


Sub-Chronic Exposure (90 Days) to Titanium Dioxide Nanoparticles (TiO₂ NPs) Induce Alterations in Hematology and Serum Biochemistry in Male Wistar Rats: Effect of Exogenous Melatonin Supplementation

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Abstract: Rapid increase in the use of metal-oxide nanoparticles in many industries increased the risk of toxicity to humans. In this regard, an experimental study was conducted in male Wistar rats to study the effect of repeated-dose sub-chronic oral exposure for 90 days to titanium dioxide nanoparticles on certain hematological and serum biochemical parameters and to evaluate the ameliorative potential of melatonin to overcome such alterations. The rats were divided randomly into four groups of fourteen each. Group-I served as vehicle control, Group-II received TiO₂ NPs (100mg.kg⁻¹); Group-III received treatment similar to that of Group-II along with melatonin @10mg.kg⁻¹, while group-IV received only melatonin. Rats exposed to TiO₂ NPs showed a significant increase in WBC count at 60days and 90 days intervals, whereas a significant decrease in RBC count, platelets count, and increase in percent granulocytes only at 90 days intervals. In addition, a significant increase in the activities of serum aminotransferases, alkaline phosphatase, and gamma-glutamyl transferase was observed in rats exposed to TiO₂ NPs. Melatonin supplementation significantly ameliorated some of these parameters on day 60 or 90 of the experimental period. Thus, melatonin can be a promising agent for therapeutic intervention to overcome titanium dioxide nanoparticles induce adverse effects after appropriate clinical studies.

Keywords: titanium dioxide (TiO₂) nanoparticles; melatonin; 90 days; hematology; serum biochemistry; Wistar rats.

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1. Introduction

The rapid development of nanotechnology has led to the use of different metal oxide nanoparticles in various fields. Among metal oxide nanoparticles, titanium dioxide nanoparticles (TiO₂ NPs) are widely employed in several industries, viz., the cosmetic industry as sunscreen, paint, ink, pharmaceutical industry as drug additives, paper, food industry as a colorant, antibacterial materials, and electronics [1] due to their unique physical and chemical properties. Nanoparticles possess a greater surface area, increased chemical reactivity, and improved cellular penetration compared to their bulk counterparts, which correlates to their toxicity [2].

Titanium dioxide nanoparticles are mainly absorbed in the intestinal tract after oral exposure and distributed to different organs and tissues through the circulatory system [1]. The circulatory system interacts mainly with red or white blood cells, platelets, plasma proteins, and coagulation factors [3]. Sub-chronic intragastric administration of TiO₂ NPs can alter the hematopoietic system [4], glucose levels, lipid profile with oxidative stress, inflammatory and DNA damage in leukocytes [5]. DNA hypomethylation is caused by TiO₂ NPs in peripheral blood mononuclear cells *in vitro* [6]. Studies in rats or mice suggest that TiO₂ NPs can induce toxicity in various organs, including the kidney [7] and liver [8,9,10]. Titanium dioxide nanoparticles can induce toxicity in the liver as it is the major organ that carries out biotransformation. Titanium dioxide nanoparticles induce hepatotoxicity mainly by oxidative stress [11, 12] and inflammation [11]. Chen *et al.* [13] reported that the liver has higher sensitivity to oxidative stress induced by orally ingested TiO₂ NPs compared to other organs. These nanoparticles can produce more dose-related harmful effects in the liver and blood as compared to their bulk counterparts [14].

The pineal gland synthesizes and releases melatonin as an endogenous neurohormone and shows circadian rhythm in release patterns with a peak at night and low levels during daytime [15]. Melatonin has been found to be crucial for regulating the mammalian biologic rhythms, such as seasonal adaptation, sleep-wake cycle, mediation of photoperiodic information, and pubertal development [16]. In addition, it has an important role in the synchronization of cell physiology, regulation of a number of physiologic processes like cardiac function, mood, anxiety, and appetite [15]. The endogenous or exogenous melatonin can have antioxidant properties and protects the cells against oxidative stress [17,18]. Melatonin has been established as a protective agent against chemical pollutants, drug, alcohol-induced liver damages [19]. Prophylactic administration of melatonin can protect TiO₂ NPs induced inflammation, oxidative stress, DNA damage, and apoptosis in rat liver [11].

However, a comprehensive investigation to study the ameliorative potential of melatonin in TiO₂ NPs induced toxic alterations in certain hematological and serum biochemical parameters is lacking. Keeping this in view, the current study was undertaken to study the ameliorative potential of melatonin against TiO₂ NPs induced alterations in certain hematologic and serum biochemical parameters following 90-days sub-chronic repeated dose oral administration in male Wistar rats.

2. Materials and Methods

2.1. Chemicals.

Titanium dioxide [Titanium (IV) oxide], anatase (TiO₂; MW: 79.87 g/mol) nanopowder (< 25 nm particle size; 99.7% purity; trace metal basis) and melatonin (C₁₃H₁₆N₂O₂; MW: 232.28 g/mol) were procured from M/s Sigma-Aldrich, St. Louis, MO, USA. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT) enzymatic assay kits, creatinine, and blood urea nitrogen (BUN) assay kits were procured from M/s Erba Mannheim, Pvt. Ltd., Mumbai. Ketamine hydrochloride, xylazine hydrochloride were procured locally. Analytical grade carboxymethyl cellulose (CMC) was procured from Ms. S.d Fine Chemicals Ltd., Mumbai, India. Millipore (reverse osmosis) water was employed for oral gavage.

