

Titanium Dioxide Nanoparticles Induce DNA Damage and Genetic Instability *In vivo* in Mice

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Abstract

Titanium dioxide (TiO₂) nanoparticles are manufactured worldwide in large quantities for use in a wide range of applications including pigment and cosmetic manufacturing. Although TiO₂ is chemically inert, TiO₂ nanoparticles can cause negative health effects, such as respiratory tract cancer in rats. However, the mechanisms involved in TiO₂-induced genotoxicity and carcinogenicity have not been clearly defined and are poorly studied *in vivo*. The present study investigates TiO₂ nanoparticles–induced genotoxicity, oxidative DNA damage, and inflammation in a mice model. We treated wild-type mice with TiO₂ nanoparticles in drinking water and determined the extent of DNA damage using the comet assay, the micronuclei assay, and the γ -H2AX immunostaining assay and by measuring 8-hydroxy-2'-deoxyguanosine levels and, as a genetic instability endpoint, DNA deletions. We also determined mRNA levels of inflammatory cytokines in the peripheral blood. Our results show that TiO₂ nanoparticles induced 8-hydroxy-2'-deoxyguanosine, γ -H2AX foci, micronuclei, and DNA deletions. The formation of γ -H2AX foci, indicative of DNA double-strand breaks, was the most sensitive parameter. Inflammation was also present as characterized by a moderate inflammatory response. Together, these results describe the first comprehensive study of TiO₂ nanoparticles–induced genotoxicity *in vivo* in mice possibly caused by a secondary genotoxic mechanism associated with inflammation and/or oxidative stress. Given the growing use of TiO₂ nanoparticles, these findings raise concern about potential health hazards associated with TiO₂ nanoparticles exposure. [Cancer Res 2009;69(22):8784–9]

Introduction

Titanium dioxide (TiO₂) accounts for 70% of the total production volume of pigments worldwide (1). It is widely used to provide whiteness and opacity to products such as paints, plastics, papers, inks, food colorants, and toothpastes. TiO₂ is also used in cosmetic and skin care products, particularly in sunblocks, where it helps to protect the skin from UV light, especially in the case of nanosized particles (<100 nm). Nevertheless, TiO₂ has recently been reclassified by the IARC as group 2B carcinogen: “possibly carcinogenic to

humans”⁶. The reason for this new classification stems from the fact that high concentrations of pigment-grade (<2.5 μ m) and ultrafine (<100 nm) TiO₂ dust can cause respiratory tract cancer in exposed rats (2, 3). However, it should be noted that epidemiologic studies of workers exposed to pigment-grade TiO₂ conducted thus far have not been able to detect an association between occupational exposure to TiO₂ and an increased risk for lung cancer (4, 5). Genotoxicity studies that measure different types of DNA damage (e.g., gene mutations, chromosomal damage, and DNA strand break formation) are an important part of cancer research and risk assessment of potential carcinogens. These studies help to understand possible mechanisms causing tumor induction. As such, *in vivo* mechanisms underlying TiO₂ nanoparticles tumor induction are still unclear.

Because nanoparticle diameter does not exceed a hundred nanometers at maximum, they are able to penetrate cells (6) and interfere with several subcellular mechanisms. Indeed, some studies show that some nanoparticles can penetrate into cell nuclei and hence may directly interfere with the structure and function of genomic DNA (7). Additionally, after oral administration in mice, TiO₂ particles were shown to translocate to systemic organs such as liver and spleen as well as lung and peritoneal tissues (8). Genotoxicity studies have been done to understand the carcinogenic potential of TiO₂ nanoparticles using assays that measure mutations in genes (e.g., Ames/*Salmonella* and hypoxanthine guanine phosphoribosyl transferase (Hprt) assays; refs. 9–11), chromosomal damage representing possible clastogenic activity of the particles (e.g., micronuclei; refs. 10, 12–15), and DNA strand breakage (e.g., alkaline comet assay; refs. 10, 13). Except for one, these studies were conducted *in vitro* in cultured cells but conflict in their results. Half of the studies show that TiO₂ nanoparticles are genotoxic in cell lines (10, 12, 13, 15), whereas the other half show that TiO₂ nanoparticles are not (9, 11, 14). The rationale for these conflicting results is not clear because different cell types, doses, and nanoparticle sizes have been used. Some studies suggest possible mechanisms for TiO₂ nanoparticles genotoxicity. TiO₂ nanoparticles might damage DNA directly or indirectly via oxidative stress and/or inflammatory responses. Two recent studies show a direct chemical interaction between TiO₂ nanoparticles and DNA, through the DNA phosphate group, but a link to mutagenesis has not been proven (16, 17). On the other hand, other studies show that TiO₂ nanoparticles can cause DNA damage indirectly through inflammation (18–21) and generation of reactive oxygen species (12, 13, 22, 23).

