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RESEARCH PAPER

# The Developmental Hepatotoxicity of Titanium Dioxide Nanoparticles in NMRI Mouse Neonates

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## ABSTRACT

This study aimed to investigate the potential hepatotoxic effects of titanium dioxide nanoparticles ($TiO_2$ NPs) on neonatal NMRI mice through maternal milk exposure. A total of 20 postpartum dams were divided into two groups: the experimental group received 30 mg/kg of $TiO_2$ NPs, while the control group received deionized water for 14 days. The offspring were analyzed for oxidative stress markers, bioaccumulation, and histopathological changes in hepatic tissues. The results showed no significant difference in body weight or liver-to-body weight ratio between the treatment and control groups. However, oxidative stress was evident in the treatment group, with a significant reduction in glutathione (GSH) levels (0.8 µmol/g tissue, $p<0.05$) and glutathione peroxidase (GPx) activity (3.68 u/g tissue, $p<0.05$), compared to the control group. Additionally, malondialdehyde (MDA) levels, indicative of lipid peroxidation, were significantly higher in the treatment group (96 nmol/g tissue, $p<0.001$). $TiO_2$ content was markedly increased in the treatment group's liver (22.4 ng/g tissue, $p<0.001$) and stomach milk (41.6 ng/g tissue, $p<0.001$), suggesting bioaccumulation. Histological analysis revealed pronounced tissue degeneration and vascular changes in the treatment group's hepatic tissues, contrasting with the normal histology observed in the control group. These findings indicate that maternal ingestion of $TiO_2$ NPs can lead to oxidative stress and potential hepatotoxicity in neonatal mice, with significant implications for environmental and consumer product safety regulations.

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## INTRODUCTION

The bactericidal efficacy of Titanium dioxide nanoparticles ($TiO_2$ NPs) has catalyzed their integration into diverse scientific and industrial domains, notably within the realms of medical science, pharmaceutics, alimentary products, cosmetology, and textile manufacturing [1–4].

In the medical field, $TiO^2$ NPs are valued for their ability to inhibit microbial growth, which is crucial for preventing infections [5–7]. For instance, studies have shown that $TiO^2$ NPs exhibit photocatalytic activity under visible light, leading to the degradation of 90% of Rhodamine B dye, a model pollutant, using an optimal condition of 10 mg/L RhB, 3.5 g/L TiO2-NPs, and a pH of 7.0 [8]. This photocatalytic property is harnessed in pharmaceutics to create self-sterilizing surfaces and equipment. In the food industry, $TiO^2$ NPs are incorporated into packaging materials to extend shelf life by preventing bacterial contamination, with surface areas of $TiO^2$-NPs reaching up to 118 $m^2/g$ [9]. The cosmetic industry benefits from the non-toxic nature of $TiO^2$ NPs, which, when used in sunscreens, provide UV protection without causing skin irritation [10]. Lastly, the textile industry utilizes $TiO_2$ NPs to produce fabrics with self-cleaning properties, where the nanoparticles' average size is about 32 nm and they exhibit a negative surface charge of -4.9 mV, enhancing the material's resistance to staining and bacterial colonization [11]. These applications underscore the versatility and efficacy of $TiO_2$ NPs in promoting hygiene and safety in everyday products.

The utilization of $TiO_2$ NPs in various applications has been accompanied by growing concerns regarding their potential health and environmental impacts [12,13]. Numerous studies have been carried out to assess the toxic impact of $TiO_2$ NPs on mammalian cells, revealing that even non-lethal concentrations can adversely affect a range of cell types, including pluripotent stem cells, murine embryonic fibroblasts, human liver cancer cells, and THP-1 monocytic cells [14]. In vitro studies have demonstrated that exposure to $TiO_2$ NPs can lead to significant cytotoxicity; for instance, concentrations as low as 5 µg/mL can reduce cell viability by up to 30% within 24 hours [15]. This highlights the nanoparticles' ability to disrupt cellular function and induce toxicity.

In vivo investigations have further shed light on the behavior of $TiO_2$ NPs within biological systems. These nanoparticles have shown a remarkable capacity to cross biological membranes, suggesting a high level of bioavailability and potential for systemic distribution [5]. Once inside the body, $TiO_2$ NPs can accumulate in various organs, with the liver being a particularly notable site of deposition. Studies have found that nanoparticles with an average size of approximately 21 nm can accumulate in the liver following oral exposure, leading to oxidative stress and inflammation [16]. The hepatic accumulation of $TiO_2$ NPs is concerning, as it may result in long-term health effects, including the potential for liver dysfunction or disease.

