorption band observed at 3029 cm-1 corresponds to the hydroxyl

(O-H) stretching vibration mode of the $TiO_2$-NPs. The second band, observed at 1622.72 cm-1, corresponds to the characteristic bending mode of the H2O molecule. The morphology of $TiO_2$-NPs was analyzed by SEM. In Figure 3, results indicate spherical and rod-like agglomerates at different magnifications, ranging from 200 nm to 2 µm.

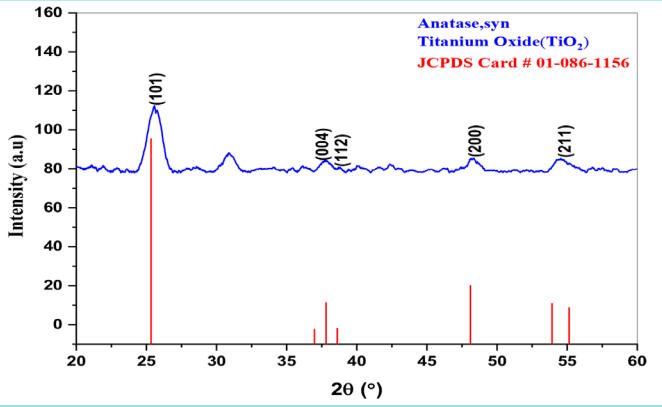


**Figure 1.** X-ray images of titanium dioxide nanoparticles ($TiO_2$-NPs) using powder diffraction (XRD).

XRD: X-ray diffraction.

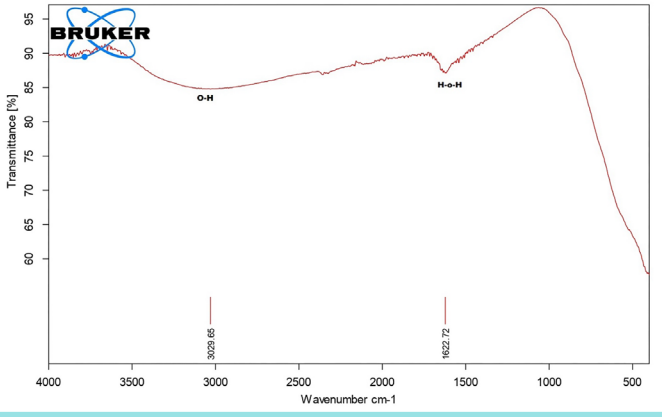


**Figure 2.** Fourier transform infrared images of ($TiO_2$-NPs).

$TiO_2$-NPs: Titanium dioxide nanoparticles.

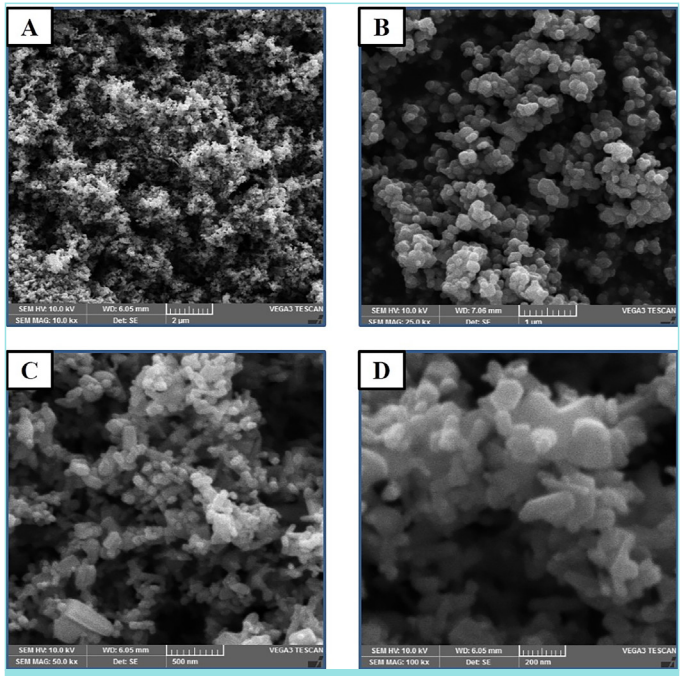


**Figure 3.** Scanning electron microscope images of ($TiO_2$-NPs). **(A)** 2 µm **(B)** 1µm **(C)** 500 nm **(D)** 200 nm.

$TiO_2$-NPs: Titanium dioxide nanoparticles.

### Behavioral Changes

Significant behavioral changes, such as aggressiveness, piloerection (fluffiness), anorexia, cannibalism, and depression, were observed at the highest dose of $TiO_2$-NPs during the study (Table 1).