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⁶ IARC. Monographs on the evaluation of carcinogenic risks to humans. Carbon black, titanium dioxide and non-asbestiform talc. Lyon (France): IARC. In press.

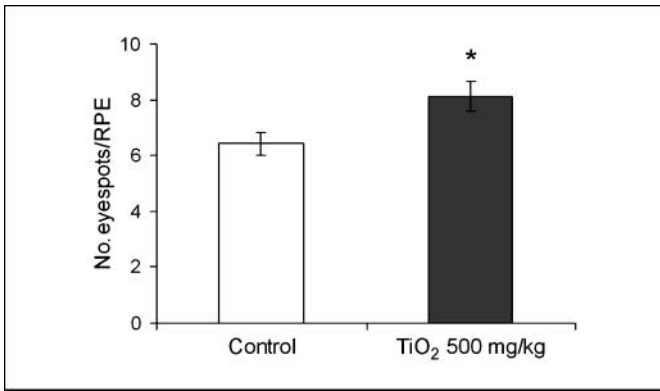


Figure 1. Frequency of DNA deletions in control and TiO₂ nanoparticles-treated mice. One RPE corresponds to one eye. Mice were treated with nanoparticles during embryonic development at a total dose of 500 mg/kg. Mean ± SE numbers of eyespots per RPE with $n = 42$ eyes for control and $n = 53$ eyes for TiO₂ nanoparticles-treated mice. *, $P < 0.05$.

Thus far, most nanoparticle genotoxicity studies have focused on cell culture systems, but confirmation from animal experiments, more relevant to human exposure, is required. To further understand TiO₂ nanoparticles toxicity *in vivo*, we studied the effect of TiO₂ nanoparticles exposure on genotoxicity, DNA damage, and inflammation in mice. To evaluate inflammation in mice, we determined mRNA expression of both proinflammatory and anti-inflammatory cytokines. To assess DNA damage, we used the γ -H2AX and the comet assays to evaluate DNA strand breaks, the micronuclei assay to estimate chromosomal damage, and the measure of 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels using high-performance liquid chromatography to determine oxidative DNA damage. We also used an *in vivo* DNA deletion assay, which allows visual detection of DNA deletion events within the *pink-eyed unstable* (p^{un}) locus in developing mouse embryos (24), which can detect environmental as well as genetic cancer predisposing factors (24). Our results show that TiO₂ P25 nanoparticles can induce 8-OHdG, γ -H2AX foci, micronuclei, DNA deletions, and inflammation markers in a mice model. Therefore, this study suggests that TiO₂ nanoparticles are genotoxic *in vivo*.

Materials and Methods

Mouse care and breeding. C57Bl/6J p^{un}/p^{un} mice were obtained from The Jackson Laboratory. The C57Bl/6J p^{un}/p^{un} background is essentially identical to C57Bl/6J, with the exception of a naturally occurring 70-kb internal duplication in the *pink-eyed dilution* (p) gene, termed the p^{un} allele. Mice were housed and cared for under standard specific pathogen-free conditions and according to the Animal Rescue Coalition and Institutional Animal Care and Use Committee regulations. Mice were given a standard, autoclaved diet from Harlan Teklad (Harlan Teklad No. 8656) and sterilized water *ad libitum*. Mice were housed in a 12-h light/dark cycle. Pregnancy was timed by checking for vaginal plugs, with noon of the day of discovery counted as 0.5 days post-coitum. Four- to 5-month-old mice were used for all experiments.