Empirical studies have reported the accumulation of $TiO_2$ NPs in hepatic tissues of murine models following oral administration, with a significant biodistribution within the liver, followed by the kidneys [17–19]. The primary mechanism of $TiO_2$ NP-induced cytotoxicity is the induction of oxidative stress, characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidative defenses [20].

The liver, being part of the reticuloendothelial system and possessing extensive vascular perfusion, is especially susceptible to oxidative damage. Experimental evidence has shown that exposure to $TiO_2$ NPs leads to an increase in ROS levels, accompanied by a reduction in glutathione reserves, lipid peroxidation, and protein carbonylation [21–23]. These changes are indicative of oxidative stress and could negatively impact the functioning of the liver. For instance, a study revealed that exposure to $TiO_2$ NPs at a concentration of 50 µg/mL resulted in a 40% increase in ROS production and a 25% decrease in glutathione levels in hepatic cells after 24 hours [24].

Furthermore, the size of $TiO_2$ NPs plays a crucial role in their cytotoxic potential [25]. Nanoparticles with a diameter of approximately 20 nm have been shown to be more readily taken up by cells and are more likely to induce oxidative stress compared to larger particles [26]. This size-dependent effect underscores the need for careful consideration of nanoparticle dimensions in risk assessments. Interestingly, the administration of antioxidants such as N-acetylcysteine (NAC) has been found to mitigate the oxidative effects induced by $TiO_2$ NPs. Pre-treatment with NAC at a dose of 5 mM was able to reduce ROS generation by 50% and restore glutathione (GSH) levels to near-normal in hepatic

cells exposed to $TiO_2$ NPs [27]. This suggests that antioxidant therapy could be a viable strategy to counteract the negative effects of $TiO_2$ NP exposure.

The exploration of developmental hepatotoxicity induced by $TiO_2$ NPs is a relatively uncharted domain, with the majority of research focusing on adult specimens. However, recent studies have highlighted the ability of $TiO_2$ NPs to cross the placental barrier and enter the fetal system, raising concerns about potential risks to developing organisms. Moreover, the possibility of $TiO_2$ NPs being transferred to neonates through lactation has been recognized, prompting investigations into the subsequent health effects on suckling offspring. This transference poses a risk of hepatotoxic effects, such as inflammation and oxidative stress, which can impair liver function and development in the neonates. The study of such developmental hepatotoxicity is crucial, as it may provide insights into the long-term health implications of early-life exposure to $TiO_2$ NPs and inform safety regulations for their use in consumer products. The current research aims to evaluate the likelihood of $TiO_2$ NPs transferring from the milk of exposed mother mice to their young, and the subsequent development of liver toxicity in these suckling pups.

## MATERIALS AND METHODS

A total of 28 NMRI mice in gestation were acquired from the animal center of University of Baghdad. Each subject was housed individually in a controlled environment with a 12-hour light/dark cycle, 20% relative humidity, and a temperature of 25±1°C. The mice had access to sterile water and standard chow ad libitum. Upon parturition, only dams with litter sizes ranging from six to eight pups were selected for the study. These were then segregated into two cohorts along with their offspring.

The experimental group, consisting of 10 postpartum dams, received 30 mg/kg of $TiO_2$ NPs, purchased from Sigma Aldrich, administered daily for a duration of 14 days [17]. The administration was performed via gavage, ensuring direct delivery to the gastrointestinal tract. Conversely, the control group, also comprising 10 dams, was treated with an equivalent volume of deionized water following the same method and schedule. The dimensions and structural characteristics of the $TiO_2$ NPs were assessed using a transmission electron microscope (TEM). The resulting images are presented in Fig. 1 for visual reference.

Upon completion of the dosing regimen, a total of 20 pups—two randomly selected from each dam—were subjected to anthropometry. Subsequently, hepatic tissues were excised, cleansed with a sodium phosphate buffer solution (pH=7.4) to maintain physiological relevance, and weighed. The hepatic mass was then expressed as a ratio to the body weight (mg/g), and the specimens were preserved at -85°C to facilitate further analyses.