### Weight Results

In our study, we observed a significant decrease in the body weight of rats treated with $TiO_2$-NPs (Table 2). Throughout our experiment, no fatalities occurred among rats treated with $TiO_2$-NPs at doses of 80, 120, and 160 mg/kg body weight. However, rats in the treatment groups (G3, G4, and G5) experienced a significant decrease in body weight over 28 days compared with the control group (G1) and the saline group (G2), indicating adverse effects of $TiO_2$-NPs (Table 2). Furthermore, in our study, both the absolute and relative weights of the brain and gonads were significantly lower in the high-dose treatment groups (G4 and G5), and measurements such as gonad length and width were also significantly reduced compared to the control and saline groups (Table 2).

**Table 1. Behavioral alterations in rats after post-exposure to $TiO_2$-NPs**

| Parameters | Groups | | | | |
|---|---|---|---|---|---|
| | G1 | G2 | G3 | G4 | G5 |
| Aggressiveness | - | - | - | ++ | +++ |
| Fluffiness | - | - | - | ++ | +++ |
| Anorexia | - | - | - | + | ++ |
| Cannibalism | - | - | - | + | ++ |
| Depression | - | - | - | + | +++ |

$TiO_2$-NPs: Titanium dioxide nanoparticles.

**Table 2. Weight Results**

| Parameters | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | p-value |
|---|---|---|---|---|---|---|
| Start of experiment AW(g) ± SD | 206.00±2.52[a] | 206.20±1.85[a] | 205.40±1.02[a] | 205.80±1.77[a] | 205.60±1.12[a] | p=0.269 |
| End of experiment AW(g) ± SD | 329.40±1.93[a] | 332.80±1.96[a] | 292.80±1.59[b] | 275.80±2.55[c] | 255.40±2.37[d] | p=0.000 |
| End of experiment OW (Brain)(g) ± SD | 0.01±0.09[a] | 0.01±0.01[a] | 3.39±0.10[b] | 7.32±0.34[c] | 13.84±0.33[d] | p=0.001 |
| End of experiment OW (Testis)(g) ± SD | 0.01±0.02[a] | 0.01±0.14[a] | 3.13±0.57[b] | 11.89±0.38[c] | 19.12±0.17[d] | p=0.000 |
| Absolute weight (Brain) (g) ± SD | 0.92±0.23[a] | 0.91±0.02[a] | 0.91±0.02[a] | 0.86±0.01[b] | 0.81±0.01[c] | p=0.000 |
| Relative weight (Brain) (%) ± SD | 0.28±0.01[a] | 0.27±0.01[a] | 0.27±0.02[b] | 0.21±0.03[b] | 0.14±0.04[c] | p=0.002 |
| Absolute weight (Testis) (g) ± SD | 1.30±0.01[a] | 1.29±0.02[a] | 1.28±0.02[a] | 1.14±0.02[b] | 1.07±0.02[c] | p=0.000 |
| Relative weight (Testis) (%) ± SD | 0.38±0.01[a] | 0.38±0.01[a] | 0.43±0.02[b] | 0.40±0.05[c] | 0.35±0.06[d] | p=0.000 |
| Gonadal measurement (Length) (mm) | 1.88±0.04[a] | 1.86±0.02[a] | 1.84±0.02[a] | 1.42±0.09[b] | 1.24±0.05[c] | p=0.000 |
| Gonadal measurement (Diameter) (mm) | 0.88±0.03[a] | 0.86±0.02[a] | 0.84±0.05[a] | 0.68±0.03[c] | 0.58±0.03[d] | p=0.000 |

[a]: Similar to each other, different from b and c, [b]: Different from a, similar within itself, [c]: Different from both a and b, [d]; Different from a, b and c.
AW: Animal weight, OW: Organ weight, SD: Standard deviation.

### Ti Accumulation

Table 3 shows the dose-dependent accumulation of Ti in the brain and testes of Sprague Dawley rats. It appeared that the Ti concentration was significantly higher in the medium- and high-dose treated groups (G4 and G5) than in the low-dose (G3), control (G2), and placebo (G1) groups.

### Hematological Parameter

In the present study, hematological parameters fluctuate due to chronic exposure to $TiO_2$-NPs. Red blood cells (RBC) count, hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), lymphocytes, and monocytes were significantly decreased (Table 4). In our study, MCV significantly increased at higher doses of $TiO_2$-NPs (Table 4). Similarly, the serological parameters ALT, AST, ALP, urea, creatinine, LDH, and total bilirubin are significantly increased with increasing dose compared with the control and saline groups (Table 5).