2.2. Experimental animals.

Male Wistar rats (N= 56) of 5-6 weeks of age weighing ~95-115g were procured from CPCSEA authorized vendor (Biogen[®] Laboratory Animal Facility, Attibele, Bengaluru-562107, K.S). The experimental protocol was conducted in accordance with the CPCSEA guidelines and Institutional Animal Ethics Committee (IAEC) with standard operating procedures. Necessary approval of (IAEC) was obtained (*vide* Letter No. VCH/IAEC/2019/115 dated 03/12/2019) to carry out the current investigation. Rats were housed in a Small Animal facility (CPCSEA Registration No. 493/GO/ReBiBt-S/Re-L/01/CPCSEA dated 16.09.2016), Veterinary College, Hebbal, Bengaluru, having a temperature of 20.2-22.8°C, relative humidity of 40-69%, light/dark cycle of about 12 hours in polypropylene cages. Animals were fed with standard rodent chow (Amrut[®], M/s. Pranav Agro Industries Ltd, Maharashtra State) *ad libitum* all time excluding scheduled fasting (overnight) and given free access to water (reverse osmosed) *ad libitum* throughout the experimental period.

2.3. Experimental protocol.

Experimental rats were randomly divided into four groups after one week of acclimatization as detailed below: Group I (n=14): Served as a vehicle control group and received 0.2% carboxymethyl cellulose (CMC) as oral gavage for a period of 90 days. Group II (n=14): Received TiO₂ NPs @ 100 mg.kg⁻¹ in 0.2% CMC through oral gavage daily during the morning hours for a period of 90 days. Group III (n=14): All the animals in this group received treatment similar to Group-II for a period of 90 days during morning hours, but in addition received melatonin @ 10 mg.kg⁻¹ in water (*per os*) daily two hours after the administration of TiO₂ NPs. Rats in Group IV (n=14) received melatonin alone @ 10 mg.kg⁻¹ as above throughout the experimental period.

2.4. Sacrifice of animals.

Four animals from each group were sacrificed on day-31 as well as on day-61, and six animals from each group were sacrificed on day-91. Before sacrifice, experimental animals were off-fed overnight but had free access to drinking water *ad libitum*. Animals were sacrificed under anaesthesia (Ketamine hydrochloride @50 mg.kg⁻¹ + Xylazine @10 mg.kg⁻¹; *i.p*). At each sacrifice, blood was withdrawn by cardiac puncture. About 0.5ml of blood was collected from each animal into separate tubes containing ethylene diamine tetraacetic acid

(EDTA) anti-coagulant for hematological studies. Hematological parameters including red blood cell (RBC) count, hemoglobin (HGB), packed cell volume (PCV), white blood cell (WBC) count, percent granulocytes, percent lymphocytes, percent monocytes, and platelets count were analyzed using an auto-hematology analyzer (MINDRAY-BC-2800, USA).

2.5. Evaluation of serum biochemical parameters.

About 2.5ml of blood from each sacrificed animal was collected in clot accelerator tubes, allowed to clot for 30min, and then centrifuged at 3000 rpm for 10min. The separated serum was collected into 2ml Eppendorf tube, and serum samples were stored at -20°C for further analysis. The serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (GGT), creatinine, and blood urea nitrogen (BUN) were measured by kinetic methods according to the instructions given by the kit supplier.

2.5.1. Alanine aminotransferase (ALT) activity.

The alanine aminotransferase (ALT) activity was determined by the variation of optical density (OD) of the reaction mixture at 365nm. The ALT value was derived according to manufacturer instructions provided with the kit.

$$\text{Activity of ALT (IU. L}^{-1}\text{)} = (\Delta A/\text{min}) \times \text{Factor (1768)}$$

where, $\Delta A/\text{min}$ = mean absorbance change/min.

2.5.2. Aspartate aminotransferase (AST) activity.

The aspartate aminotransferase (AST) activity was determined by the variation of OD of the reaction mixture at 365nm. The AST value was calculated according to manufacturer instructions provided with the kit.

$$\text{Activity of AST (IU. L}^{-1}\text{)} = (\Delta A/\text{min}) \times 1768$$

where, $\Delta A/\text{min}$ = mean absorbance change/min

2.5.3. Alkaline phosphatase (ALP) activity.

The alkaline phosphatase (ALP) activity was determined by the variation of OD of the reaction mixture at 405nm, and ALP value was calculated by using the formula provided in the commercial kit.

Activity of ALP (IU. L⁻¹) = f x $\Delta A/\text{min}$ where, f = factor (2764), $\Delta A/\text{min}$ = mean absorbance change/min.