To elucidate the histopathological alterations, eight hepatic samples—one from each randomly chosen pup—were fixed in 10% neutral-buffered formalin for 48 hours. Post-fixation, the tissues were processed, embedded in paraffin, and sectioned into 5 µm thick slices using a digital microtome. Four sections per liver were then stained utilizing the hematoxylin and eosin (H&E) technique. The prepared slides were subsequently examined under a light microscope to assess cellular morphology [28].

For the assessment of oxidative stress markers,

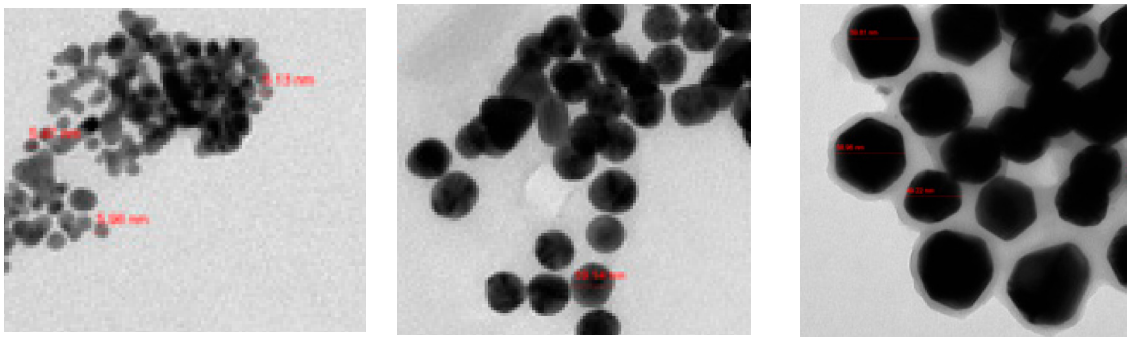



Fig. 1. Visualization of the $TiO_2$ NPs dimensions and structural form through high-resolution TEM.

hepatic tissues from 20 neonates in each group—control and $TiO_2$ NP-treated—were randomly selected. The tissues were homogenized in a chilled 0.1 M phosphate buffer (pH=7.4). The homogenates were then centrifuged at 2500 rpm for 10 min using a microcentrifuge, and the supernatant was carefully collected. This supernatant served as the sample for quantifying the levels of GSH, glutathione peroxidase (GPx) activity, and malondialdehyde (MDA) concentration.

The quantification of GSH was conducted via the Ellman's reagent (DTNB) method [29]. Initially, to precipitate proteins, 1 ml of 5% trichloroacetic acid (TCA) was added to 1 ml of the supernatant. Following centrifugation, 0.1 ml of the resultant supernatant was mixed with 2 ml of phosphate buffer (pH 8.4) and 0.5 ml of DTNB. The mixture was then incubated, and the absorbance was measured at 468 nm using a spectrophotometer after 12 min, providing an index of the GSH content within the samples. This meticulous procedure ensures the accuracy of the biomarker analysis, which is pivotal for understanding the biochemical impact of $TiO_2$ NP exposure on neonatal mice.

In the enzymatic assay to determine GPx activity, 1 ml of reaction buffer—comprised of 0.4 M potassium phosphate (pH=7.0), 8 mM reduced GSH, 0.5 mM EDTA, and 5 mM sodium azide—was combined with 0.2 ml of the hepatic supernatant. The mixture was incubated at 37°C for 10 min to allow for enzymatic reactions. Subsequently, 1 ml of 8 mM hydrogen peroxide ($H_2O_2$) was added, and the incubation continued for an additional 10 min at 37°C to measure the consumption of GSH by GPx.

To precipitate proteins, 1 ml of 5% TCA was introduced to the reaction mixture. Following centrifugation, the remaining reduced GSH in the supernatant was quantified, as previously described. It is noteworthy that one unit of GPx is defined as the amount of enzyme that catalyzes the oxidation of one nanomole of GSH per minute under the assay conditions [23].

The thiobarbituric acid reactive substances (TBARS) was used to determine the concentration of MDA for assessing lipid peroxidation [30]. To the supernatant volume of 0.2 ml, 1 ml of 50% TCA in 0.1 M hydrochloric acid (HCl) and 1 ml of 26 mM thiobarbituric acid (TBA) were mixed in. The mixture was then heated in a water bath at 95°C for 20 min. After cooling, the samples were centrifuged at 1000 x g for 10 min, and the absorbance of the supernatant was read at 561 nm to determine the MDA levels.