TiO₂ nanoparticles preparation and exposure. "Aeroxide" P25 TiO₂ (Degussa, now Evonik) nanoparticles were chosen for this study. The crystal structure is a mixture of 75% anatase and 25% rutile TiO₂, purity was at least 99.5% TiO₂, and primary particle size was 21 nm with a specific surface area of 50 ± 15 m²/g. These nanoparticles have been used in many of the previous mammalian studies (13, 14, 22, 25–28). Using dynamic light scattering in water revealed that the size of TiO₂ nanoparticles agglomerates ranged from 21 to 1,446 nm and the mean size was

160 ± 5 nm. About 70% of particles have a size of 160 nm. Solutions of dispersed TiO₂ nanoparticles were prepared by ultrasonication (Solid State/Ultrasonic FS-14; Fisher Scientific) for 15 min in drinking water at 60, 120, 300, and 600 μ g/mL concentrations just before use. We measured TiO₂ nanoparticles-supplemented water intake at the end of experiments in each cage, which housed 2 to 3 mice, and calculated an average daily water intake per mouse. Daily TiO₂ nanoparticles-supplemented water intake ranged from 3 to 7 mL/mouse, consistent with normal daily water intake. Doses were calculated using a 30 g average weight per mouse, and an average of 5 mL water intake per day. The exposure was 5 days in adult males. For *in utero* exposure, pregnant dams were given nanoparticles-supplemented drinking water for 10 days from 8.5 to 18.5 days post-coitum at a concentration of 300 μ g/mL. Water was used as negative control.

***In vivo* DNA deletion assay.** To evaluate genotoxicity of TiO₂ nanoparticles, we employed an intrachromosomal duplication of 70-kb fragment spanning exons 6 to 18 of the p gene in mice (termed p^{un} mutation). When a DNA deletion event occurs between these duplications, the p^{un} allele reverts to the wild-type p gene. Reconstitution of the wild-type p gene can be seen as a single pigmented cell or a clone of pigmented cells on the unpigmented retinal pigment epithelium (RPE) in the transgenic mice and represents a DNA deletion as a permanent genotoxic event (29). Pregnant mice were treated with TiO₂ nanoparticles, and offspring were sacrificed at age 20 days. Their eyes were extracted and dissected to display the RPE for the deletion/eyespot assay as described previously (24). One RPE corresponds to one eye.

Alkaline comet assay. Peripheral blood was collected by submandibular vein puncture (before treatment and after treatment) in an EDTA-coated tube. The comet assay was done as described previously (30). On average, from three slides, 150 to 200 randomly captured comets per sample were analyzed. Results were expressed as average ± SE tail moment.

Micronuclei assay. The micronuclei assay was done as described elsewhere (31). Three microliter aliquots of the peripheral blood were collected as described above and smeared on slides and stained into Giemsa stain for 1.5 min. Approximately 2,000 erythrocytes were scored per animal to estimate the frequency of micronucleated erythrocytes.

Bone marrow preparation. Animals were sacrificed with an overdose of isoflurane after 5 days of treatment with TiO₂ nanoparticles in drinking water. Both femora were dissected, and marrow cells were flushed out with 1 mL PBS and pipetted several times. The cell suspension was centrifuged at 1,000 rpm for 5 min, the supernatant was withdrawn, and the cell pellet was resuspended and placed on a clean glass slide.

