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Review Article

Genotoxicity of titanium dioxide nanoparticles ☆

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ABSTRACT

Titanium dioxide nanoparticles (TiO$_2$-NPs, <100 nm) are increasingly being used in pharmaceuticals and cosmetics due to the unique properties derived from their small sizes. However, their large surface-area to mass ratio and high redox potential may negatively impact human health and the environment. TiO$_2$-NPs can cause inflammation, pulmonary damage, fibrosis, and lung tumors and they are possibly carcinogenic to humans. Because cancer is a disease involving mutation, there are a large number of studies on the genotoxicity of TiO$_2$-NPs. In this article, we review the results that have been reported in the literature, with a focus on data generated from the standard genotoxicity assays. The data include genotoxicity results from the Ames test, *in vitro* and *in vivo* Comet assay, *in vitro* and *in vivo* micronucleus assay, sister chromatid exchange assay, mammalian cell hypoxanthine-guanine phosphoribosyl transferase gene assay, the wing somatic mutation and recombination assay, and the mouse *phosphatidylinositol glycan*, class A gene assay. Inconsistent results have been found in these assays, with both positive and negative responses being reported. The *in vitro* systems for assessing the genotoxicity of TiO$_2$-NPs have generated a greater number of positive results than the *in vivo* systems, and tests for DNA and chromosome damage have produced more positive results than the assays measuring gene mutation. Nearly all tests for measuring the mutagenicity of TiO$_2$-NPs were negative. The current data indicate that the genotoxicity of TiO$_2$-NPs is mediated mainly through the generation of oxidative stress in cells.

1. Introduction

Titanium dioxide (TiO$_2$) is the naturally occurring oxide of titanium. It has several different crystalline structures. Rutile is the most common natural form of TiO$_2$, whereas anatase and brookite are two more rare polymorphs. TiO$_2$ has been used widely in pigments, accounting for 70% of the total production volume of pigments worldwide. It provides whiteness and opacity to products such as paints, plastics, papers, inks, foods, and toothpastes. It can also be found in pharmaceuticals and cosmetic products such as sunblock [1] due to its

photocatalytic, biocidal, and/or antiproliferative properties [2]. Until recently, the use had been limited to coarse and fine (both diameters > 100 nm) TiO_2 particles. Coarse and fine particles of TiO_2 have been investigated and declared biologically inert in humans and animals [3,4]. More recently, TiO_2 nanoparticles (TiO_2-NPs, <100 nm) have increasingly been used in pharmaceuticals and cosmetics due to the unique properties derived from their small sizes [5,6]. These new applications of TiO_2-NPs, however, call into question their biological inertness.

TiO_2-NPs have a large surface-area to mass ratio [7] and a high redox potential, which can cause undesirable effects on human health and the environment. Recent studies have revealed that exposure to TiO_2-NPs can cause inflammation, pulmonary damage, fibrosis, and lung tumors in rodents [7–9]. TiO_2-NPs are possibly carcinogenic to humans (Group 2B) based on sufficient evidence in experimental animals and inadequate evidence from epidemiology studies, according to a report from the International Agency for Research on Cancer [10]. The National Institute for Occupational Safety and Health (NIOSH) [11] also concluded that TiO_2-NPs were a potential occupational carcinogen, acting through a secondary genotoxicity mechanism primarily related to particle size and surface area.

Genotoxicity data are important for nanotechnology regulation and risk assessment. Recently, the genotoxicity of TiO_2-NPs has been intensively studied due to their carcinogenicity. Although a large number of reports on the genotoxicity of TiO_2-NPs and its underlying mechanisms have been published, there has been no review article specific to the genotoxicity of TiO_2-NPs. The purpose of this review is to present up-to-date knowledge regarding the genotoxicity of TiO_2-NPs, with a focus on results from standard genotoxicity assays.

2.   *In vitro* studies

Results on genotoxicity studies on TiO_2-NPs were identified through Medline database searches. The data from the studies using the standard genotoxicity assays including the Ames test, Comet assay, micronucleus assay, sister chromatid exchange (SCE) assay, and mammalian cell gene mutation assay, are summarized in Table 1.

2.1.   Ames test

The Ames test is formally called the *Salmonella typhimurium* reversion assay. This test is used worldwide as an initial screen to determine the mutagenic potential of agents and the assay identifies point mutagens [12,13].

Four different types of TiO_2-NPs have been evaluated by the Ames assay and all of them were negative in the standard mutation assay. However, two showed positive responses when evaluated with a modified fluctuation test procedure (Table 1).