**Table 3. Titanium accumulation in selected organ (brain and testis)**

| Parameters | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | p-value |
|---|---|---|---|---|---|---|
| Ti accumulation in brain | 0.01±0.09[a] | 0.01±0.01[a] | 3.39±0.10[b] | 7.32±0.34[c] | 13.84±0.33[d] | p=0.000 |
| Ti accumulation in testis | 0.01±0.02[a] | 0.01±0.14[a] | 3.13±0.57[b] | 11.89±0.38[c] | 19.12±0.17[d] | p=0.000 |

[a]: Similar to each other, different from b and c, [b]: Different from a, similar within itself, [c]: Different from both a and b, [d]: Different from a, b and c.
Ti: Titanium, SD: Standard deviation.

**Table 4. Hematological parameters in rats by subcutaneous administration with anatase $TiO_2$-NPs for alternately "28 days"**

| Parameters | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | p-value |
|---|---|---|---|---|---|---|
| **Erythrocytes (1012/L)** | 8.65±0.32[a] | 8.64±0.14[a] | 7.64±0.17[ab] | 6.62±0.26[b] | 5.09±0.22[c] | p=0.000 |
| **Hemoglobin (g/dL)** | 13.28±0.23[a] | 13.10±0.24[a] | 12.52±0.20[a] | 10.82±0.22[b] | 8.86±0.19[c] | p=0.001 |
| **Hematocrit (%)** | 46.18±0.29[a] | 46.76±0.41[a] | 46.04±0.37[a] | 42.09±0.52[b] | 37.98±0.81[c] | p=0.000 |
| **Mean corpuscular volume (fL)** | 49.60±0.50[a] | 50.00±0.70[a] | 50.00±0.70[a] | 45.60±0.67[b] | 42.40±1.02[c] | p=0.002 |
| **Mean corpuscular hemoglobin (pg)** | 15.10±0.29[a] | 14.70±0.40[a] | 15.56±0.22[ab] | 16.44±0.29[b] | 19.50±0.54[c] | p=0.000 |
| **Mean corpuscular hemoglobin concentration (g/dL)** | 41.64±0.87[a] | 41.47±0.67[a] | 40.44±0.86[ab] | 31.02±0.77[b] | 29.50±0.87[c] | p=0.001 |
| **Leucocytes (109/l)** | 10.42±0.32[a] | 10.64±0.55[a] | 10.78±0.62[a] | 13.34±0.34[b] | 10.02±0.24[b] | p=0.000 |
| **Lymphocytes (%)** | 64.62±0.85[a] | 64.52±0.67[a] | 62.24±0.56[ab] | 48.32±0.76[b] | 40.96±0.97[c] | p=0.001 |
| **Monocytes (%)** | 7.06±0.19[a] | 7.04±0.19[a] | 6.18±0.38[a] | 3.93±0.50[b] | 2.83±0.24[b] | p=0.000 |

[a]: Similar to each other, different from b and c, [b]: Different from a, similar within itself, [c]: Different from both a and b, [ab]: Similar to both a and.
$TiO_2$-NPs: Titanium dioxide nanoparticles.

**Table 5. Serological parameters in rats by subcutaneous administration with anatase $TiO_2$-NPs for alternately "28 days"**

| Parameters | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | p-value |
|---|---|---|---|---|---|---|
| Alanine transaminase (IU/L) | 41.40±2.90[a] | 42.20±1.93[a] | 53.80±2.67[b] | 78.40±3.21[c] | 82.00±2.55[d] | p=0.000 |
| Aspartate transaminase (IU/L) | 52.40±2.50[a] | 52.80±2.41[a] | 53.00±1.70[a] | 61.00±1.94[b] | 60.60±2.34[b] | p=0.002 |
| Alanine phosphatase (IU/L) | 266.80±4.35[a] | 241.00±3.45[a] | 249.30±4.24[b] | 275.80±3.20[c] | 307.80±4.21[d] | p=0.000 |
| Urea | 41.20±1.01[a] | 42.40±0.87[a] | 52.40±1.07[b] | 58.40±0.92[c] | 70.00±1.30[d] | p=0.002 |
| Creatinine | 0.84±0.01[a] | 0.87±0.02[a] | 0.91±0.01[b] | 1.03±0.05[bc] | 1.17±0.07[c] | p=0.005 |
| Lectate dehydrogenase | 177.40±1.93[a] | 179.20±1.16[a] | 188.00±1.22[b] | 288.00±11.84[c] | 332.20±16.71[d] | p=0.000 |
| Total bilirubin | 0.41±0.05[a] | 0.41±0.06[a] | 0.55±0.05[b] | 0.87±0.04[c] | 0.90±0.05[d] | p=0.000 |

[a]: Similar to each other, different from b and c, [b]: Different from a, similar within itself, [c]: Different from both a and b, [d]: Different from a, b and c, [bc]: Similar to both b and c.
$TiO_2$-NPs: Titanium dioxide nanoparticles.