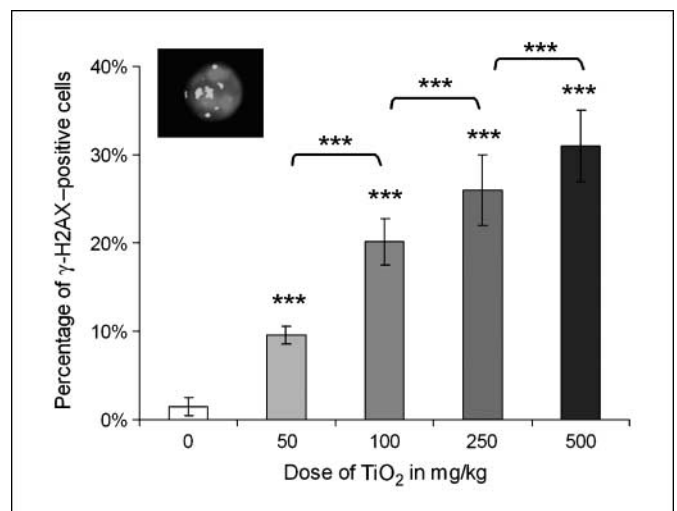


Figure 2. Percentage of γ -H2AX-positive cells in bone marrow in untreated and TiO₂ nanoparticles-treated mice and a picture of a γ -H2AX-positive cell with more than four foci. Columns, mean of 5 mice; bars, SE. ***, $P < 0.001$, TiO₂ nanoparticles-treated versus control.

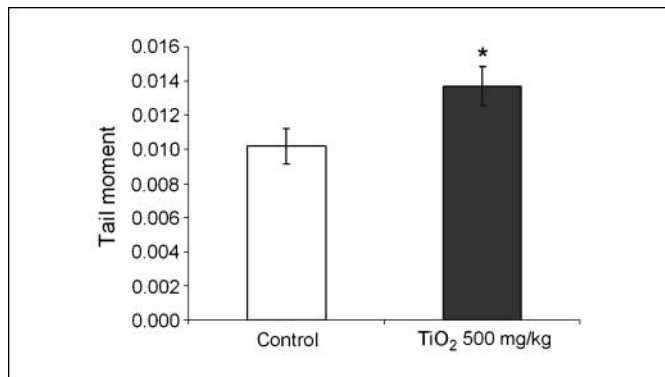


Figure 3. Frequency of DNA strand breaks in mice before and after treatment with 500 mg/kg TiO₂ nanoparticles. DNA damage is represented by the tail moment. Mean ± SE ($n = 5$ mice/group). *, $P < 0.05$, compared with untreated mice.

γ -H2AX assay, RNA isolation, and quantitative real-time PCR. The γ -H2AX assay was done with bone marrow cells, and the RNA isolation for quantitative real-time PCR was carried out on peripheral blood. These assays were done as described elsewhere (31).

Determination of oxidative DNA damage by measuring 8-OHdG. Mouse livers were isolated just after 5 days of treatment with nanoparticles and immediately frozen in liquid nitrogen and homogenized under liquid nitrogen. 8-OHdG level was measured using high-performance liquid chromatography with electron capture detection system as described previously (32).

Statistical analysis. For the deletion assay, the comet assay, the micronuclei assay, and 8-OHdG and mRNA levels of cytokines, we used the Student's t test to compare untreated mice with treated mice. For the γ -H2AX experiment, the percentage of positive cells for control groups versus treated groups was compared via χ^2 test. In addition to the t test, for comet assay data, the Wilcoxon test for matched paired data was also used to compare the effect of TiO₂ nanoparticles on the tail moment before and after nanoparticle treatment. The difference was considered significant at the 95% confidence level ($P < 0.05$) and highly significant at the 99% confidence level ($P \leq 0.01$).

Results

TiO₂ nanoparticles increased the frequency of DNA deletions. We used the DNA deletion assay to evaluate *in vivo* genotoxicity of TiO₂ nanoparticles. We quantified the number of eyespots per RPE as a measure of DNA deletions in *in utero* exposed mice. TiO₂ nanoparticles-treated mice had an average of 8.13 ± 1.70 eyespots per RPE versus 6.42 ± 1.47 eyespots per RPE in nontreated mice (Fig. 1). TiO₂ nanoparticles-exposed mice displayed a significant increase in eyespots (27%) compared with unexposed mice ($P = 0.019$), suggesting that, after maternal oral exposure, TiO₂ nanoparticles increased DNA deletion frequency in fetuses.