Jomini et al [14] used the standard fluctuation test and a modified fluctuation test procedure with the *S. typhimurium* strains TA97, TA98, TA100, and TA102 to measure the mutagenic potential of two types of TiO_2-NPs. The test was negative when the normal assay was used. However, when they applied a simple pre-exposure of bacteria to the NPs in a low ionic strength solution (NaCl, 10 mM) at a pH below the nanoparticles isoelectric points (pH 5.5), the results were positive. They concluded that a simple pre-exposure step in a low ionic-strength solution, at a pH below the nanoparticle isoelectric points (NaCl, 10 mM, pH 5.5) could increase bacterial uptake of the nanoparticles and improve the accuracy of the test.

In another two tests, TiO_2-NPs were negative in different *Salmonella* strains. Landsiedel et al [15] evaluated several TiO_2-NPs used for sunscreen products using Ames test. *S. typhimurium* TA1535, TA100, TA1537, TA98, and TA102 were treated with the NPs at 20–5000 μg/plate both with or without metabolic activation. No mutagenicity was found. In the other study, the bacteria were preincubated with eight different concentrations of 10 nm anatase TiO_2-NPs up to 5000 μg/plate. No mutation induction was found. Analyses with transmission electron microscopy and energy-dispersive X-ray spectroscopy show that the TiO_2-NPs are not able to enter the bacterial cells [16].

2.2.   Comet assay

The Comet assay is a method for measuring DNA strand breaks in eukaryotic cells. The Comet assay is also called the single-cell gel electrophoresis assay due to its working principle. After treatment, single cell suspensions are embedded in agarose on a microscope slide and lysed. Electrophoresis at high pH results in structures resembling comets when observed by fluorescence microscopy. The intensity of the comet tail relative to the head is proportional to the number of DNA breaks. For detecting oxidative DNA damage, cells embedded in agarose on microscope slides can be further treated with nucleases such as formamidopyrimidine DNA-glycosylase (Fpg), endonuclease III (Endo III), and 8-hydroxyguanine DNA-glycosylase to generate secondary DNA breaks at the sites with oxidative DNA adducts. The Comet assay has been widely used to assess genotoxicity of nanomaterials due to its sensitivity and simplicity. The results from *in vitro* Comet assays on TiO_2-NPs are summarized in Table 1.

Among 24 Comet assay tests, 17 of them showed positive responses to treatments of different types of TiO_2-NPs (Table 1). Bottlenose dolphin leukocytes were treated with smaller than 25 nm anatase TiO_2-NPs and the Comet assay was performed to measure the genotoxicity of the NPs. The results showed that the NPs were genotoxic for the cells after exposure to concentrations of 50 μg/mL and 100 μg/mL for 24 hours and 48 hours, respectively [17]. AGS human gastric epithelial cells treated with 21 nm TiO_2-NPs caused DNA damage. The tail intensity increased 1.88-fold in 150 mg/mL of TiO_2-NPs treated cells compared to the control cells [18]. Human peripheral blood lymphocytes and cultured human embryonic kidney (HEK293) cells were treated with 1 μg/mL, 10 μg/mL, and 100 μg/mL of 2.3 nm TiO_2-NPs and the DNA breaks were measured using the Comet assay with or without the Fpg and Endo III enzymes. The 100 μg/mL of TiO_2-NPs significantly increase the DNA damage with or without the Fpg and Endo III enzymes in both the cell lines [19]. The Comet assays were conducted using human bronchial epithelial BEAS 2B cells to

Table 1 — *In vitro* studies on genotoxicity of titanium dioxide nanoparticles.