### Oxidative Stress Analysis

The values of LPO, MDA, CAT and GSH were significantly increased in the high-dose treated groups (G4 and G5) compared with the control and saline groups (G1 and G2) (Table 6).

### Genotoxicity by Commet Assay

In our study, both blood and organ samples showed significant decreases in head length and head DNA percentage and significant increases in tail length, comet length, tail DNA percentage, and tail moment (Tables 7-9). These abnormalities were not significant in the low-dose-treated and normal-saline-treated groups compared with the control group, suggesting that they were caused by DNA breakage, lagging acentric chromosomes, and chromatid fragments induced by the toxicant (Figure 4).

**Table 6. $TiO_2$-NPs induced alteration in oxidative stress enzymes of brain tissue after "28 days" of exposure**

| Parameters | Group 1 | Group2 | Group 3 | Group 4 | Group 5 | p-value |
|---|---|---|---|---|---|---|
| Lipid peroxidase mm/100g | 18.40±2.90[a] | 18.20±1.93[a] | 23.80±2.67[b] | 53.40±3.21[c] | 69.00±2.55[d] | p=0.001 |
| Malondialdehyde nM/g | 135.40±2.50[a] | 134.80±2.41[a] | 151.00±1.70[b] | 176.00±1.94[c] | 195.60±2.34[d] | p=0.000 |
| Catalase (unit/mL) | 348.80±4.35[a] | 347.00±3.45[a] | 384.30±4.24[b] | 411.80±3.20[c] | 474.80±4.21[d] | p=0.000 |
| Glutathione (uM/g) | 461.20±1.01[a] | 460.40±0.87[a] | 492.40±1.07[b] | 540.40±0.92[c] | 593.00±1.30[d] | p=0.001 |

[a]: Similar to each other, different from b and c, [b]: Different from a, similar within itself, [c]: Different from both a and b, [d]: Different from a, b and c.
$TiO_2$-NPs: Titanium dioxide nanoparticles.

**Table 7. Genotoxic abnormalities in blood of rats treated with anatase $TiO_2$-NPs assessed by commet assay**

| Parameters | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | p-value |
|---|---|---|---|---|---|---|
| Length head | 41.40±1.36[a] | 40.40±0.67[a] | 39.80±2.74[a] | 16.40±2.08[b] | 7.60±1.93[c] | p=0.002 |
| Length tail | 7.60±1.43[a] | 7.40±0.60[a] | 8.00±0.89[a] | 37.40±1.80[b] | 49.00±1.76[c] | p=0.001 |
| Length commet | 46.60±0.87[a] | 46.20±0.96[a] | 46.59±0.65[a] | 53.80±1.24[b] | 56.80±1.29[c] | p=0.000 |
| Head DNA (%) | 84.98±0.76[a] | 84.91±0.57[a] | 81.19±0.59[a] | 43.22±0.49[b] | 11.84±0.41[c] | p=0.000 |
| Tail DNA (%) | 15.01±0.11[a] | 15.08±0.16[a] | 17.21±0.23[a] | 56.83±0.56[b] | 88.29±0.98[c] | p=0.001 |
| Tail Moment | 0.78±0.01[a] | 0.76±0.05[a] | 2.39±0.11[a] | 14.20±0.90[b] | 29.72±1.37[c] | p=0.000 |

[a]: Similar to each other, different from b and c, [b]: Different from a, similar within itself, [c]: Different from both a and b.
DNA: Deoxyribonucleic acid, $TiO_2$-NPs: Titanium dioxide nanoparticles.

**Table 8. Genotoxic abnormalities in Brain of rats treated with anatase $TiO_2$-NPs assessed by commet assay**

| Parameters | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | p-value |
|---|---|---|---|---|---|---|
| Length head | 44.80±1.2[a] | 43.80±0.8[a] | 42.40±0.8[a] | 21.80±0.5[b] | 7.40±0.92[c] | p=0.001 |
| Length tail | 5.40±0.50[a] | 6.40±0.50[a] | 6.40±0.60[a] | 35.00±1.5[b] | 55.00±1.6[c] | p=0.000 |
| Length commet | 50.20±1.3[a] | 50.30±0.8[a] | 51.20±0.3[a] | 56.80±1.3[b] | 62.40±1.4[c] | p=0.000 |
| Head DNA (%) | 90.95±0.6[a] | 90.77±0.6[a] | 89.62±0.6[a] | 61.01±0.4[b] | 36.83±0.6[c] | p=0.001 |
| Tail DNA (%) | 8.83±0.54[a] | 8.88±0.57[a] | 8.99±0.22[a] | 38.98±0.4[b] | 63.16±0.6[c] | p=0.000 |
| Tail moment | 0.76±0.03[a] | 0.79±0.02[a] | 2.25±0.05[a] | 11.43±0.1[b] | 21.59±0.9[c] | p=0.000 |