TiO₂ nanoparticles induced γ -H2AX foci. Phosphorylation of histone H2AX on serine 139 occurs at sites flanking DNA double-strand breaks (DSB), providing a measure of the number of DSBs within a cell (33). We used this assay to compare DSB formation in bone marrow of mice with and without TiO₂ nanoparticles treatment.

The γ -H2AX foci formation increased by ~10%, 20%, 25%, and 30% following treatment with 50, 100, 250, and 500 mg/kg TiO₂ nanoparticles, respectively, compared with untreated mice (Fig. 2). Percentage of γ -H2AX-positive cells increased with TiO₂ nanoparticles concentration in a clear dose-dependent manner ($P < 0.001$).

These data provided evidence that, after oral administration, TiO₂ nanoparticles induce DSBs in bone marrow cells.

TiO₂ nanoparticles increased DNA strand breaks. DNA strand breaks (DSBs, single-strand breaks, and/or strand breaks induced by alkali-labile sites) were measured by the alkaline comet assay in mice peripheral blood before and after treatment. Tail moment significantly increased after TiO₂ nanoparticles treatment (Fig. 3). The average tail moment was 0.0102 ± 0.001 before treatment and 0.0137 ± 0.0011 after TiO₂ nanoparticles treatment. TiO₂ nanoparticles increased DNA strand breaks in WBCs from peripheral blood by 34% ($P = 0.001$, t test, and $P = 0.04$, Wilcoxon test).

TiO₂ nanoparticles induced micronuclei. The micronuclei assay was used to detect chromosomal damage in erythrocytes from peripheral blood. The incidence of micronuclei serves as an index of clastogenicity. Micronuclei frequency increased significantly only at the highest (500 mg/kg) dose of TiO₂ nanoparticles used ($P = 0.009$; Fig. 4). At this dose, the average micronuclei frequencies for untreated mice were 4.3 ± 0.93 versus 9.2 ± 1.07 per 2,000 RBC for TiO₂ nanoparticles-treated mice, which resulted in a 2.1-fold increase in micronuclei formation. This result showed that, at high dose, TiO₂ nanoparticles induced detectable clastogenicity in mice peripheral blood.

TiO₂ nanoparticles induced oxidative DNA damage. We examined the degree of oxidative DNA damage by measuring the level of 8-OHdG in DNA isolated from TiO₂ nanoparticles-treated and untreated mouse livers. The level of 8-OHdG was significantly higher in TiO₂ nanoparticles-treated than untreated mice ($P = 0.04$; Fig. 5). The average number of 8-OHdG per 10⁶ dG was 4.25 ± 0.66 for untreated mice and 6.43 ± 0.58 for TiO₂ nanoparticles-treated mice resulting in a 1.5-fold increase at 500 mg/kg TiO₂ nanoparticles. This suggested that TiO₂ nanoparticles induced oxidative DNA damage in liver.

TiO₂ nanoparticles induced a proinflammatory response. We quantified mRNA transcripts of three Th1/proinflammatory cytokines (T-helper cell type 1) and three Th2/anti-inflammatory cytokines (T-helper cell type 2) in the peripheral blood. After treatment, the proinflammatory cytokines tumor necrosis factor- α , IFN- γ , and the mouse orthologue of interleukin-8 (KC) were significantly upregulated ($P = 0.01$, 0.02, and 0.05, respectively; Fig. 6A).