Size and crystalline structure	Dose	Test system	Result	Author and Reference
Ames test				
23 nm; 84% anatase and 16% rutile	1–100 μg/mL	A modified fluctuation test procedure for the Ames test	Positive	Jomini et al, 2012 [14]
5.7 nm; 86% anatase and 14% brookite	1–100 μg/mL	A modified fluctuation test procedure for the Ames test	Positive	Jomini et al, 2012 [14]
23 nm; 84% anatase and 16% rutile	1–100 μg/mL	Fluctuation Ames test	Negative	Jomini et al, 2012 [14]
5.7 nm; 86% anatase and 14% brookite	1–100 μg/mL	Fluctuation Ames test	Negative	Jomini et al, 2012 [14]
10 × 50 nm; rutile in T-Lite	Up to 5 mg/plate	Ames test	Negative	Landsiedel et al, 2010 [15]
10 nm; anatase	5 mg/plate	Ames test	Negative	Woodruff et al, 2012 [16]
Comet assay				
<25 nm; anatase	50 μg/mL and 100 μg/mL	Bottlenose dolphin leukocytes	Positive	Bernardeschi et al, 2010 [17]
21 nm; 80%/20% anatase/rutile	150 mg/mL	AGS human gastric epithelial cell line	Positive	Botelho et al, 2013 [18]
2.3 nm	100 μg/mL	With or without Fpg and End III in Human peripheral blood lymphocytes and cultured human embryonic kidney cells (HEK293)	Positive	Demir et al, 2013a [19]
50 nm	25 μg/mL	Human lymphocytes	Positive	Ghosh et al, 2013 [21]
14 nm; anatase	50 μg/mL	Syrian hamster embryo cells	Positive	Guichard et al, 2012 [22]
25 nm; 80%/20% anatase/rutile	10 μg/mL, 25 μg/mL, and 50 μg/mL	Syrian hamster embryo cells	Positive	Guichard et al, 2012 [22]
5.9 nm; anatase	100 mg/mL	Chinese hamster lung fibroblast cells	Positive	Hamzeh and Sunahara, 2013 [23]
34.1 nm; 83% anatase and 17% rutile	100 mg/mL	Chinese hamster lung fibroblast cells	Positive	Hamzeh and Sunahara, 2013 [23]
1.5 nm; rutile	100 mg/mL	Chinese hamster lung fibroblast cells	Positive	Hamzeh and Sunahara, 2013 [23]
Varying sizes 12–140 nm; anatase or rutile	100 μg/mL	A549 human lung carcinoma cells	Positive	Jugan et al, 2012 [24]
7 nm anatase or 10 nm rutile	0.5–256 μg/mL	Human hepatoblastoma C3A cells	Positive	Kermanizadeh et al, 2012 [25]
27.5 nm; 86% anatase/14% rutile	20–100 μg/mL	Human bronchial epithelial cell	Positive	Prasad et al, 2013 [26]
30 nm	20 μg/mL	Human amnion epithelial (WISH) cells	Positive	Saquib et al, 2012 [27]
10 nm; anatase	0.8–80 μg/mL	Human epidermal cells (A431)	Positive	Shukla et al, 2011 [29]
30–70 nm	1–80 μg/mL	HepG2 cells	Positive	Shukla et al, 2013 [28]
<100 nm	3μM, 5 μM, and 10 μM	Human peripheral blood lymphocytes	Positive	Turkez 2011 [30]
25 nm anatase; 25 nm 80% anatase and 20% rutile	80 μg/mL, 120 μg/mL, and 150 μg/mL	Human SHSY5Y neuronal cells	Positive	Valdiglesias et al, 2013 [31]
<100 nm anatase	Up to 50 μg/cm^2	Human lung fibroblasts and human bronchial fibroblasts	Negative	Bhattacharya et al, 2009 [32]
62 nm; rutile	Up to 50 μg/mL	Syrian hamster embryo cells	Negative	Guichard et al, 2012 [22]
15–30 nm; anatase	20–200 μg/mL	Human peripheral blood lymphocytes	Negative	Hackenberg et al, 2011 [33]
1–10 nm; polyacrylate-coated	100 mg/mL	Chinese hamster lung fibroblast cells	Negative	Hamzeh and Sunahara, 2013 [23]
10 × 50 nm; rutile in T-Lite	Up to 600 μg/mL for 4 h exposure and up to 150 μg/mL for 24 h exposure	V79 cells	Negative	Landsiedel et al, 2010 [15]

(continued on next page)

Table 1 – (continued)

