TiO_2 nanoparticles induced oxidative stress mediated DNA damage in the liver of adult male Wistar rats

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Abstract

Nanoparticles (NPs) are extensively being used in modern life due to their distinctive properties like small size having large surface area. Titanium dioxide (TiO_2) NPs are mostly used in cosmetic products, food additives, pharmaceuticals and electronics. They are capable of inducing oxidative stress in both animals and human. The intention of this study was to find out the hepatotoxic effect of TiO_2 NPs on the male Wistar rats. The animals were divided in to three groups. First group received normal saline; Second group received TiO_2 NPs (50 mg/kg/bw) for 14 days continuously, while third group received TiO_2 NPs (100 mg/kg/bw) for the same duration. The increased levels of specific markers AST, ALT, ALP, LDH and GGT along with the TBARS, LOOH, CD and PC in the liver clearly shows the hepatotoxic action of TiO_2. The hepatotoxic nature of TiO_2 NPs was more evidenced by the diminished activity of antioxidant enzymes levels and also showed augmented DNA damage and fragmentation in hepatocytes. In conclusion, the data indicated that TiO_2 NPs induced oxidative stress which produces hepatotoxicity in the rat liver.

Keywords: TiO_2 NPs, rat liver, ROS, DNA damage, hepatotoxicity.

Introduction

Nanotechnology is a promising field in molecular technology that spans many areas of sciences, which may present a variety of uses in medical, agriculture, industrial, manufacturing and military sectors. Recently there is a spurt in the usage of NPs in cosmetics as well as in the area of biomedicine [**1-4**]. The uses of engineered or manufactured NPs are manipulation of materials at the nanoscale level, typically ranging from 1 to 100 nm [**5, 6**]. Synthesis and its applications are the major part of nanotechnology. NPs differ from their bulk particles in several physical and chemical characters like size, surface area and dispersal capacity [**7, 8**]. Such properties lead to the generation of several avenues in nanotechnology in human health care aspects [**9**]. The spurt in nanotechnology is also responsible for several unexpected adverse effects give birth to another field like nanotoxicology [**10**].

TiO_2 is one among the widely used NPs, which is non-flammable white powder produce abundantly in past two decades. The applications of NPs also responsible for health risk to human beings [**5-11**]. The nanoparticles destabilize membrane characteristic [**12, 13**]. One of the mechanisms underlining by which nanoparticulate matter induced toxicity is by generating ROS promoting oxidative stress, which damages proteins, lipids and DNA [**10, 14**]. Moreover, TiO_2 NPs develops the formation of ROS, which is one of the major toxic mechanisms observed in organisms [**15**]. The toxicity of TiO_2 NPs is also more evident in aquatic animals as demonstrated by Mansouri *et al.* [**16**].

Liver is most vulnerable organs, which play a vital role in detoxification of tissues. Such biochemical markers are measurable responses to the exposure of an aquatic organism as well as terrestrial [**17, 18**]. The colour additives in food, tooth paste and drug capsules are major sources of entry of TiO_2 NPs through oral route [**19**]. In toxicological aspects, the changes of enzyme activities further leads to damage in cells and organs [**20**]. For instance, aspartic aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) is good indicator for the liver disorders when the animals exposed to heavy metal toxicity [**21**]. These enzymes are biomarkers of acute hepatic damage and assessing for necrosis of the liver cells [**22-24**]. Several studies have reported that injected or inhaled TiO_2 NPs can migrate to several organs through circulation and imposes adverse effects of organisms [**11, 25-30**]. Recently, Zaki *et al.* [**31**]

observed that TiO_2 NPs causes much toxic effects like inflammation, cytotoxicity and genomic instability in mammals, plants, and micro-organisms. However, oral administration of TiO_2 NPs at the dose of 5g/kg/bw of mice significantly increased liver weight and produced hepatocyte necrosis [**30**]. Meantime, some other study also reported that TiO_2 anatase NPs could cause liver injury of treated mice daily for 14 days [**27**].

As on date, most of the studies of TiO_2 NPs in mammalian models have noted via inhalation or dermal exposure. Recently, TiO_2 NPs has been broadly used as food additives, which is easily entering the human body via oral ingestion [**32**]. Hence, oral ingestion of TiO_2 NPs is a potential route of exposure for several organisms including human. To our knowledge, only few reports are available on oral toxicity of TiO_2 NPs in mice and rats. Therefore, in the present study was intended to investigate the hepatotoxic effects of mixed rutile and anatase TiO_2 NPs on mammalian model of adult male Wistar rats.