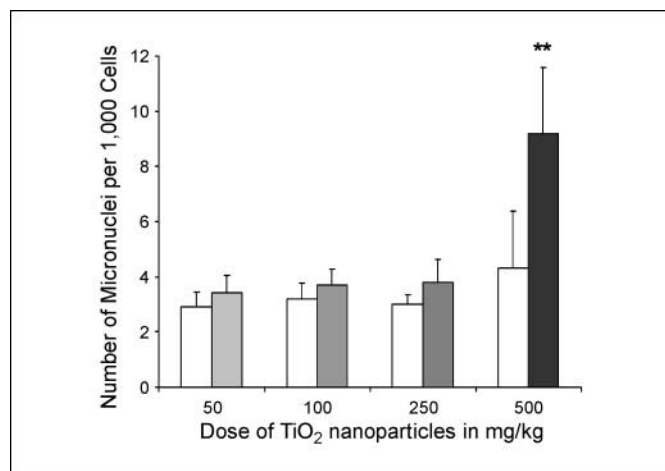


Figure 4. Frequency of micronuclei in mice before and after TiO₂ nanoparticles treatment in peripheral blood erythrocytes. Open columns, untreated controls; gray columns, TiO₂ nanoparticles-treated mice. Columns, mean of 5 mice; bars, SE. **, $P < 0.01$, compared with untreated mice.

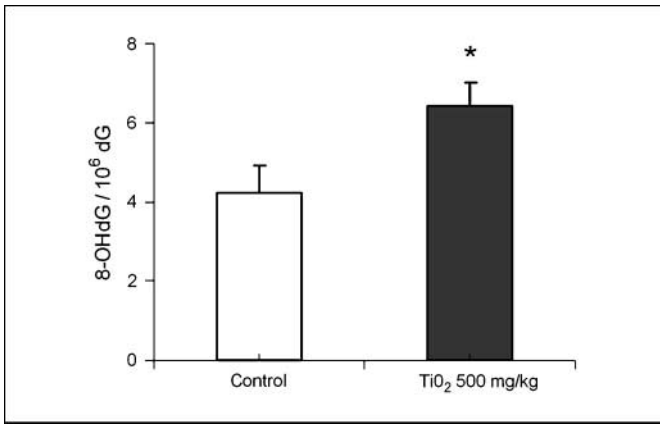


Figure 5. Level of 8-OHdG in untreated and 500 mg/kg TiO₂ nanoparticles-treated mouse livers. Mean ± SE ($n = 5$ mice/group). *, $P < 0.05$, compared with untreated mice.

A general upregulation of these cytokines may be due to the effects of circulating TiO₂ nanoparticles directly in the peripheral blood, suggesting systemic distribution, and direct activation of a proinflammatory response. In the contrary, anti-inflammatory cytokines with generally opposing function were not upregulated, including transforming growth factor- β , interleukin-10, and interleukin-4 (Fig. 6B). TiO₂ nanoparticles did not induce an anti-inflammatory response, which mean they did not inhibit the production and release of proinflammatory mediators.

Discussion

Here, we report for the first time that TiO₂ nanoparticles are genotoxic and clastogenic *in vivo* in mice. We showed that TiO₂ nanoparticles (500 mg/kg) induce not only DNA single-strand breaks and DSBs but also chromosomal damage. The formation of γ -H2AX foci, which show DSB formation, was the most sensitive parameter and showed a consistent dose-dependent response. Concerning health relevance, DSBs are much more damaging in terms of genetic instability than single-strand breaks and oxidative DNA damage, which are transient. Our results extend previous *in vitro* findings with the micronuclei and comet assays in several human cells and Syrian hamster embryo cells (10, 12, 13, 15),

although they have not been detected in some studies (11, 14). Differences in response between studies may be due to how TiO₂ nanoparticles differ in terms of TiO₂ production, particle size, degree of aggregation, preparation method (sonication), incubation conditions, dose, and susceptibility between cell types (34, 35), implying that more studies are needed to determine the conditions in which TiO₂ nanoparticles genotoxicity occurs.