Size and crystalline structure	Dose	Test system	Result	Author and Reference
10 nm; anatase	Up to 40 µg/mL	Chinese hamster ovary cells (CHO-K1)	Negative	Wang et al, 2011 [34]
10 nm; anatase	Up to 200 µg/mL	TK6 human lymphocytes	Negative	Woodruff et al, 2012 [16]
Micronucleus assay				
20 nm	0.5 µg/mL and 1 µg/mL	Chinese hamster ovary cells	Positive	Di Virgilio et al, 2010 [35]
27.5 nm; 86% anatase/14% rutile	20–100 µg/mL	Human bronchial epithelial cell	Positive	Prasad et al, 2013 [26]
≤20 nm	1.0 µg/cm^2	Syrian hamster embryo cells	Positive	Rahman et al, 2002 [36]
30–70 nm	1–80 µg/mL	HepG2 cells	Positive	Shukla et al, 2013 [28]
10 nm; anatase	0.8–80 µg/mL	Human epidermal cells (A431)	Positive	Shukla et al, 2011 [29]
<25 nm	10 and 50 µg/mL	Human lung cancer cells (A549)	Positive	Srivastava et al, 2011 [37]; Srivastava et al, 2013 [38]
21 nm; anatase;	5–125 µg/mL	Human lymphocytes	Positive	Tavares et al, 2013 [39]
22 nm; hydrophobic rutile	5–125 µg/mL	Human lymphocytes	Positive	Tavares et al, 2013 [39]
19 nm; hydrophilic rutile	5–125 µg/mL	Human lymphocytes	Positive	Tavares et al, 2013 [39]
<100 nm	3 µM, 5 µM, and 10 µM	Human peripheral blood lymphocytes	Positive	Turkez, 2008 [30]; Turkez, 2011 [51]
25 nm; anatase	80 µg/mL, 120 µg/mL, and 150 µg/mL	Human SHSY5Y neuronal cells	Positive	Valdiglesias et al, 2013 [31]
25 nm; 80% anatase and 20% rutile	80 µg/mL, 120 µg/mL, and 150 µg/mL	Human SHSY5Y neuronal cells	Positive	Valdiglesias et al, 2013 [31]
25 nm; 80% anatase and 20% rutile	Up to 50 µg/mL	Syrian hamster embryo cells	Negative	Guichard et al, 2012 [22]
14 nm; anatase	Up to 50 µg/mL	Syrian hamster embryo cells	Negative	Guichard et al, 2012 [22]
62 nm; rutile	Up to 50 µg/mL	Syrian hamster embryo cells	Negative	Guichard et al, 2012 [22]
20 nm; 85% anatase and 15% rutile	Up to 250 µg/mL	Human lymphocytes	Negative	Tavares et al, 2013 [39]
Sister chromatid exchange assay				
20 nm	1–5 µg/mL	Chinese hamster ovary cells	Positive	Di Virgilio et al, 2010 [35]
<100 nm	3µM, 5 µM and 10 µM	Human peripheral blood lymphocytes	Positive	Turkez, 2008 [30]; Turkez, 2011 [51]
Hprt mutation assay				
10 nm; anatase	Up to 40 µg/mL	*Hprt* mutation assay in Chinese hamster ovary cells (CHO-K1)	Negative	Wang et al, 2011 [34]

evaluate genotoxicity of <25 nm uncoated anatase TiO$_2$-NPs and 10 × 40 nm SiO$_2$-coated rutile TiO$_2$-NPs. The results showed that both the two types of TiO$_2$-NPs induced DNA breaks [20]. DNA fragmentation was induced by TiO$_2$-NPs in human lymphocytes at a concentration of 25 μg/mL [21]. Guichard et al [22] compared genotoxicity of nanosized and non-nanosized anatase and rutile TiO$_2$ particles in Syrian hamster embryo (SHE) cells. Although TiO$_2$-NPs in both forms induced higher cytotoxicity than their bulk counterparts after 72 hours of exposure, the anatase NPs induced similar levels of DNA damage in the Comet assay after 24 hours of exposure as the bulk particles. Chinese hamster lung fibroblast cells were treated with 100 mg/mL of 5.9 nm anatase, 34.1 nm 83% anatase and 17% rutile mixture, and 1.5 nm rutile TiO$_2$-NPs, respectively. DNA double strand breaks were measured using the Comet assay. All types of the NPs were positive in the test [23]. Jugan et al [24] evaluated genotoxicity of varying sizes of NPs in A549 human lung carcinoma cells and found that they were genotoxic when assayed with the Comet assay. Following 4 hours' exposure of the human hepatoblastoma C3A cells to sublethal levels of the TiO$_2$-NPs, DNA damage measured by the Comet assay was significantly induced [25]. Prasad et al [26] evaluated effects of TiO$_2$-NP agglomeration on their genotoxicity using three different nanoparticle-treatment media. They found that TiO$_2$-NPs induced similar amounts of DNA damage measured by the Comet assay in all three media, independent of the amount of agglomeration, cellular interaction, or cell-cycle changes [26]. Human amnion epithelial (WISH) cells were exposed to varying concentrations of 30 nm TiO$_2$-NPs for 6 hours. The comet results exhibited a significant induction of DNA damage at 20 μg/mL of the particles [27]. TiO$_2$-NPs (30–70 nm) induced significant oxidative DNA damage in HepG2 cells measured with the Fpg-Comet assay even at 1 μg/mL [28]. Human epidermal cells (A431) were treated with 50 nm anatase TiO$_2$-NPs at doses 0.008–80 μg/mL. The treatment resulted in a significant DNA damage in the cells [29]. Human peripheral blood lymphocytes were treated with 3 μM, 5 μM, and 10 μM TiO$_2$-NPs (<100 nm). DNA damage measured using the Comet assay was increased by the treatment and addition of ascorbic acid prevented the induction [30]. Two types of TiO$_2$-NPs, 25-nm anatase and 25 nm with 80% anatase and 20% rutile, were used for the treatment of human SHSY5Y neuronal cells. The Comet assay was conducted after treatment for 3 hours or 6 hours of the two types of particles. Positive results were obtained for both types of NP [31].