2.5.4. Gamma-glutamyl transferase (GGT) activity.

The gamma-glutamyl transferase (GGT) activity was determined by the variation of OD of the reaction mixture at 405nm, and GGT value was calculated by using the formula provided with the commercial kit.

$$\text{GGT (IU. L}^{-1}\text{)} = f \times \Delta A/\text{min}$$

where, f = factor (1158), $\Delta A/\text{min}$ = mean absorbance change/min.

2.5.5. Creatinine level.

Kinetic determination of creatinine was performed according to the instructions given by the kit supplier. The creatinine level was determined by the variation of OD of the reaction mixture at 505nm, and creatinine value was calculated by the formula, as prescribed by the kit manufacturer

$$\text{Creatinine (mg. dl}^{-1}\text{)} = \left(\frac{\Delta A \text{ of test}}{\Delta A \text{ of standard}} \right) \times \text{Concentration of standard}$$

where, $\Delta A = A_2 - A_1$, Concentration of standard (mg.dl⁻¹) = 2

2.5.6. Blood urea nitrogen (BUN) level.

Kinetic determination of BUN was performed according to the instructions given manufacture of the kit. The BUN level was determined by the variation of OD of the reaction mixture at 340nm, and the BUN value was calculated by the formula, as prescribed by the kit manufacturer

$$\text{BUN (mg. dl}^{-1}\text{)} = \left(\frac{\Delta A \text{ of test}}{\Delta A \text{ of standard}} \right) \times \text{Concentration of standard}$$

where, $\Delta A = A_2 - A_1$, Concentration of standard (mg.dl⁻¹) = 23.4.

2.6. Statistical analysis.

The values obtained from the various experiments were expressed as Mean \pm S.E. with 'n' equal to the number of animals or samples. Data obtained were statistically subjected to mixed-effects- analysis followed by Tukey's post hoc multiple comparison test using GraphPad Prism software program (GraphPad® software Inc., Version 8.4.3; San Diego, CA, USA). The difference was considered significant at $p < 0.05$ or lower.

3. Results and Discussion

The current study was carried out to investigate the toxic effects of TiO₂ NPs on certain hematological and serum biochemical parameters in Wistar rats following 90 days of repeated oral administration and to examine the potential role of melatonin under such circumstances.

3.1. Hematological parameters.

3.1.1. Red blood cell (RBC) count.

The mean (\pm SE) RBC count of different experimental groups at different time intervals is depicted in Table 1. There was no significant ($p > 0.05$) difference in the RBC count between all the groups at both 30 days and 60 days intervals. Whereas, at 90 days interval, the RBC count in TiO₂ NPs exposed animals (Group-II) ($8.24 \pm 0.09 \times 10^6 \cdot \mu\text{l}^{-1}$) was significantly ($p < 0.05$) less compared to both vehicle control (Group-I) ($8.95 \pm 0.15 \times 10^6 \cdot \mu\text{l}^{-1}$) and melatonin alone (Group-IV) ($9.02 \pm 0.19 \times 10^6 \cdot \mu\text{l}^{-1}$). Grissa *et al.* [4] exposed (intra-gastric; 60 days) rats to TiO₂ NPs (50, 100 or 200mg.kg⁻¹) and reported dose-dependent decrease in the RBC count. The present result is in line with the findings of Chakrabarti *et al.* [20], who orally exposed albino mice to TiO₂ NPs (200 or 500mg.kg⁻¹ x 90 days) and reported a significant decrease in RBC count in higher dose group. On the contrary, Ranjan *et al.* [21] and Han *et al.* [22] reported

non-significant changes in the RBC count in rats following exposure to TiO₂ NPs for 12 weeks and 90 days, respectively.

The present study's reduction in RBC count may be due to TiO₂ NPs induced adverse effects on red blood cells production and increased red blood cells destruction in hemopoietic organs [4]. RBC count in the remaining groups differed non-significantly ($p > 0.05$).

Table 1. The mean (\pm SE) RBC count ($\times 10^6, \mu\text{l}^{-1}$) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	8.52 \pm 0.06	8.40 \pm 0.15	8.43 \pm 0.12	8.63 \pm 0.13
Day 60	8.61 \pm 0.18	8.31 \pm 0.11	8.42 \pm 0.21	8.68 \pm 0.24
Day 90	8.95 \pm 0.15 ^a	8.24 \pm 0.09 ^b	8.64 \pm 0.13 ^{ab}	9.02 \pm 0.19 ^a

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin].

3.1.2. Hemoglobin (HGB).

The mean (\pm SE) HGB content of different experimental groups at different time intervals is depicted in Table 2. The HGB values of all the experimental groups differed non-significantly ($p > 0.05$) throughout the experiment. This finding correlates with the findings of Ranjan *et al.* [21] following oral administration of TiO₂ NPs (20, 40, 60, 80, or 100mg.kg⁻¹) to Wistar rats for 12 weeks. Similar findings were also reported by Han *et al.* [22] in specific pathogen-free Sprague Dawley rats following oral administration of food-grade TiO₂ NPs (10, 100, or 1000mg.kg⁻¹; 90 days). Whereas on the contrary, Grissa *et al.* [4] reported a non-significant decrease in the HGB in rats exposed (intragastric x 60 days) to TiO₂ NPs.