Materials and methods

Nanomaterials and preparation of treated dose suspension

The TiO_2 NPs (< 100 nm, 99.5% purity, CAS NO: 13463-67-7) was obtained from Sigma-Aldrich Co. (St, Louis, MO 63103, USA). The TiO_2 NPs was suspended in 0.9 % physiological saline solution and the suspension was ultrasonically vibrated for 10 minutes before oral administration.

Animals and diet

Adult male Wistar rats of equal weight (250-260g) were purchased and housed in polypropylene cages lined with paddy husk and kept in a semi natural light/dark condition animals house (Siddha Central Research Institute (SCRI), Chennai (TN), India). The animals had free access to water and standard pellet diet. Animal handling and experimental procedures were approved by the Institutional Animal Ethical Committee, SCRI, Chennai, India (Registration Number: 138/PHARMA/ SCRI, 2013).

Experimental design

Experimental design as follows:
Group 1: Control group (n=6) administered with 0.9 % saline as a vehicle, daily for 14 days
Group 2: Rats (n=6) administered daily with TiO_2 NPs (50mg/kg/bw) dissolved in normal saline solution (0.9% NaCl)
Group 3: Rats (n=6) administered daily with TiO_2 NPs (100mg/kg/bw) dissolved in normal saline solution (0.9% NaCl)

The rats were administered with TiO_2 NPs daily in the morning 8-9 AM. After last treatment (15^{th} day), all animals were euthanized by cervical decapitation. Liver was removed, cleared off the adhering tissues and weighed. Liver was dissected out and processed immediately for biochemical and histological analysis. All the biochemical estimations were carried out by standard spectrophotometric techniques. For the comet assay, the liver tissue suspension was obtained by enzymatic digestion at 37 °C. After digestion, the tissue extract was centrifuged for ten minutes at 3000*g*. The tissue pellet was then suspended in PBS and used for the DNA damage analysis.

Hepato-specific biochemical assays

The activity of alanine transaminase (ALT) and aspartate transaminase (AST) were assayed by the method of Reitman and Frankel [**33**]. The activity of alkaline phosphatase (ALP) was assayed by the method of Bessey *et al.* [**34**]. The activity of γ-glutamyle trans peptidase (GGT) was assayed by the method of Orlowski and Meister, [**35**]. The activity of lactate dehydrogenase (LDH) was assayed by the method of King, [**36**].

Estimation of LPO

LPO contents were measured by the method of Niehaus and Samuelsson **[37]** and Jiang *et al.* **[38]**, respectively. The protein oxidation, total protein carbonyl contents were determined by Levine *et al.* **[39]**.

Determination of non-enzymatic antioxidants

Reduced glutathione (GSH) **[40]**, Total sulfhydryl (TSH) [**41**], vitamin C **[42]** and vitamin E **[43]** were measured by the standard spectrophotometric methods.

Determination of enzymatic antioxidants

Superoxide dismutase (SOD) [**44**], catalase (CAT) [**45**], glutatnione peroxidase (GPx) [**46**], glutathione-s-transferase (GST) [**47**], glutathione reductase (GR) [**48**] and total protein [**49**] were measured by standard spectrophotometric methods.

Comet and DNA fragmentation assays

Hepatocytes were secluded from experimental and control groups and processed for the alkaline comet assay as described previously [**50-52**]. After electrophoresis, the slides were prepared by ethidium bromide and the images were captured using a fluorescence microscope (Eclipse TS100, USA). The images were analyzed for tail length and olive tail moment using by CASP software (version 1.2.2). For DNA fragmentation, Agarose gel electrophoresis was performed to determine the DNA fragmentation as described previously [**53**].

Histopathological studies in the liver

For histological analysis, the liver tissue was fixed in ten percent formalin solution and dehydrated by ethyl alcohol mixture, cleaned in xylene, and then embedded in paraffin wax. Sections of the tissues were prepared by microtome (5µm), stained with haematoxylin and

eosin (H & E) and then mounted in a neutral deparaffinized xylene medium for microscopic examinations.