[a]: Similar to each other, different from b and c, [b]: Different from a, similar within itself, [c]: Different from both a and b.
DNA: Deoxyribonucleic acid, $TiO_2$-NPs: Titanium dioxide nanoparticles.

**Table 9. Genotoxic abnormalities in testis of rats treated with anatase $TiO_2$-NPs assessed by commet assay**

| Parameters | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | p-value |
|---|---|---|---|---|---|---|
| Length head | 45.20±0.71[a] | 45.00±0.70[a] | 44.20±1.21[a] | 21.20±1.09[b] | 9.00±0.70[c] | p=0.000 |
| Length tail | 5.00±0.70[a] | 5.20±0.37[a] | 5.80±0.37[a] | 41.40±0.81[b] | 62.20±1.23[c] | p=0.001 |
| Length commet | 50.20±0.42[a] | 49.60±0.52[a] | 50.80±0.61[a] | 62.60±1.22[b] | 71.20±1.24[c] | p=0.002 |
| Head DNA (%) | 90.39±0.22[a] | 90.28±0.34[a] | 89.50±0.62[a] | 45.56±1.67[b] | 27.13±0.72[c] | p=0.000 |
| Tail DNA (%) | 9.60±0.27[a] | 9.72±0.32[a] | 9.82±0.31[a] | 54.43±1.62[b] | 72.86±1.72[c] | p=0.000 |
| Tail moment | 0.48±0.01[a] | 0.49±0.01[a] | 1.95±0.30[a] | 15.30±0.41[b] | 43.48±1.14[c] | p=0.000 |

[a]: Similar to each other, different from b and c, [b]: Different from a, similar within itself, [c]: Different from both a and b.
DNA: Deoxyribonucleic acid, $TiO_2$-NPs: Titanium dioxide nanoparticles.

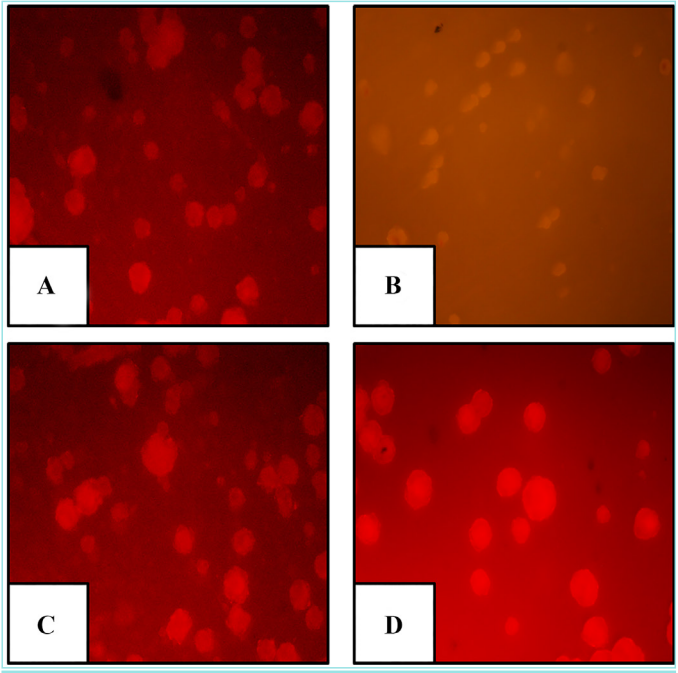


**Figure 4.** DNA damage induced by $TiO_2$-NPs in Sprague Dawley rats. **(A)** The control group shows normal nuclei with intact DNA and minimal or no comet tail formation, indicating no DNA damage. **(B)** Blood cells showing comet formation with visible DNA tail migration, representing $TiO_2$-NP-induced DNA strand breaks. **(C)** Gonadal cells exhibiting DNA damage as evidenced by increased comet tail length and head deformation, indicating significant genotoxic effects of $TiO_2$-NPs on reproductive tissues. **(D)** Brain cells showing pronounced comet tails, suggesting extensive DNA fragmentation and high levels of $TiO_2$-NP-induced DNA damage.