Table 2. The mean (\pm SE) hemoglobin (HGB) values (g.dl⁻¹) in experimental groups of rats at different time intervals

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	15.85 \pm 0.10	15.80 \pm 0.11	15.70 \pm 0.13	15.90 \pm 0.13
Day 60	15.93 \pm 0.15	15.53 \pm 0.15	15.70 \pm 0.20	15.88 \pm 0.16
Day 90	15.93 \pm 0.23	15.40 \pm 0.22	15.78 \pm 0.13	15.92 \pm 0.19

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin].

3.1.3. Packed cell volume (PCV).

The mean (\pm SE) PCV of different experimental groups at different time intervals is depicted in Table 3.

Table 3. The mean (\pm SE) packed cell volume (PCV) values (%) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	51.60 \pm 1.96	50.93 \pm 1.80	50.73 \pm 1.77	52.70 \pm 2.17
Day 60	52.00 \pm 1.72	50.60 \pm 1.25	50.75 \pm 0.62	52.08 \pm 0.89
Day 90	52.12 \pm 0.53	49.88 \pm 0.80	49.98 \pm 1.51	52.60 \pm 1.07

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin].

The PCV values showed a non-significant ($p>0.05$) difference between all the experimental groups throughout the experiment. This result agrees with the findings of Chakrabarti *et al.* [20] in mice, Ranjan *et al.* [21], and Han *et al.* [22] in rats. On the other hand, Grissa *et al.* [4] reported a significant decrease in the PCV in rats exposed (intragastric x 60 days) to TiO₂ NPs.

3.1.4. White blood cell (WBC) count.

The mean (\pm SE) WBC count of different experimental groups at different time intervals is depicted in Table 4. At 30 days intervals, there was no significant ($p>0.05$) change in the WBC count between all the experimental groups. At 60 days interval, TiO₂ NPs exposed animals (Group-II) ($11.98\pm 0.52 \times 10^3 \cdot \mu\text{l}^{-1}$) showed a significant ($p<0.05$) increase in WBC count compared to vehicle control (Group-I) ($9.70\pm 0.27 \times 10^3 \cdot \mu\text{l}^{-1}$) and melatonin alone group (Group-IV) ($9.73\pm 0.27 \times 10^3 \cdot \mu\text{l}^{-1}$). This result agrees with Grissa *et al.* [4], who reported a significant increase in the WBC count in rats exposed to TiO₂ NPs for 60 days. At 90 days interval, WBC count in Group-II ($12.63\pm 0.51 \times 10^3 \cdot \mu\text{l}^{-1}$) showed a significant ($p<0.05$) increase compared to both Group-I ($10.07\pm 0.37 \times 10^3 \cdot \mu\text{l}^{-1}$) and Group-IV ($10.13\pm 0.26 \times 10^3 \cdot \mu\text{l}^{-1}$). Similar findings were observed by Chakrabarti *et al.* [20] in mice following exposure to a high dose of TiO₂ NPs ($500\text{mg}\cdot\text{kg}^{-1}$) for 90 days and by Ranjan *et al.* [21] in rats orally exposed to TiO₂ NPs ($100\text{mg}\cdot\text{kg}^{-1}\times 12$ weeks). Similarly, Chen *et al.* [23] evaluated cardiovascular effects of TiO₂ NPs (2, 10, or 50 $\text{mg}\cdot\text{kg}^{-1}$ x 90 days; *p.o*) and found increased WBC count in higher dose in female Sprague Dawley rats. White blood cells play an important role in the body's defense system. An increase of WBC in the present study indicates that TiO₂ NPs can activate the body's immune system.

Table 4. The mean (\pm SE) white blood cell (WBC) count ($\times 10^3 \cdot \mu\text{l}^{-1}$) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	9.48 \pm 0.42	10.03 \pm 0.46	9.60 \pm 0.45	9.38 \pm 0.40
Day 60	9.70 \pm 0.27 ^a	11.98 \pm 0.52 ^b	10.25 \pm 0.60 ^{ab}	9.73 \pm 0.27 ^a
Day 90	10.07 \pm 0.37 ^a	12.63 \pm 0.51 ^b	10.35 \pm 0.47 ^a	10.13 \pm 0.26 ^a

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin].

On the contrary, Cui *et al.* [8] and Hashem *et al.* [24] reported a significant decrease in WBC count in mice and rats, respectively, following exposure to TiO₂ NPs for 90 days. This discrepancy may be due to differences in the species of animal or the dose of the TiO₂ NPs used in the study. Han *et al.* [22] reported a non-significant change in WBC count in TiO₂ NPs exposed rats.

Melatonin supplementation (Group-III) ($10.35\pm 0.47 \times 10^3 \cdot \mu\text{l}^{-1}$) significantly ($p<0.05$) decreased WBC count compared to Group-II ($12.63\pm 0.51 \times 10^3 \cdot \mu\text{l}^{-1}$), and non-significantly ($p > 0.05$) increased compared to both Group-I and Group-IV. Similar ameliorative potential of melatonin ($5\text{mg}\cdot\text{kg}^{-1}\times 21$ days; *p.o*) against acrylamide ($5\text{mg}\cdot\text{kg}^{-1}\times 60$ days; *p.o*) induced increase in WBC count in Wistar rats was reported by Alwan and Ghadhban [25]. The antioxidant nature of melatonin might have reversed TiO₂ NPs induced an increase in WBC count.