For the bioaccumulation study, 10 pups from each group—control and $TiO_2$ NP-treated—were randomly selected. The livers and the casein clumps formed in the stomachs from milk digestion were meticulously extracted. These samples were then placed in a solution of perchloric acid and concentrated nitric acid at a 1:4 ratio for complete digestion over 24 hours [16]. The digested samples were then heated at 120°C until complete evaporation of the acids. Finally, the digested tissues were analyzed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to quantify the titanium content, replacing the original measurement of $TiO_2$.

In this investigation, quantitative data were expressed as the mean ± standard deviation (SD). To compare the metrics between the control and the NMRI mouse treatment groups, the independent-samples t-test was employed. Statistical evaluations were performed with SPSS software, version 23.0. The thresholds for significance were established at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, where smaller p-values denote greater statistical significance of the noted disparities.

To ensure the robustness of the experimental outcomes and to mitigate any potential bias due to litter effects, a random selection protocol was implemented. Specifically, one to two pups from each litter were chosen at random for each assay performed. This approach promotes the generalizability of the results by reducing the likelihood of skewing data due to intra-litter variability. Such a sampling strategy is crucial in translational research where findings are intended to be extrapolated to broader populations.

## RESULTS AND DISCUSSION

According to Fig. 2, the body weight of neonates in the treatment group, which were exposed to $TiO_2$ NPs through maternal milk, did not significantly differ from that of the control group neonates. Furthermore, the analysis revealed that the weight-to-body weight ratio of the treatment group's offspring was not significantly reduced when compared to the control group.

The data indicate that there was a statistically significant reduction in the regeneration of GSH ($p<0.05$) and a marked decrease in the activity of

GPx enzyme within the hepatic tissues of the NMRI mouse neonates in the treatment group ($p<0.05$). These findings suggest a compromised antioxidant defense system in the livers of pups exposed to $TiO_2$ NPs through maternal milk.

Additionally, the analysis of MDA levels, a marker of lipid peroxidation, revealed a significant increase ($p<0.001$) in this biomarker in the liver tissues of the treatment group's offspring. This elevation in MDA concentration is indicative of heightened oxidative stress and potential cellular damage in the neonates that consumed $TiO_2$ NP-contaminated maternal milk, compared to the control group's infants. The result illustrates that the concentration of titanium, as a result of $TiO_2$ NP exposure, was significantly higher in both the stomach milk and liver tissues of the treatment group's neonates when contrasted with the control group's offspring ($p<0.001$). Notably, the titanium levels were more pronounced in the stomach milk than in the liver tissue. This disparity suggests a differential bioaccumulation pattern of $TiO_2$ NPs, with a higher propensity for retention in the ingested milk within the gastrointestinal tract before systemic distribution to the liver. The statistical significance denoted by the p-values underscores the reliability of the observed differences between the control and treatment groups in terms of $TiO_2$ NP exposure and its biological implications.

Histological examination of the hepatic tissue sections demonstrated that in the control group, hepatocytes were uniformly distributed with no evidence of cellular or tissue damage (Fig. 3). In contrast, the treatment group's liver sections exhibited numerous vacuolar structures, indicative of hepatocyte malnutrition and tissue degradation. Additionally, these sections showed pronounced sinusoidal dilation and vascular hyperemia, suggesting alterations in hepatic microcirculation potentially attributable to $TiO_2$ NP exposure. These histopathological findings provide insights into the subcellular impact of $TiO_2$ NPs on neonatal hepatic architecture.