Statistical analysis

Statistical analysis of all data are expressed as mean ± standard deviation (SD) and also followed by one-way analysis of variance (ANOVA) using SPSS version 19. And the individual comparisons were obtained by Duncan's multiple range test. The value of $p< 0.05$ was considered as statistical significant.

Results and discussion

The liver is a major organ for detoxification of unsafe materials circulating in the body. A vast verity of chemicals, drugs and metals have been shown to amend the structural and functional integrity of the liver. The level of hepatic damage by TiO_2 NPs depends on the characteristic features of materials, dose, route, and exposure duration [**54**]. In the present study indicate that the intragastric intubation of TiO_2 NPs can increase liver damage and induce histopathological and molecular changes in the liver.

Table 1. Changes in the activities of serum AST, ALT, ALP, LDH, and GGT in control and experimental rats.

Para meters	Group 1 (Control)	Group 2 (50mg/kg/bw)	Group 3 (100mg/kg/bw)
ALT ($IU.L^{-1}$)	47.17 ± 1.970^a	53.39 ± 2.462^b	59.43 ± 2.914^c
AST ($IU.L^{-1}$)	32.63 ± 1.738^a	36.23 ± 2.624^b	40.31 ± 2.846^c
ALP ($IU.L^{-1}$)	19.49 ± 0.890^a	13.74 ± 0.868^b	16.61 ± 1.138^c
LDH ($IU.L^{-1}$)	54.36 ± 3.248^a	56.74 ± 2.254^b	63.38 ± 3.428^c
GGT ($IU.L^{-1}$)	3.97 ± 0.184^a	4.16 ± 0.124^b	4.49 ± 0.118^c

Values are the mean ± SD for 6 rats in each group. Values not sharing a common letter (a–c) differ significantly at $P < 0.05$ (Duncan's multiple range test).

Table 1 shows the level of hepato-specific marker enzymes such as ALT, AST, ALP, LDH and GGT in control and experimental rats. The levels of ALT, AST, ALP, LDH and GGT were significantly ($P<0.05$) increased in the 50 and 100 mg/kg administrated TiO_2 NPs when compared to the control rats. Outflow of hepato-specific marker enzymes into blood has been measured as an indicator of liver malfunction as well as hepatic cells damage. An increase in the activities of these enzymes in liver tissue is pinpointing of hepatic cells degeneration or damage, and thus causes severe dysfunction of the liver. Fatemeh and Fazilati, reported that hepatic damage interrelated as well with the leakage these enzymes [**55**]. Therefore, cellular damage caused by toxic materials is often accompanied by increasing cell membrane permeability [**56**]. In the current exploration, the increased activities of liver AST, ALT, ALP, and LDH in clearly shows that the liver is highly vulnerable to TiO_2 NPs toxicity which is in line with existing report of Fatemeh and Fazilati [**55**]. This may be due to increased ROS levels in the liver which ascribed to liver damage resulting either in increased release of hepatocyte specific biomarkers from of the liver. GGT has been commonly used as an indicator of liver malfunction marker. Current works have noted that measuring liver GGT might be affirmative in studying oxidative stress mechanism in different organs including liver [**57**].

Oxidative stress mediated liver damage induced by TiO_2 NPs can be examined in experimental animals by detecting LPO markers. TiO_2 NPs is generating oxidative stress in the liver by increasing LPO by free radicals [**58**]. In addition, LPO causes impaired membrane functions, structural integrity, reduced membrane volatility, and inactivation of number of membrane bound enzymes may lead to decreased ATP production and increased production of ROS in liver [**59**]. In the present study, significant increase levels of LPO markers were observed in the liver of TiO_2 NPs treated rats (**Fig. 1**). This result was corroborating with the previous report of Praveen *et al.* [**60**]. Present study further confirms with earlier report of Vasantharaja *et al.* reported that the oral administration of TiO_2 NPs have possible accumulation in the liver which induce adverse effects in the rat liver [**54**].