Seven out of 24 Comet assay tests gave negative responses to TiO$_2$-NPs (Table 1). TiO$_2$-NPs (anatase, <100 nm) did not induce DNA-breakage measured by the Comet assay in human lung fibroblasts and human bronchial fibroblasts [32]. Guichard et al [22] found that 60-nm rutile TiO$_2$-NPs did not increase DNA damage after 24 hours of exposure to SHE cells at concentrations up to 50 μg/mL. Human peripheral blood lymphocytes from 10 male donors were exposed to 15–30 nm anatase TiO$_2$-NPs at concentrations 20 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL for 24 hours. Although the NPs were detected in the cytoplasm and nucleus of the lymphocytes, they did not induce genotoxicity in the cells measured with the Comet assay [33]. Landsiedel et al [15] treated V79 cells with 10 nm × 50 nm rutile TiO$_2$-NPs at concentrations up to

600 μg/mL for 4 hours and up to 150 μg/mL for 24 hours' exposure in T-Lite and measured the DNA damage with the Comet assay. The result was negative for the test. Chinese hamster lung fibroblast cells were treated with 1–10 nm polyacrylate-coated TiO$_2$-NPs and the Comet assay was performed. The NPs were negative in the test [23]. Chinese hamster ovary cells (CHO–K1) were exposed to < 25 nm anatase TiO$_2$-NPs at concentrations 0 μg/mL, 10 μg/mL, 20 μg/mL, or 40 μg/mL for 60 days. Immediately after the treatment, the alkaline Comet assay was performed and the result was negative [34]. The genotoxicity of 10 nm anatase TiO$_2$-NPs was assessed with the Comet assay. TK6 cells were treated with 0–200 μg/mL TiO$_2$-NPs for 24 hours. Although the TK6 cells did take up the particles, no significant induction of DNA breakage or oxidative DNA damage was observed in the treated cells using the standard alkaline assay or the EndoIII and 8-hydroxyguanine DNA-glycosylase-modified assay [16].

2.3. Micronucleus assay

The micronucleus test measures damage to the chromosomes and mitotic apparatus of cells. An increase in the frequency of micronucleated cells is an indication of induced chromosome damage. Studies on 16 types of TiO$_2$-NPs using the micronucleus assay are summarized in Table 1. Twelve of them were positive in the test, and four were negative. Genotoxic effects of 20 nm TiO$_2$-NPs were evaluated using Chinese hamster ovary (CHO-K1) cells. Micronucleus frequencies were significantly increased by 0.5 μg/mL and 1 μg/mL of the NPs [35]. Human bronchial epithelial BEAS 2B cells were treated with smaller than 25 nm uncoated anatase TiO$_2$-NPs and 10 nm × 40 nm SiO$_2$-coated rutile TiO$_2$-NPs, respectively. Although the uncoated TiO$_2$-NPs increased the micronucleus frequency, the SiO$_2$-coated NPs did not [20]. Guichard et al [22] found that none of the TiO$_2$-NPs or TiO$_2$ bulk particles showed significant induction of micronuclei formation after 24 hours' exposure of these particles to SHE cells. Prasad et al [26] found that TiO$_2$-NPs induced micronuclei only in a medium that facilitated the lowest amount of agglomeration, the greatest amount of NP-cellular interaction, and the highest population of cells accumulating in S phase. The genotoxic potential of ≤20 nm TiO$_2$-NPs was assessed in SHE cells. The cells were treated with 1.0 μg/cm^2 of the particles for 12 hours, 24 hours, 48 hours, 66 hours, and 72 hours. The micronucleus frequencies were increased by the treatment in a time-dependent manner [36]. HepG2 cells were treated with low doses of 30–70 nm TiO$_2$-NPs and a significant increase in the micronucleus frequency was observed in the treated cells [28]. Human epidermal cells (A431) were treated with 50 nm anatase TiO$_2$-NPs at doses of 0.008–80 μg/mL. The treatment resulted in significant chromosome alteration at doses 0.8–80 μg/mL [29]. Human lung cancer cells, A549, were treated with 10 μg/mL and 50 μg/mL of TiO$_2$-NPs for 24 hours. Micronucleus assay was conducted to determine the genotoxicity of the particles and there was a positive response in the micronucleus induction for both of the treatment concentrations [37,38]. Tavares et al [39] evaluated genotoxicity of different types of TiO$_2$-NPs using in vitro micronucleus assay in human lymphocytes. They found that 21 nm anatase TiO$_2$-NPs at 125 μg/mL dose, 22 nm hydrophobic rutile TiO$_2$-NPs at