3.1.5. Percent granulocytes.

The mean (\pm SE) percent granulocytes count of different experimental groups at different time intervals is depicted in Table 5. There was no significant ($p>0.05$) difference in the percent granulocytes count between all the experimental groups at both 30 day and 60 days intervals. At 90 days intervals, the percent granulocytes count in TiO₂ NPs exposed animals (Group-II) ($24.45\pm 2.05\%$) showed a significant ($p<0.05$) increase compared to that of vehicle control (Group-I) ($16.58\pm 1.09\%$) and melatonin alone group (Group-IV) ($16.50\pm 1.13\%$). Ranjan *et al.* [21] reported a significant increase in percent neutrophils in the TiO₂ NPs ($100\text{mg.kg}^{-1}\times 12$ weeks) rats. Similarly, Hashem *et al.* [24] also reported neutrophilia in TiO₂ NPs (20 or 40 $\text{mg.kg}^{-1}\times 90$ days; *p.o*) exposed rats.

Table 5. The mean (\pm SE) granulocytes count (%) in experimental groups of rats at different time intervals

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	18.50 \pm 0.91	20.60 \pm 1.60	19.28 \pm 1.42	17.25 \pm 1.02
Day 60	17.35 \pm 0.70	21.13 \pm 1.13	17.90 \pm 1.36	16.13 \pm 1.30
Day 90	16.58 \pm 1.09 ^a	24.45 \pm 2.05 ^b	16.95 \pm 0.95 ^a	16.50 \pm 1.13 ^a

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin].

An increase in percent granulocytes count in the present study might be due to TiO₂ NPs induced inflammation. As the major inflammatory cells among granulocytes, TiO₂ NPs can exert neutrophil agonistic properties and activate certain cytokines [26]. On the contrary, Han *et al.* [22] reported a non-significant change in percent granulocytes in TiO₂ NPs exposed rats. This difference in the result could be attributable to a change in the laboratory animals or the type of TiO₂ NPs used in the study. The percent granulocytes count in melatonin supplementation (Group-III) differed non-significantly ($p>0.05$) from that of remaining groups at both 30 days and 60 days intervals. Whereas, at 90 days intervals, there was a significant ($p<0.05$) decrease in percent granulocytes count in Group-III ($16.95\pm 0.95\%$) compared to Group-II ($24.45\pm 2.05\%$).

3.1.6. Percent lymphocytes.

The mean (\pm SE) percent lymphocytes count of different experimental groups at different time intervals is depicted in Table 6. The percent lymphocytes count showed a non-significant ($p>0.05$) difference between all the experimental groups throughout the experiment. Similar findings were observed by Ranjan *et al.* [21] in rats following 12 weeks of exposure.

Table 6. The mean (\pm SE) lymphocytes count (%) in experimental groups of rats at different time intervals

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	78.75 \pm 1.24	76.28 \pm 1.31	77.90 \pm 1.41	80.18 \pm 1.34
Day 60	79.53 \pm 1.04	75.48 \pm 1.06	78.78 \pm 1.59	81.13 \pm 1.75
Day 90	80.32 \pm 1.53	72.37 \pm 2.55	79.77 \pm 1.43	80.62 \pm 1.34

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin].

3.1.7. Percent monocytes.

The mean (\pm SE) percent monocytes count of different experimental groups at different time intervals is depicted in Table 7. The percent monocytes count showed a non-significant ($p>0.05$) difference between all the experimental groups throughout the experiment. The result is in line with the findings of Ranjan *et al.* [21] and Han *et al.* [22] in rats following 12 weeks and 90 days of exposure, respectively.

Table 7. The mean (\pm SE) monocytes count (%) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	2.75 \pm 0.34	3.13 \pm 0.31	2.83 \pm 0.19	2.58 \pm 0.35
Day 60	3.13 \pm 0.35	3.40 \pm 0.37	3.33 \pm 0.38	2.75 \pm 0.46
Day 90	3.10 \pm 0.49	3.18 \pm 0.60	3.28 \pm 0.53	2.88 \pm 0.35

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin]

3.1.8. Platelets count.

The mean (\pm SE) platelets count of different experimental groups at different time intervals is depicted in Table 8. At both 30 days and 60 days intervals, there was no significant difference ($p>0.05$) in the platelet count between different groups. But at 90 days interval, the platelets count in TiO₂ NPs exposed animals (Group-II) ($709.50 \pm 25.42 \times 10^3 \cdot \mu\text{l}^{-1}$) was significantly ($p < 0.05$) lower compared to that of vehicle control (Group-I) ($823.33 \pm 16.61 \times 10^3 \cdot \mu\text{l}^{-1}$) and melatonin alone (Group-IV) ($837.17 \pm 17.37 \times 10^3 \cdot \mu\text{l}^{-1}$).