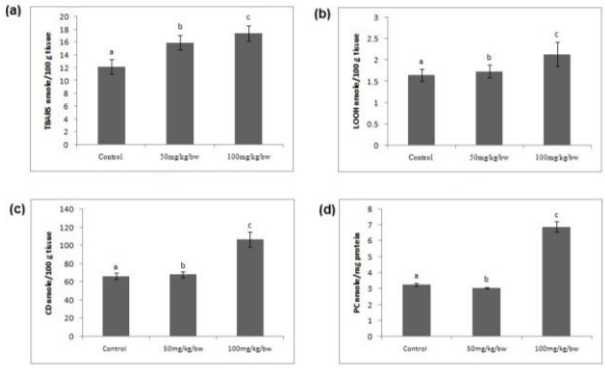

Fig. 1. The changes in the levels of hepatic (a) lipid peroxidation (TBARS), (b) lipid hydroperoxides (LOOH), (c) conjugated dienes (CD) and (d) protein carbonyl (PC) content in the liver of control and experimental rats. Values are the mean ± SD for 6 rats in each group. Values not sharing a common letter (a–c) differ significantly at $P < 0.05$ (Duncan's multiple range test).

Mutilation of the antioxidant defense system is measured to be vitally concerned in TiO_2 NPs induced toxic effects. Vitamin C is a dietary antioxidant that plays a vital role in concert with vitamin E, which is a chain breaking antioxidant that prevents the free radicals. Several reports have revealed that the positive effect of vitamin C as a scavenger of free radicals [**61**]. GSH, which has a sulfhydryl group in its peptide, is an important antioxidant largely present in living systems. In the present study, our result represent the depleted levels of non-enzymatic antioxidants in the TiO_2 NPs intoxicated rat liver that leads to oxidative stress (**Fig. 2**), which corroborated by Liang *et al.* [**59**]. This could be due to over accumulation of NPs induced ROS in the liver which led to organ dysfunctions.

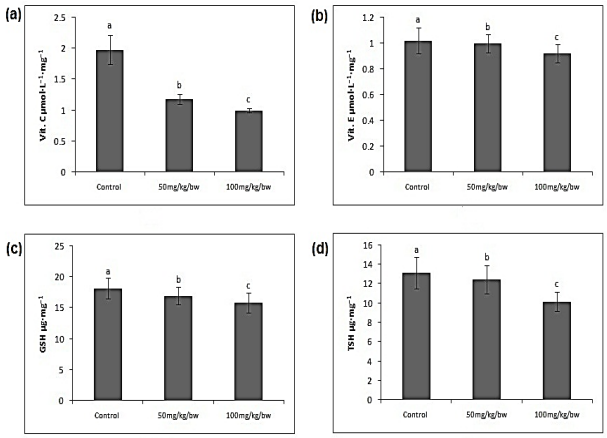

Fig. 2. The changes in the levels of (a) vitamin C, (b) vitamin E, (c) reduced glutathione (GSH), and (d) total sulfhydryl groups (TSH) in the liver of control and experimental rats. Values are the mean ± SD for 6 rats in each group. Values not sharing a common letter (a–c) differ significantly at $P < 0.05$ (Duncan's multiple range test).

The amount of antioxidant enzymes is the proper indirect way to assess the pro-oxidant–antioxidant condition in TiO_2 NPs induced toxicity. Both SOD and CAT are involved in elimination of ROS. SOD is an enzyme accountable for the transformation of O^- into less unsafe yields like H_2O_2, whereas CAT brings about the reduction of H_2O_2 and protects tissues from the highly reactive hydroxyl radicals [**62**]. Glutathione-related enzymes such as GPx, GR, and GST function either directly or indirectly as antioxidants. GPx is a selenium containing enzyme that uses glutathione in decomposing H_2O_2 to nontoxic products. In the present study, TiO_2 NPs administration decreases the activities of enzymatic antioxidant in liver (**Table. 2**) indicated that the occurrence of oxidative stress and LPO response were generated by the NPs accumulation. Our result of the current study corroborated with the earlier report of Oberdorster *et al.* [**63**].

Table 2. The changes in the activities of SOD, CAT, GPX, GST, and GR in the liver of control and experimental rats.