To date, very few *in vivo* genotoxicity studies have been carried out with nanoparticles. A chronic exposure to TiO₂ nanoparticles at concentrations that produce chronic pulmonary inflammation was associated with an increased incidence of tumors in rat lungs (3). Thus far, only two *in vivo* genotoxicity studies have been reported, which showed that *in vivo* TiO₂ nanoparticles increased *Hprt* mutation frequency in alveolar epithelial cells (9) but did not induce DNA adduct formation in rat lungs (26). Our study showed for the first time that, *in vivo* after oral exposure, TiO₂ nanoparticles induce DNA strand breaks and chromosomal damage in bone marrow and/or peripheral blood, which may help to further understand potential mechanisms of TiO₂ nanoparticles carcinogenicity. We also found that maternal exposure to 500 mg/kg TiO₂ nanoparticles during gestation results in significantly elevated frequencies of DNA deletions in offspring. This result is a major finding because it shows for the first time that *in utero* exposure of fetuses via the mother causes an increase in large deletions in offspring. Taken together, our findings show that TiO₂ nanoparticles, orally administrated, induce genotoxicity systemically in organs, such as blood, bone marrow, and even the embryo.

Surprisingly, human studies have not been able to detect any relation between TiO₂ occupational exposure and cancer risk (4, 5, 36), but these studies have methodologic and epidemiologic limitations as reviewed by the National Institute of Occupational Safety and Health (37). In addition, these studies were not designed to investigate the relationship between TiO₂ particle size and lung cancer risk, an important question for assessing the potential occupational carcinogenicity of TiO₂. Indeed, Dankovic and colleagues, comparing several tumor studies, pointed out that TiO₂ nanoparticles produced lung tumors in rats at a much lower dose than fine particles (<250 nm; 10 and 250 mg/m³ for nanoparticles and fine particles, respectively; ref. 3). Although TiO₂ nanoparticles are prone to forming agglomerates of >100 nm in solution, these agglomerates are apparently not stable and appear to dissociate in

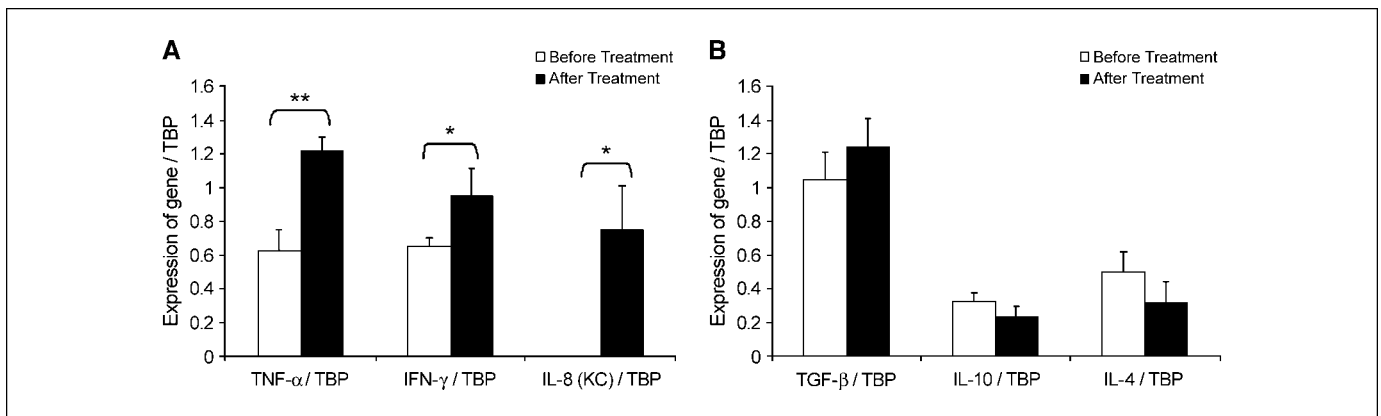


Figure 6. TiO₂ nanoparticles at 500 mg/kg induce proinflammatory but not anti-inflammatory cytokines. Open columns, untreated controls; black columns, TiO₂ nanoparticles-treated mice. A, expression of proinflammatory cytokine panel relative to TBP, the internal control gene. *, $P < 0.05$; **, $P < 0.01$, Student's *t* test for treatment comparisons. B, expression of anti-inflammatory cytokine panel relative to TBP. Student's *t* test revealed no significant differences. For each graph, $n = 5$ mice/group.