DNA: Deoxyribonucleic acid, $TiO_2$-NPs: Titanium dioxide nanoparticles.

## DISCUSSION

The present study, entitled "Genotoxic and Hemato-biochemical effects induced by Anatase Titanium dioxide Nano Particles in Sprague Dawley rats", was conducted. The benefits of $TiO_2$-NPs are diverse in application, but vigorous use of $TiO_2$-NPs induces health abnormalities, such as behavioral changes, antioxidant imbalance, impairment of biochemical indices, cellular membrane damage, DNA damage, and reduced growth rate. The average size of $TiO_2$-NPs used in the current study was 25 nm, as confirmed by XRD and SEM. The findings of the current study are in good agreement with the study by[21], who used $TiO_2$-NPs of sizes 25 nm and 25-50 nm in albino rats and found that $TiO_2$-NPs of these sizes were toxic to their model organism. In the current study, $TiO_2$-NPs of 25 nm were toxic to Sprague Dawley rats. The anatase morphology of $TiO_2$-NPs is associated with greater toxicity than other polymorphs, such as rutile, due to its increased surface reactivity and its ability to generate ROS, which can lead to oxidative stress and cellular damage.[22]

Significant behavioral changes such as increased aggression, excessive grooming, piloerection (fluffed fur), anorexia, cannibalism, and signs of depression were observed in the group exposed to the highest dose of $TiO_2$-NPs. These alterations suggest a potential neurotoxic effect associated with high-level exposure. Such behaviors may indicate stress, neurological disruption, and systemic toxicity induced by nanoparticles. The presence of anorexia and cannibalism further reflects impaired physiological and social functioning. These findings highlight the need for careful evaluation of $TiO_2$-NPs safety, particularly at higher concentrations. This result is consistent with those reported by[23], who observed the same behavioral changes in rats due to $TiO_2$-NPs. Exposure nanoparticles have the potential to accumulate in sensitive organs such as the brain and testes, where they may disrupt normal physiological functions. This accumulation can result in neurotoxicity or reproductive toxicity by interfering with cellular mechanisms. Our study demonstrated a dose-dependent increase in Ti accumulation within the brain and testes, with significantly higher concentrations observed in the medium- and high-dose groups. These findings align with the results of El-Shafai et al.[24], Bermudez et al.[25], and Mahrousa[26], who also reported greater Ti accumulation at higher exposure levels. This consistency reinforces the evidence that Ti exhibits bioaccumulative properties in sensitive organs.

Exposure to $TiO_2$-NPs led to a significant reduction in body weight and the somatic indices of organs, suggesting potential toxic effects that may be linked to organ stress or damage. Such outcomes indicate that $TiO_2$-NPs can induce oxidative stress, inflammation, impaired organ function, and raise serious concerns about their safety under long-term exposure. Similar decreases in body weight following $TiO_2$-NP exposure have been documented in earlier studies by Geraets et al.[27], Faucher and Lespes[28], and Tian et al.[29] In the present study, no mortality was observed among rats treated with $TiO_2$-NPs at doses of 80, 120, and 160 mg/kg body weight.

However, animals in experimental groups (G3, G4, and G5) exhibited a significant decrease in body weight after 28 days compared with the control and saline-treated groups ($p<0.05$), indicating adverse effects of $TiO_2$-NPs (Table 3). These findings are consistent with reports by El-Sharkawy et al.[30] and El-Sheikh et al.[31] who also observed marked

reductions in body weight in rats exposed to $TiO_2$-NPs for 45 days. Likewise, other studies[32] have confirmed notable declines in body weight associated with $TiO_2$-NP exposure.

A reduction in both relative and absolute organ weights may indicate toxicity of $TiO_2$-NPs. Such alterations can disrupt normal organ function and may signify tissue damage or physiological stress, ultimately affecting overall health and homeostasis. In our study, the high-dose treatment groups (G4 and G5) showed a significant decrease in both absolute and relative weights of the brain and gonads. Additionally, gonad dimensions, including length and width, were markedly reduced compared to the control and saline groups. Similar findings have been reported in other studies involving rats exposed to $TiO_2$-NPs[33], reinforcing that prolonged exposure to excessive amounts of $TiO_2$ can lead to adverse effects.