Parameters	Group 1(Control)	Group 2 (50mg/kg/bw)	Group 3 (100mg/kg/bw)
SOD (U/mg protein)	7.04 ± 0.459^a	5.96 ± 0.534^b	5.12 ± 0.412^c
CAT (U/mg protein)	98.24 ± 4.73^a	82.69 ± 4.23^b	79.85 ± 4.42^c
GPx (U/mg protein)	8.72 ± 0.502^a	7.27 ± 0.434^b	6.18 ± 0.431^c
GST (U/mg protein)	8.09 ± 0.514^a	6.52 ± 0.550^b	5.71 ± 0.410^c
GR (U/mg protein)	2.34 ± 0.142^a	1.57 ± 0.195^b	1.42 ± 0.198^c

Values are the mean ± SD for 6 rats in each group. Values not sharing a common letter (a–c) differ significantly at $P < 0.05$ (Duncan's multiple range test).

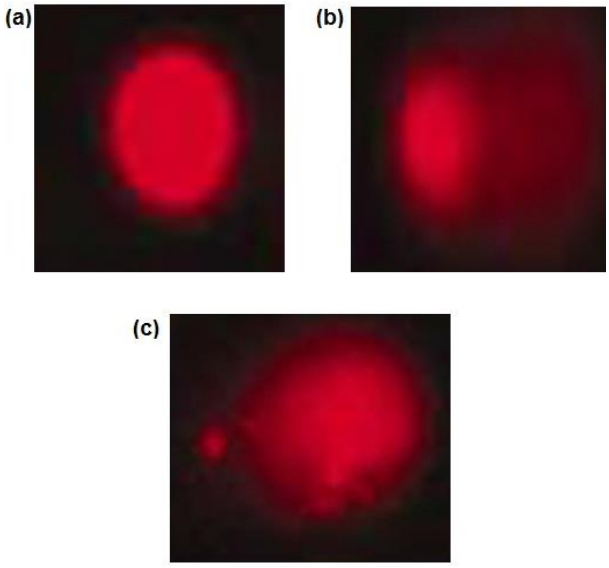

Fig. 3. Photomicrographs of comets assay showing the DNA migration pattern in the liver of rats caused by oral administration with TiO_2 NP_S for 14 consecutive days. (a) Control group (200x) shows no DNA migration. (b) 50 mg/kg body weight of TiO_2 NPs group (200x) shows minimal DNA with migration. (c) 100 mg/kg of bodyweight TiO_2 NPs group (200x) shows wide-ranging of DNA damage with migration. The photomicrographs were stained with thidium bromide.

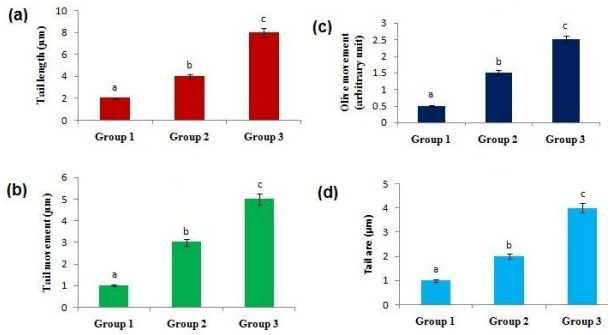

Fig. 4. Effect of TiO_2 NPs on DNA damage (in terms of tail length (a), tail moment (b), olive tail moment (c), and % tail area (d)) in the liver of control and experimental rats. Arbitrary unit = percentage tail DNA × tail length. Values are the mean ± SD for 6 rats in each group. Values not sharing a common letter (a, b, and c) differ significantly at $P < 0.05$ (DMRT).

The comet assay can be used to perceive DNA damage caused by single or double strand breaks, alkali labile sites, oxidative stress stand damage, and DNA cross-linking with DNA or protein. The comet assay is a quick, sensitive, and versatile technique for the quantification of DNA damages in cells [**64**]. As such, this technique is mostly used in the cancer biology for the evaluation of genotoxicity. The resulting image that is obtained resembles a "comet" with a distinct head and tail. The head is composed of intact DNA, while the tail consists of damaged or broken pieces of DNA. In the present study, the levels of percentage tail DNA, tail length, and tail movement drastically elevated in the liver of TiO_2 NPs treated rats (**Fig. 3 & 4**). Prior study has confirmed that LPO products of PUFA

(polyunsaturated fatty acids) play a vital role in the genotoxicity of cells [**65**]. Meena and Paulraj, reported that the administration of 25 and 50 mg per kilogram of TiO_2 NPs damage the DNA of liver cells in the rats [**58**]. Our result also strongly revealed that the administration of both the TiO_2 NPs groups significantly damage the DNA of rat liver when compared with control. This result may be due to the TiO_2 NPs induced oxidative stress mediated inflammation, necrosis that damages the DNA [**30, 66**].