Given the heightened susceptibility of infants compared to adults, attributed to their lower plasma protein levels, diminished enzymatic activity, and less robust immune defenses against

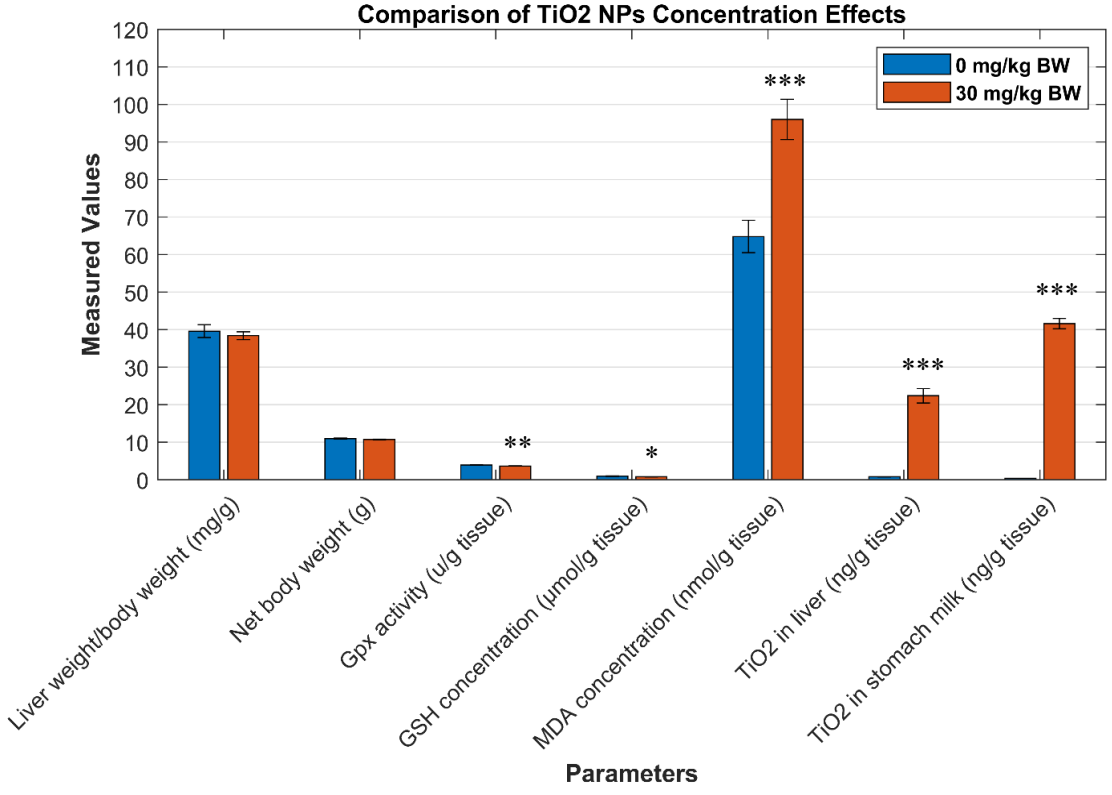



Fig. 2. Impact of differing concentrations of $TiO_2$ NPs on selected parameters (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

various stresses [31], it is crucial to understand the impact of maternal exposure to environmental agents. This study investigated the influence of $TiO_2$ NPs on the hepatic health of NMRI mouse pups following exposure through lactation. This research focused on assessing whether the $TiO_2$ NPs ingested by the nursing mothers can be transmitted to their offspring and the potential hepatotoxic effects this exposure may have on the developing livers of the neonatal mice.

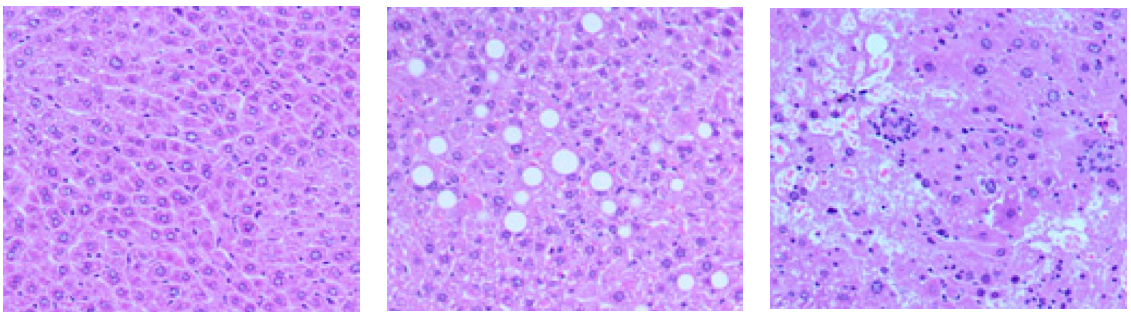
Initial findings indicate that the body weight and the liver-to-body weight ratio of infant NMRI mice, whose mothers were exposed to $TiO_2$ NPs, did not significantly differ from those of the control group's offspring. Various studies have reported inconsistent outcomes regarding the impact of different nanoparticles on weight metrics and organ-to-body weight ratios [32]. These discrepancies may be attributed to factors such as the nanoparticle type, concentration, exposure duration, and even the specific animal species under investigation.