Hematological parameters are essential for evaluating the overall health and detecting a wide range of disorders, such as anemia and infections. They help monitor disease progression and the effectiveness of treatments. In the present study hematological parameters fluctuate due to chronic exposure of $TiO_2$-NPs. RBC count, Hb, HCT, MCV, MCHC, lymphocytes, and monocytes were significantly decreased, and white blood cells (WBC) were significantly increased at higher doses of $TiO_2$-NPs.[34] Reported a decrease in RBC count and Hb in female mice after $TiO_2$-NPs, which is in accordance with our results. The observed decrease in these parameters indicates anemia. This decrease may be due to the production of immature RBCs, the suppressive impact of $TiO_2$-NPs on stem cells in the bone marrow, and malfunction of erythropoiesis.[35] The study is also similar to our study, but differs in the mode of administration of NPs. An experiment conducted by[36] reported fluctuations in hematological parameters induced by $TiO_2$-NPs in pregnant mice, consistent with our study.[37] Performed a similar study on male Sprague Dawley rats, and the results are consistent with our findings.[38] Also reported similar hematological findings in rats. WBC levels in our study increased significantly at higher doses of $TiO_2$ -NPs (Table 4). This increase is may be due to innate defense system activation.[39] A similar increase in WBC was also reported by Smith et al.[40], Shakeel et al.[41], and Heo et al.[42] in their studies on rats. In this study, lymphocyte and monocyte counts were significantly reduced (Table 4). The decrease in lymphocytes, also reported by[43] after oral administration of $TiO_2$-NPs, is in accordance with our results. The significant decrease in monocytes observed in our study is consistent with[44], who administered $TiO_2$-NPs to albino rats.

In this study, $TiO_2$-NPs doses of 80, 120, and 160 mg/kg were injected subcutaneously. Administered doses of 120 mg/kg and 160 mg/kg body weight alter serum parameters, as supported by[45], who orally administered $TiO_2$-NPs at 62.5 mg/kg, 125 mg/kg, and 250 mg/kg body weight and reported alterations at doses higher than 125 mg/kg body weight. In study[46], $TiO_2$-NPs doses of 10, 50, 100, and 200 mg/kg body weight were administered for 60 days. “100 mg/kg” and “200 mg/kg” showed significant fluctuations in serum biochemical parameters, supporting our dose-dependent study.

Toxic chemicals can cause hepatotoxicity. The liver protects the body from toxic chemicals and serves as an important site for detecting biological changes. The liver secretes its substances into bile, resulting in nanoparticle distribution throughout the animal body. In[47], hepatic enzymes (ALT, AST, and ALP) were significantly increased after 28 days of chronic exposure to $TiO_2$-NPs in high-dose groups. ALT, AST, and ALP are present in the liver, and fluctuations in their levels indicate liver injury. Similar studies related to AST, ALT, ALP fluctuations were supported by.[48]

In our study, a significant increase in hepatic enzymes (Table 6) may be due to liver injury, as supported by previous work of Sadiq et al.[49], Meena and Paulraj[50], Rizk et al.[51], and Relier et al.[52] Similar studies related to AST, ALT, ALP fluctuations were supported by Abu-Dief et al.[53], Sheydaei et al.[54], Chen et al.[55] and Yang et al.[56]

Following exposure to toxicants, LDH activity increased in various organs, including the liver, heart, and lungs. LDH is an isoenzyme that plays a key role in glycolysis and gluconeogenesis. Toxicant-induced injury causes LDH to be released from damaged cells or organs into the bloodstream, resulting in elevated serum LDH activity.[57] In the present study, LDH activity in rats increased in proportion to $TiO_2$ nanoparticle exposure (Table 6). Similar findings were reported in[58], who observed a significant increase in LDH activity after oral administration of $TiO_2$ nanoparticles to female mice, consistent with our results.

The kidneys are responsible for removing nitrogenous waste from the blood. $TiO_2$-NPs circulating in the bloodstream can lead to renal dysfunction and damage. In this study, rats treated with $TiO_2$-NPs exhibited renal impairment, as evidenced by significant increases in urea and creatinine levels (Table 5) compared with the control group, particularly at doses of 120 and 160 mg/kg body weight. The observed rise in urea and creatinine may be attributed to nephron damage, glomerular toxicity, congestion of renal tubules with protein-rich fluid, and glomerular swelling, all due to the accumulation of $TiO_2$-NPs in the kidneys. These findings are consistent with previous studies[59], which reported similar increases in urea and creatinine following exposure to $TiO_2$-NPs, and with studies[60] confirming renal toxicity. Additionally, total bilirubin levels in this study were significantly elevated in the high-dose groups G4 and G5 ($p<0.05$) (Table 6). This increase may result from $TiO_2$-NP-induced RBC destruction, potentially leading to anemia and liver dysfunction, a possibility further supported by fluctuations in hepatic enzyme levels observed in this study. The results for total bilirubin agree with earlier research[61], which reported similar increases following $TiO_2$-NPs exposure.