The DNA fragmentation produce a typical ladder pattern of multiple sized nucleosomal nucleotides; a hallmark of apoptosis. In this study, increased DNA damage in the liver of TiO_2 NPs treated rats was observed compared with that of control (**Fig. 5**). In this study, we demonstrated that the administration of 50 and 100 mg per kg body weight of TiO_2 NPs significantly increased the DNA fragmentation which was corroborated by Meena and Paulraj [**58**]. This may be due to the over production of ROS which induce the LPO and protein oxidation of hepatocytes in TiO_2 NPs treated rats.

The histoarchitectual results also strongly hold up our biochemical markers that the administration of both dose of TiO_2 NPs treated rats showed inflammation, vacuolization, dilation of sinusoidal spaces, and severe necrosis in the liver (**Fig. 6**). This could be due to the increased formation of LPO end products and protein carbonylation in the liver which leads to membrane integrity and other pathological changes in the liver of TiO_2 NPs intoxicated rats.

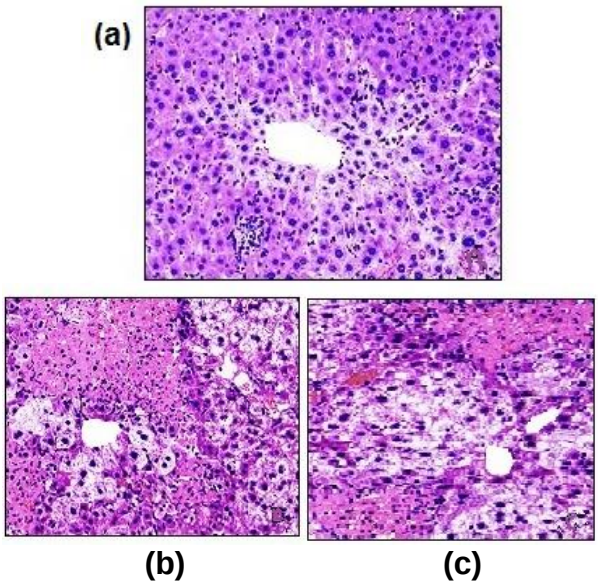

Fig. 6. Histology of the liver tissue sections in rats caused by oral administration with TiO_2 NPs for consecutive 14 days. (a) control group (100x) shows normal hepatocyte architecture; (b) 50 mg/kg/bw TiO_2 NPs group shows inflammation, dilated sinusoids, and degeneration of hepatocytes; (c) 100 mg/kg/bw TiO_2 NPs group shows the severe necrosis, vacuolization, and inflammatory cell infiltration with degenerated hepatocytes. The section was stained with Haematoxylin and eosin (H & E) and examined by light microscopy.

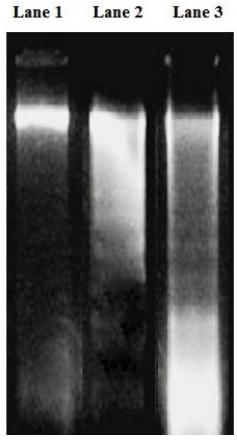

Fig. 5. Effect of TiO_2 NPs on DNA fragmentation in hepatocytes of control and experimental rats. Lane 1, Control (normal DNA); lane 2, 50mg/kg/bw TiO_2 NPs treated group (minimal DNA fragmentation); lane 3, 100mg/kg/bw TiO_2 NPs treated group (severe DNA fragmentation).

Conclusion

So with paying attention to the NPs which are less than 100 nm have the most application in the cosmetics, food additives, medical and biological contexts. From this study, it is concluded that the oral administration of TiO_2 NPs may be accumulated in liver via gastrointestinal tract to circulation. The accumulation of TiO_2 NPs might relate to the pathological and biochemical changes showed with 50 and 100 mg/kg/BW of TiO_2 NPs treatment. NPs of TiO_2 induce production of intracellular ROS which were responsible for DNA damage and dysfunction of antioxidative system in the liver. The results show that, the liver is more sensitive to NPs toxicity compared to any other organs. Further studies are needed to understand the molecular mechanism of TiO_2 NPs and its toxic impact on different organs.

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