Similar findings were reported by Chakrabarti *et al.* [20] in mice, Ranjan *et al.* [21], and Hashem *et al.* [24] in rats following sub-chronic exposure to TiO₂ NPs. Contrary to this, Grissa *et al.* [4] reported a dose-dependent increase in platelets count. In contrast, Han *et al.* [22] reported non-significant changes in platelet count following sub-chronic exposure to TiO₂ NPs.

Table 8. The mean (\pm SE) platelets count ($\times 10^3 \cdot \mu\text{l}^{-1}$) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	788.75 \pm 26.97	801.50 \pm 33.71	770.75 \pm 28.37	779.25 \pm 17.76
Day 60	809.25 \pm 14.86	750.25 \pm 29.76	796.75 \pm 16.65	817.00 \pm 34.34
Day 90	823.33 \pm 16.61 ^a	709.50 \pm 25.42 ^b	775.50 \pm 24.65 ^{ab}	837.17 \pm 17.37 ^a

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin]

3.2. Serum biochemistry.

The nanoparticles can accumulate in the liver, impairing the structure and function of hepatic cells. [27]. Injury to hepatic tissue can be primarily identified by activities of aminotransferases (ALT and AST) in serum. ALT is only found in the cytoplasm in the liver, whereas AST is found in both the cytosol and the mitochondria. Whenever acute or chronic injury to the liver occurs, resulting in the death of hepatocytes or membrane damage, the release of these enzymes into the blood circulation, thus increasing serum aminotransferases, indicates hepatic damage. On the surface of the bile duct epithelia, hepatic ALP is present. If the bile duct is damaged or injured, ALP can leak from the liver into the bloodstream [28]. Hence, the

level of these enzymes in serum can be considered quantitative biomarkers for the extent of liver damage. In this regard, the possible effect of TiO₂ NPs and melatonin on the function of the liver was evaluated by monitoring the serum level of biomarkers like ALT, AST, ALP, and GGT.

3.2.1. Alanine aminotransferase (ALT).

The mean (\pm SE) serum alanine aminotransferase (ALT) activities of different experimental groups at different time intervals are depicted in Table 9. The serum ALT activities of all groups were comparable at a 30 days interval. There was a significant ($p < 0.05$) increase in the ALT activity in TiO₂ exposed (Group-II) (58.35 ± 2.28 IU.L⁻¹) compared to both vehicle control (Group-I) (45.97 ± 1.61 IU.L⁻¹) and melatonin alone (Group-IV) (44.20 ± 2.28 IU.L⁻¹) at 60-days interval. Whereas, at 90 days intervals, the increase in the ALT activity in TiO₂ exposed (Group-II) (70.72 ± 3.26 IU.L⁻¹) was significant ($p < 0.01$) with that of Group-I (47.74 ± 2.09 IU.L⁻¹) and Group-IV (48.92 ± 2.17 IU.L⁻¹). An increase in the serum ALT activity in TiO₂ exposed (Group-II) (70.72 ± 3.26 IU.L⁻¹) at 90 days intervals was significant ($p < 0.05$) with that of both 30 days (48.62 ± 2.34 IU.L⁻¹) and 60 days interval (58.35 ± 2.28 IU.L⁻¹). Similar findings were reported by Cui *et al.* [8], Chakrabarti *et al.* [20] in mice and Jafari *et al.* [9], Ranjan *et al.* [21] in rats following sub-chronic exposure to 10mg.kg⁻¹, 500mg.kg⁻¹ and 100mg.kg⁻¹, 100mg.kg⁻¹ TiO₂ NPs, respectively.

Melatonin supplementation significantly ($p < 0.05$) decreased the serum ALT activity in (Group-III) (48.18 ± 1.51 and 56.58 ± 2.46 IU.L⁻¹) compared to Group-II (58.35 ± 2.28 and 70.72 ± 3.26 IU.L⁻¹) at 60 days and 90 days intervals, respectively. Othman *et al.* [29] reported that melatonin (5mg.kg⁻¹ x 8 weeks) pretreatment could decrease aluminum chloride-induced elevations in serum ALT and AST levels.

Table 9. The mean (\pm SE) serum alanine aminotransferase (ALT) activity (IU.L⁻¹) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	44.64 \pm 2.21	48.62 \pm 2.34 ^X	45.08 \pm 1.84	42.43 \pm 2.28
Day 60	45.97 \pm 1.61 ^a	58.35 \pm 2.28 ^{bX}	48.18 \pm 1.51 ^a	44.20 \pm 2.28 ^a
Day 90	47.74 \pm 2.09 ^a	70.72 \pm 3.26 ^{bY}	56.58 \pm 2.46 ^a	48.92 \pm 2.17 ^a

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin]

3.2.2. Aspartate aminotransferase (AST).

The mean (\pm SE) serum aspartate aminotransferase (AST) activities of different experimental groups at different time intervals are depicted in Table 10. At a 30 days interval, the AST activities of all the groups differed non-significantly ($p > 0.05$). On the contrary, Azim *et al.* [10] reported a significant increase in serum ALT and AST activities in mice exposed to TiO₂ NPs (150mg.kg⁻¹ x 2 weeks; $p.o$). This discrepancy could be attributable to either species of laboratory animal used or a dose of TiO₂ NPs employed in the current study.