A key parameter for assessing the potential toxicity of nanoparticles is oxidative stress. Antioxidants play a crucial role in protecting the body from the harmful effects of free radicals and ROS, which can damage cells and tissues, by neutralizing them. Insufficient antioxidant levels increase vulnerability to oxidative stress and toxicity, highlighting their importance in mitigating these effects Selamoglu et al.[62], Adnan et al.[63] $TiO_2$-NPs can induce fenton-type reactions, leading to ROS generation, including oxyradicals, and modulate antioxidant defenses, resulting in altered CAT and GSH levels and increased oxidative stress markers such as LPO and MDA (Salam et al.[64], Ibrahim et al.[65] In the present study, levels of GSH, LPO, and MDA were significantly elevated in the medium- and high-dose-treated groups, indicating activation of the antioxidant defense system in response to nanoparticle-induced toxicity. These findings are consistent with previous reports on alterations in CAT, GSH, and LPO levels Ishak et al.[66], Ahmad et al.[67]

In our experiment, we investigated genotoxic abnormalities using the comet assay. Damage to bone marrow DNA can result in alterations in the number or types of cells present in the bloodstream. The comet assay evaluates DNA damage by producing images that reveal various

forms of damage, including characteristic comet-like structures in cells. We analyzed several parameters measured in this assay, including head length, tail length, comet length, percentage of DNA in the head and tail, and tail moment, in the blood, brain, and gonads of rats. Previous in vivo studies have demonstrated the genotoxic potential of $TiO_2$-NPs. For instance[68], conducted research in mice treated with the same toxicant, reporting significant DNA damage in the blood, although their results differ from ours. Our genotoxicity findings, particularly those from the comet assay, are consistent with studies by Chen et al.[69], and Valentini et al.[70] Moreover, studies evaluating DNA damage in the brains of mice, such as[71], reported results similar to our comet assay observations.

### Study Limitations

This study provides valuable insights into the genotoxic and hemato-biochemical effects of anatase $TiO_2$-NPs. The study was limited by a small sample size and short exposure duration, but this study highlights the acute toxicological impacts of short-term exposure to small-sized anatase $TiO_2$-NPs in a limited population, enabling focused observation of dose-dependent effects.

## CONCLUSION

In conclusion, our study shows that $TiO_2$-NPs are highly toxic at the moderate and high doses tested, causing genotoxic and hemato-biochemical effects in Sprague Dawley rats.

## MAIN POINTS

- Titanium dioxide nanoparticles ($TiO_2$-NPs) showed significant accumulation in brain and gonads (especially in G4 and G5 groups) confirmed by inductively coupled plasma mass spectrometry.
- Body and organ weights were significantly decreased (brain and gonads) in the medium- to high-dose groups. Gonadal measurements (length and diameter) were also reduced significantly, indicating possible reproductive toxicity.
- Exposure to $TiO_2$-NPs caused decreases in red blood cells, hemoglobin, hematocrit, mean corpuscular hemoglobin concentration, lymphocytes, and monocytes. Liver and kidney function markers (alanine transaminase, aspartate aminotransferase, urea, creatinine, lactate dehydrogenase, bilirubin) were increased significantly in the high-dose groups, indicating systemic toxicity and organ stress.
- High-dose groups (G4 and G5) exhibited significant increases in oxidative stress markers, including lipid peroxidase, malondialdehyde, catalase, and glutathione. This suggests reactive oxygen species -mediated cellular damage as a key mechanism of toxicity.
- Comet assay results from blood, brain, and gonads showed increased deoxyribonucleic acid (DNA) fragmentation in high-dose groups, evidenced by longer tail lengths, higher tail DNA %, and increased tail moment.

## ETHICS

**Ethics Committee Approval:** Ethical approval was obtained on Government College University Faisalabad Ethics Review Committee (approval number: GCUF/ERC/976, date: 24.09.2019), but due to COVID-19 the study commenced in 2021. The trial was conducted from March to April 2021, after which bioassays were completed.

**Informed Consent:** Patient approval has not been obtained as it is performed on animals.

### Footnotes

### Authorship Contributions

Concept: M.A.L., F.J., Design: M.A.L., F.J., Data Collection and/or Processing: M.A.L., Z.I., A.K., Analysis and/or Interpretation: M.A.L., F.J., Literature Search: M.A.L., Z.I., A.K., Writing: M.A.L., F.J.

## DISCLOSURES

**Conflict of Interest:** No conflict of interest was declared by the authors.

**Financial Disclosure:** The authors declared that this study received no financial support.

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