5 μg/mL and 45 μg/mL doses, and 19 nm hydrophilic rutile TiO$_2$-NPs at 15 μg/mL and 45 μg/mL doses significantly increased the frequencies of micronucleated binucleated cells except for 20 nm uncoated 15% rutile and 85% anatase TiO$_2$-NPs. Human peripheral blood lymphocytes were treated with 3 μM, 5 μM, and 10 μM TiO$_2$-NPs (<100 nm). A positive response of TiO$_2$-NPs in micronucleus assay was found and the addition of ascorbic acid decreased the micronucleus induction [30]. Human SHSY5Y neuronal cells were treated with 25 nm anatase TiO$_2$-NPs and 25 nm TiO$_2$-NPs with 80% anatase and 20% rutile. Results from the micronucleus test showed that both types of the TiO$_2$-NPs induced a dose-dependent micronucleus formation after 6 hours' exposure [31].

2.4. SCE assay

The SCE assay is a short-term test for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome. A positive response of this assay indicates certain types of chromosome damage. Only two studies on SCE analysis of TiO$_2$-NPs have been reported (Table 1). Genotoxic effects of 20 nm TiO$_2$-NPs were evaluated by conducting SCE assay in Chinese hamster ovary (CHO—K1) cells. SCE frequencies were significantly increased by 1—5 μg/mL of the TiO$_2$-NPs [35]. In the second study, human peripheral blood lymphocytes were treated with 3 μM, 5 μM, and 10 μM TiO$_2$-NPs (<100 nm). The SCE assay was conducted and the genotoxicity of the NPs tested was positive. Addition of ascorbic acid resulted in decreasing the SCE frequencies [30].

2.5. Mammalian cell mutation assay

The in vitro mammalian cell mutation assays detect gene mutations induced by test agents. The most commonly-used genes for measurement of mutations are the thymidine kinase and hypoxanthine-guanine phosphoribosyl transferase (Hprt) genes. The mutation tests detect different spectra of mutational events. Only one in vitro mammalian cells mutation study was found (Table 1). The Hprt gene mutation assay was conducted on chronically exposed cells for 60 days to 0—40 μg/mL < 25 nm anatase TiO$_2$-NPs. The results showed no mutation induction [34].

3. In vivo studies

The in vivo genotoxicity of TiO$_2$-NPs has been investigated in several studies (Table 2) using the in vivo Comet assay, micronucleus assay, wing somatic mutation and recombination assay, and phosphatidylinositol glycan, class A gene (Pig-a) mutation assay.

3.1. The in vivo Comet assay

Five studies have been performed using the in vivo Comet assay in different tissues. Two showed positive responses to TiO$_2$-NP treatment. CBAB6F1 mice were treated via gavage with 33 nm TiO$_2$-NPs at doses of 40 mg/kg, 200 mg/kg, and 1000 mg/kg body weight, daily for 7 days. Genotoxic effects in brain, liver, and bone marrow were evaluated by the Comet

assay. The NPs induced DNA damage in bone marrow and liver, but not in the brain [40]. In the other positive comet study, mice were treated through drinking water containing a 21-nm mixture of 75% anatase and 25% rutile TiO$_2$-NPs and DNA damage measured by the Comet assay in the blood cells. DNA strand breaks were significantly increased by 500 mg/kg TiO$_2$-NPs [41].