bodily fluids and tissues, which could be an explanation for TiO₂ nanoparticles higher toxicity. It has also been reported that TiO₂ nanoparticles surface interactions are weak (20). In addition, an inhalation study showed that TiO₂ nanoparticles agglomerates of ~700 nm dissociate into smaller units after deposition in the lung (21). Even if nanoparticles aggregate into larger-sized agglomerates, their primary particle sizes remain a significant trait that affects their toxicity. Further human studies would be necessary to understand nanoparticle health effects. For instance, one could use blood-based assays similar to those done in this study in a future molecular epidemiology study in occupational settings. Furthermore, TiO₂ is also used in food colorants and toothpaste. This supports the notion that nanoparticle ingestion constitutes a relevant route of exposure in humans and underscores the significance of the findings of our study. In addition, given the capability of nanoparticles to enter the systemic blood circulation, nanoparticles may pose hazard to a variety of other organs as we have shown here.

As suggested previously, a possible mechanism for nanoparticles-caused genotoxicity might be via oxidative stress (38). Indeed, previous studies showed that TiO₂ nanoparticles have hydroxyl radical activity (39–41) and can also trigger reactive oxygen species production in different cell lines (13, 42) on interaction with the cell membrane or even in cell-free environment (23, 43). We confirmed these results in our *in vivo* experiment by showing oxidative DNA damage (8-OHdG) increase in mouse livers after TiO₂ nanoparticles treatment. Also, TiO₂ nanoparticles–increased DNA deletions during fetal development might be a result of oxidative genome damage. As discussed previously (44), oxidative stress is particularly hazardous in replicating cells. For instance, oxidative DNA lesions (e.g., 8-OHdG, single-strand breaks, or stalled replication forks) can lead to DSBs after replication, which can result in recombination, thus producing permanent genome rearrangements. As shown in yeast, oxidative mutagens might be powerful inducers of DNA deletions (45). Because embryonic cells are generally characterized by a high replication index, they might be particularly susceptible to oxidative genome damage.

We have also observed an inflammatory reaction as shown by changes in cytokine expression in peripheral blood, in which TiO₂ nanoparticles could be exerting direct inflammatory effects on circulating innate cells and Th1 effector cells. The inflammatory response involving recruitment and activation of phagocytes is

capable of causing oxidative bursts that may serve as a possible explanation for the observed genotoxicity to peripheral leukocytes, micronuclei formation, oxidative DNA damage in liver cells, and DNA deletion induction in fetal RPE. Because we showed that in mice TiO₂ nanoparticles induce an inflammatory reaction and oxidative DNA damage, it is tempting to speculate that the mechanism underlying TiO₂ nanoparticles genotoxicity might be a secondary (indirect) genotoxicity pathway as suggested by Dankovic and colleagues (3). Secondary genotoxicity is considered to be the key aspect of some particle toxicity (e.g., quartz and silica) because of their ability to elicit persistent inflammation *in vivo* (9, 46). This implies that particles have prooxidant and proinflammatory activity, leading to genotoxicity.

In summary, our study showed for the first time that TiO₂ nanoparticles induce clastogenicity, genotoxicity, oxidative DNA damage, and inflammation *in vivo* in mice. These results have been observed after only 5 days of treatment via drinking water and in multiple organs suggesting a systemic effect. We also showed that *in utero* exposure to TiO₂ nanoparticles results in an increased frequency in DNA deletions in the fetus. These results represent the first comprehensive *in vivo* genotoxicity study of TiO₂ nanoparticles. These data suggest that we should be concerned about a potential risk of cancer or genetic disorders especially for people occupationally exposed to high concentrations of TiO₂ nanoparticles and that it might be prudent to limit ingestion of TiO₂ nanoparticles through nonessential drug additives, food colors, etc.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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