There was a significant ($p < 0.05$) increase in the serum AST activity in TiO₂ exposed (Group-II) (101.22 ± 3.56 IU.L⁻¹) with that of both vehicle control (Group-I) (80.00 ± 3.34 IU.L⁻¹) and melatonin alone (Group-IV) (78.68 ± 2.34 IU.L⁻¹) at 60 days interval. Whereas, at 90 days intervals, the increase in the AST activity in Group-II (117.57 ± 5.45 IU.L⁻¹) was significant ($p < 0.01$) compared to both Group-I (81.62 ± 3.04 IU.L⁻¹) and Group-IV (83.39 ± 4.51 IU.L⁻¹).

An increase in the serum AST activities in Group-II from 30 days (86.19±4.53 IU.L⁻¹) to 90 days interval (117.57±5.45 IU.L⁻¹) was significant ($p<0.05$). Similar findings were reported by Cui *et al.* [8], Chakrabarti *et al.* [20] in mice and Jafari *et al.* [9], Ranjan *et al.* [21] in rats following sub-chronic exposure to 10mg.kg⁻¹, 500mg.kg⁻¹ and 100mg.kg⁻¹, 100mg.kg⁻¹ TiO₂ NPs, respectively. Increased serum activities of ALT and AST in the TiO₂ NPs exposed animals could be due to hepatic cell damage, cellular leakage, and loss of functional integrity of liver cell membranes [10].

Melatonin supplementation (Group-III) (85.75±2.10 IU.L⁻¹ and 94.00±3.69 IU.L⁻¹) significantly ($p<0.05$) decreased the serum AST activities compared to Group-II (101.22±3.56 IU.L⁻¹ and 117.57±5.45 IU.L⁻¹) at both 60 days and 90 days intervals, respectively. The AST levels of Group-IV were comparable with that of both Group-I and Group-III. Similarly, Pan *et al.* [30] reported that melatonin (5 or 10mg.kg⁻¹ x12 weeks; *i.p*) could reduce high-fat diet-induced hepatic steatosis, inflammation, and lower serum ALT, AST, cholesterol, and triglycerides levels in rats. The melatonin's anti-oxidative, anti-apoptotic, and membrane-protective properties can reduce hepatocyte death as well as serum ALT and AST levels. [31].

Table 10. The mean (±SE) serum aspartate aminotransferase (AST) activity (IU.L⁻¹) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	78.23±2.64	86.19±4.53 ^X	79.12±4.11	74.26±3.68
Day 60	80.00±3.34 ^a	101.22±3.56 ^{bXY}	85.75±2.10 ^a	78.68±2.34 ^a
Day 90	81.62±3.04 ^a	117.57±5.45 ^{bY}	94.00±3.69 ^a	83.39±4.51 ^a

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin]

3.2.3. Alkaline phosphatase (ALP).

The mean (±SE) alkaline phosphatase (ALP) levels of different experimental groups at different time intervals are depicted in Table 11. At a 30 days interval, the serum ALP activities in all the groups differed non-significantly ($p>0.05$).

Table 11. The mean (± SE) serum alkaline phosphatase (ALP) activity (IU.L⁻¹) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	129.91±5.41	147.88±6.43 ^X	133.36±5.90	121.62±5.41
Day 60	131.98±8.77 ^a	174.13±6.58 ^{bY}	140.27±2.36 ^a	126.45±7.52 ^a
Day 90	134.98±4.65 ^a	198.09±9.21 ^{bY}	146.49±8.44 ^a	132.67±7.03 ^a

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin]

At 60 days intervals, an increase in the serum ALP activity in TiO₂ NPs exposed (Group-II) (174.13±6.58 IU.L⁻¹) was significant ($p<0.05$) with that of vehicle control (Group-I) (131.98±8.77 IU.L⁻¹) and melatonin alone (Group-IV) (126.45±7.52 IU.L⁻¹). At 90 days intervals, there was a significant increase ($p<0.01$) in the ALP activity in Group-II (198.09±9.21 IU.L⁻¹) compared to both Group-I (134.98±4.65 IU.L⁻¹) and Group-IV (132.67±7.03 IU.L⁻¹). Increase in the ALP activities in Group-II from 30 days (147.88±6.43 IU.L⁻¹) to 60 days (174.13±6.58 IU.L⁻¹) and 90 days (198.09±9.21 IU.L⁻¹) intervals were significant ($p<0.05$) and ($p<0.01$), respectively. This is in agreement with the findings of Jafari *et al.* [9], Ranjan *et al.* [21], and Han *et al.* [22] in TiO₂ NPs (100mg.kg⁻¹) exposed rats for 60

days, 12 weeks, and 90 days, respectively. Similar findings were also observed by Cui *et al.* [8] and Chakrabarti *et al.* [20] in mice following 90-days exposure to 10mg.kg⁻¹ and 500mg.kg⁻¹, respectively.