In the first negative study, rats were exposed by inhalation with T-Lite SF containing 79—89% TiO$_2$-NP (10 nm × 50 nm rutile) and T-Lite Max containing 69—73% TiO$_2$-NPs, used as UV protecting agents in sunscreens, and genotoxicity of the particles were investigated in the lung by the in vivo Comet assay. No DNA damage was found in the lung [15]. C57BL/6J mice were treated with freshly generated TiO$_2$-NPs (74% anatase, 26% brookite at approximately 80 nm in size) for 5 days, 4 hours/day by inhalation at doses of 0.8 mg/m^3, 7.2 mg/m^3, and 28.5 mg/m^3. DNA damage was assessed by the Comet assay in lung epithelial alveolar type II and Clara cells sampled immediately following the exposure. Although a dose-dependent deposition of Ti in lung tissue was seen, no significant effect was observed on the level of DNA damage in lung epithelial cells [42]. The genotoxicity of 5 nm anatase TiO$_2$-NPs was evaluated using the Comet assay after a single or repeated intratracheal instillation at doses of 1.0 mg/kg or 5.0 mg/kg body weight or 0.2 mg/kg or 1.0 mg/kg body weight once a week for 5 weeks. The lung cells were used for the assay and there was no increase in % tail DNA in any of the treatment groups [43].

3.2. The in vivo micronucleus assay

Four in vivo micronucleus tests of TiO$_2$-NPs have been reported: one was positive and three of them were negative. C57BL/6J mice were treated with NPs and micronuclei were analyzed in peripheral blood polychromatic erythrocytes (PCEs) collected 48 hours after the last exposure. No significant effect on micronucleated PCEs was observed [42]. In vivo micronucleus assay was conducted to evaluate the genotoxicity of 10 nm TiO$_2$ anatase NPs in mice. B6C3F1 mice were treated intravenously for 3 consecutive days with 0.5 mg/kg, 5.0 mg/kg, and 50 mg/kg TiO$_2$-NPs. The mouse blood was assayed and no induction of micronuclei was found although the NPs reached the bone marrow and induced cytotoxicity [44]. Finally, there was no induction of micronuclei in the PCE of mice 14 days after a single intravenous injection of different doses of 40 nm anatase TiO$_2$-NPs [45].

3.3. In vivo mutation assay

Two studies have been reported on the mutagenicity of TiO$_2$-NPs in vivo and both of them had negative results. Drosophila melanogaster third instar larvae were fed with the medium containing 0.1—10 mM TiO$_2$-NPs and the SMART assay was conducted. The results showed no significant increases in the frequency of all measured spots, indicating that these NPs were not able to induce mutations or recombination [46]. The Pig-a mutation assay was performed at different sampling times after mice were treated with different doses of 10 nm anatase TiO$_2$-NPs. The NPs did not increase mutant frequency in the gene at any time point or dose [44].

Size and crystalline structure	Dose	Test system	Result	Reference
In vivo Comet assay				
33 nm	40–1000 mg/kg	CBAB6F1 mice were gavaged daily for 7 d, and their bone marrow, liver and brain were assayed	Positive in bone marrow and liver; negative in brain	Sycheva et al, 2011 [40]
21 nm; 75% anatase and 25% rutile	500 mg/kg	Mice were exposed via drinking water for 5 d, and their blood cells were used for the assay	Positive	Trouiller et al, 2009 [41]
80 nm; 74% anatase and 26% brookite	Up to 28.5 mg/m^3	Rats were exposed via inhalation for 5 d, 4 h/d and their lung cells were assayed	Negative	Lindberg et al, 2012 [42]
10 × 50 nm; rutile in T-Lite	10 mg/kg	Rats were exposed by inhalation, and their lung cells were assayed	Negative	Landsiedel et al, 2010 [15]
5 nm; anatase	Up to 5 mg/kg	Intratracheally exposure to male Sprague–Dawley rats, and the lung cells was assayed	Negative	Naya et al, 2012 [43]
Micronucleus assay				
21 nm; 75% anatase and 25% rutile	500 mg/kg	Mice were exposed via drinking water for 5 d. The mouse blood cells were tested	Positive	Trouiller et al, 2009 [41]
80 nm; 74% anatase and 26% brookite	Up to 28.5 mg/m^3	Rats were exposed via inhalation for 5 d, 4 h/d. The peripheral blood polychromatic erythrocytes were tested	Negative	Lindberg et al, 2012 [42]
10 nm; anatase	Three daily dose of 50 mg/kg	Intravenously exposure to male B6C3F1 mice; the blood cells was assayed	Negative	Sadiq et al, 2012 [44]
40 nm; anatase	Up to 1387 mg/kg	One dose intravenous injection to mice, and the blood cells were assayed	Negative	Xu et al, 2013 [45]
Mutation assay				
2.3 nm; anatase	Fed, 0.1–10 mM	*Drosophila melanogaster*, the wing somatic mutation and recombination assay	Negative	Demir et al, 2013b [46]
10 nm; anatase	Three daily dose of 50 mg/kg	Intravenously exposure to male B6C3F1 mice and *Pig-a* mutation assay in blood cells was conducted	Negative	Sadiq et al, 2012 [44]

Table 2 – *In vivo* studies on genotoxicity of titanium dioxide nanoparticles.