Although there was no observed change in the liver-to-body weight ratio in the current investigation, microscopic examination of the liver from the treated group's offspring revealed pronounced vascular dilation, increased blood flow within the liver sinusoids, and a proliferation of vacuolar formations. These symptoms may indicate significant lipid peroxidation. In analogous research, where rats were exposed to $TiO_2$ nanoparticle inhalation over a period of 28 days, there was a noted rise in both vacuolation and cell death within the liver hepatocytes [30]. Further studies have reported similar outcomes, such as enhanced activity of liver enzymes like alkaline phosphatase, central vein dilation, and bile duct proliferation upon $TiO_2$ NP exposure. Comparable structural changes in the liver were also seen in rainbow trout when their aquatic environment was infused with $TiO_2$ NPs at certain dosages [13,22,31].

It is suggested that these histological alterations in the liver of mature animals due to $TiO_2$ NP exposure may be linked to oxidative stress induction [26]. However, data on the hepatotoxic effects of these nanoparticles during lactation is scarce. Pursuing this line of inquiry, this study measured several oxidative stress markers, revealing a marked reduction in both GSH levels and GPx enzyme activity in the treated group's offspring compared to controls. These antioxidants are crucial for liver protection. The likely cause for this depletion is the generation of ROS in the liver due to $TiO_2$ NP exposure, which could impact everything from gene expression to enzyme function [28].

Supporting this hypothesis, other research has shown that exposure to $TiO_2$ NPs can lead to a decrease in GSH concentration and an increase in mitochondrial membrane potential, indicating elevated ROS levels [10,12,13]. Additionally, exposure to these nanoparticles has been linked to the inhibition of key enzymes involved in GSH biosynthesis and changes in gene expression related to antioxidant enzymes.

Oxidative stress can lead to the destruction of vital macromolecules such as proteins, DNA, and lipids [30]. This was evidenced by a significant increase in MDA, a marker of lipid oxidation, in the liver tissue of offspring consuming milk contaminated with $TiO_2$ NPs. These findings are



(a) (b) (c)

Fig. 3. Comparative histopathological analysis of neonatal mice liver tissue. (a) Control Group: Normal histological architecture of liver hepatocytes, exhibiting no signs of tissue pathology; (b) Treatment Group: Pronounced tissue degeneration characterized by the formation of numerous vacuolar structures within hepatocytes; (c) Treatment Group: Marked dilation of hepatic sinusoids and hyperemia, evidenced by the substantial presence of erythrocytes within the sinusoidal spaces.

consistent with previous studies where exposure to nanoparticles resulted in similar biochemical and tissue alterations [21,24].

This study also investigated the potential direct transfer of TiO2 NPs through breast milk to the offspring by measuring the nanoparticle concentration in the milk and the livers of the newborns. Significant levels of $TiO_2$ were detected, confirming the direct impact of these nanoparticles on the liver tissue of the infants. This aligns with other research demonstrating the transfer and effects of various nanoparticles through lactation [16].

## CONCLUSION

This study provides compelling evidence of the hepatotoxic effects of $TiO_2$ NPs on neonatal NMRI mice through maternal milk exposure. Despite no significant differences in body weight or liver-to-body weight ratios between the control and treatment groups, the oxidative stress markers indicated a compromised antioxidant defense system in the treatment group. The significant reduction in GSH levels and GPx activity, coupled with increased MDA levels, suggests that $TiO_2$ NPs induce oxidative stress in neonatal hepatic tissues. The bioaccumulation of $TiO_2$ NPs in the liver and stomach milk of the treatment group further underscores the potential for maternal transfer of nanoparticles and their retention in the offspring. Histopathological findings, including vacuolation, sinusoidal dilation, and vascular hyperemia, reveal the subcellular impact on hepatic architecture and function. These results highlight the need for cautious use of $TiO_2$ NPs, particularly in products that may affect pregnant and nursing mothers, to safeguard the health of neonates who are more vulnerable to environmental toxins.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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