At a 60 days interval, melatonin supplementation (Group-III) (140.27±2.36 IU.L⁻¹) significantly ($p<0.05$) decreased the ALP activity compared to Group-II (174.13±6.58 IU.L⁻¹). Whereas, at 90 days intervals, melatonin supplementation (Group-III) (146.49±8.44 IU.L⁻¹) significantly ($p<0.01$) decreased the serum ALP activity compared to Group-II (198.09±9.21 IU.L⁻¹). Banerjee *et al.* [32] reported that melatonin (10mg.kg⁻¹ x 60 days) could lower chromium-induced elevated serum levels of ALP and ALT in rats. The antioxidant nature of melatonin might have reduced serum ALP levels.

3.2.4. Gamma-glutamyl transferase (GGT).

The mean (±SE) gamma-glutamyl transferase (GGT) activities of different experimental groups at different time intervals are depicted in Table 12. There was a non-significant ($p>0.05$) difference in the serum GGT activities between all the groups at a 30 days interval. An increase in the GGT activities in TiO₂ NPs exposed (Group-II) from 30 days (1.62±0.15 IU.L⁻¹) to 90 days interval (2.84±0.19 IU.L⁻¹) was significant ($p<0.05$). At 60 days intervals, an increase in the serum GGT activity in TiO₂ NPs exposed (Group-II) (2.2±0.15 IU.L⁻¹) was significant ($p<0.05$) with that of both vehicle control (Group-I) (1.51±0.13 IU.L⁻¹) and melatonin alone (Group-IV) (1.45±0.08 IU.L⁻¹). Other groups were comparable with each other.

Table 12. The mean (± SE) serum gamma-glutamyl transferase (GGT) activity (IU.L⁻¹) in experimental groups of rats at different time intervals

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	1.30±0.10	1.62±0.15 ^X	1.57±0.08	1.27±0.13
Day 60	1.51±0.13 ^a	2.2±0.15 ^{bY}	1.74±0.15 ^{ab}	1.45±0.08 ^a
Day 90	1.78±0.13 ^a	2.84±0.19 ^{bZ}	2.03±0.16 ^a	1.70±0.19 ^a

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin]

At 90 days intervals, an increase in the serum GGT activity of Group-II (2.84±0.19 IU.L⁻¹) was significant ($p<0.01$) with that of both Group-I (1.78±0.13 IU.L⁻¹) and Group-IV (1.70±0.19 IU.L⁻¹), respectively. Ranjan *et al.* [21] reported TiO₂ NPs induced an increase in GGTP in rats. Melatonin supplementation in Group-III significantly ($p<0.05$) decreased the GGT activity (2.03±0.16 IU.L⁻¹) compared to Group-II (2.84±0.19 IU.L⁻¹).

Table 13. The mean (± SE) serum creatinine levels (mg/dl) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	0.66±0.05	0.71±0.04	0.73±0.05	0.66±0.02
Day 60	0.68±0.04	0.85±0.07	0.75±0.06	0.64±0.04
Day 90	0.69±0.03	0.92±0.08	0.78±0.10	0.67±0.04

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin]

3.2.5. Creatinine.

The mean (\pm SE) creatinine levels of different experimental groups at different time intervals are depicted in Table 13. The creatinine levels of all the experimental groups differed non-significantly ($p>0.05$) between each other throughout the experiment. Similar findings were reported by Chakrabarti *et al.* [20] in mice, Ranjan *et al.* [21] and Han *et al.* [22] in rats following sub-chronic exposure.

3.2.6. Blood urea nitrogen (BUN).

The mean (\pm SE) blood urea nitrogen (BUN) levels of different experimental groups at different time intervals are depicted in Table 14. The BUN levels in all the experimental groups differed non-significantly ($p>0.05$) between each other throughout the experiment. Similar findings were reported by Han *et al.* [22] in rats following 90-days exposure.

Table 14. The mean (\pm SE) blood urea nitrogen (BUN) values (mg/dl) in experimental groups of rats at different time intervals.

No. of days of exposure	C	TiO ₂ NPs	TiO ₂ NPs + MLT	MLT
Day 30	16.06 \pm 0.87	16.56 \pm 0.87	16.43 \pm 0.79	15.93 \pm 0.93
Day 60	16.68 \pm 0.72	19.29 \pm 1.14	19.54 \pm 1.53	16.56 \pm 0.87
Day 90	17.01 \pm 0.81	20.75 \pm 1.01	19.67 \pm 1.20	17.34 \pm 0.72

Note: Data were analysed by mixed effects-analysis followed by Tukey's *post hoc* multiple comparison test; Values bearing dissimilar small alphabets within a row and capital alphabets with in a column vary significantly at $p < 0.05$ [C = Control, TiO₂ NPs = Titanium dioxide nanoparticles, TiO₂ NPs + MLT = Titanium dioxide nanoparticles + melatonin, MLT = melatonin]

4. Conclusions

It can be concluded from the experimental study that sub-chronic exposure (*per os*) to titanium dioxide nanoparticles (TiO₂ NPs) can lead to significant alterations in hematological and serum biochemical parameters in male Wistar rats. Further, melatonin supplementation can ameliorate some of these hematologic and serum biochemical alterations induced by titanium dioxide nanoparticles under experiential conditions.

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Conflicts of Interest

The authors declare no conflict of interest.

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