4. Mechanisms underlying the genotoxicity of TiO₂-NPs

4.1. Oxidative stress

A number of studies suggest that TiO$_2$-NPs induce toxicity via oxidative stress due to their relatively large surface area and greater reactive activity than bulk TiO$_2$ particles. Jugan et al [24] found that TiO$_2$-NPs caused an early intracellular accumulation of reactive oxygen species (ROS) and that addition of glutathione significantly reduced the ROS, suggesting that this molecular antioxidant detoxified ROS overproduction caused by TiO$_2$-NPs. Evidence for the induction of oxidative damage to DNA was also gained from the measurement of the level of 8-oxo-7,8-dihydro-2′-deoxyguanosine [24]. TiO$_2$-NPs induced a significant reduction in mitochondrial dehydrogenase activity in human lymphocytes, resulting in the generation of ROS and an alteration of mitochondrial membrane potential [21]. Bhattacharya et al [32] showed that TiO$_2$-NPs were able to generate free radicals and induce indirect genotoxicity mainly by DNA-adduct formation.

However, Jugan et al [24] reported that if experiments were processed in the dark to avoid any photocatalytic effects on TiO$_2$-NPs, ROS accumulation decreased, suggesting that TiO$_2$-NPs generated ROS in cells via photocatalysis.

4.2. Size, structure, and agglomeration effects

Smaller TiO$_2$-NPs were more genotoxic than larger TiO$_2$-NPs, regardless of their crystalline phases. It is likely that the smaller the NPs are, the easier for them to enter cells and accumulate inside the cells, both in the cytoplasm and the nucleus [47]. Some studies show that anatase NPs induce more deleterious effects than rutile NPs because of the photocatalytic properties of anatase TiO$_2$. TiO$_2$-NPs forming large agglomerates induced DNA damage in different cell lines, whereas NPs that formed smaller agglomerates (200 nm) had no effect on genotoxicity [48].

4.3. Effects on DNA repair, proliferation, and apoptosis

Evidence indicates that TiO$_2$-NPs can impair cellular DNA repair, by inactivation of both the NER and BER pathways. The inactivation could result from structural modification of NER and BER proteins by oxidation caused by TiO$_2$-NPs induced ROS accumulation. Protein oxidation/reduction has been regarded as a critical mechanism for the modulation of repair pathway. Botelho et al [18] reported that 21 nm TiO$_2$-NPs increased oxidative stress and cell proliferation, and decreased apoptosis in the AGS human gastric epithelial cell line. Huang et al [49] showed that short-term exposure to TiO$_2$-NPs enhanced cell proliferation, survival, ERK signaling activation, and ROS production in cultured fibroblasts whereas long-term exposure to TiO$_2$-NPs not only increased cell survival and growth but also increased the numbers of multinucleated cells and micronuclei. Their results suggest that long-term exposure to TiO$_2$-NPs disturbs cell cycle progression and genome segregation, leading to chromosomal instability and cell transformation.

5. Conclusions

As with many other nanomaterials, controversial results have been reported on the genotoxicity of TiO$_2$-NPs. A large number of tests on TiO$_2$-NPs have been conducted and many have detected positive responses. However, there remain clear inconsistencies in the reported results. The conflicting results reported from different studies could have arisen from inconsistencies in characteristics of the test materials, such as size, shape, and crystalline structure. The different results may also have been a function of the different test systems and different genotoxicity endpoints that have been employed. Generally, in vitro systems for assessing genotoxicity of TiO$_2$-NPs generated more positive results than the in vivo systems, whereas DNA and chromosome damage tests generated more positive results than the assays measuring gene mutations. Nearly all tests for the mutagenicity of TiO$_2$-NPs were negative, except for a few studies using the Ames test, which has been suggested to be unsuitable for detecting the genotoxicity of nanoparticles [50]. The available evidence indicates that TiO$_2$-NPs induce genotoxicity mainly via generating oxidative stress in cells. The ROS generation is dependent on the size, structure, and aggregation of the TiO$_2$-NPs. The oxidative stress produced by the NPs could affect DNA repair, cell cycle progression, cell proliferation, and apoptosis by affecting protein structure.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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