ROS

REVIEW ARTICLE

Toxicity of Titanium Dioxide Nanoparticles Induced by Reactive Oxygen Species

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ABSTRACT | Titanium dioxide nanoparticles (TiO_2 NPs) are one of the most widely used types of nanoparticles which can be used in food additives and drugs. However, the safety of TiO_2 NPs is still controversial and the biological mechanism of TiO_2 NPs-induced toxicity is not clear yet. We reviewed the research about toxicity of TiO_2 NPs induced by reactive oxygen species (ROS) in vitro or in vivo. TiO_2 NPs could induce significant increase of ROS and excessive free radicals, destroying redox balance. ROS-mediated oxidative stress leads to the occurrence of lipid and protein peroxidation, which in turn would induce autophagy, apoptosis, and necrosis of cells. Increased ROS production induced by TiO_2 NPs was generally associated with inflammatory response, mitochondrial dysfunction, and genetic damage in vitro and in vivo. Although ROS may play an important role in TiO_2 NPs-induced biological effects, the specific way it produces and its complex relationship with subsequent biological effects need to be further clarified.

KEYWORDS | Nanomaterial; Oxidative stress; Reactive oxide species; Titanium dioxide nanoparticle; Toxicity

ABBREVIATIONS | GSH, reduced glutathione; MDA, malondialdehyde; MPTP, mitochondrial permeability transition pore; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; RIRR, ROS-induced ROS release; ROS, reactive oxygen species; TiO_2 NPs, titanium dioxide nanoparticles

CONTENTS

1. INTRODUCTION

Titanium dioxide nanoparticles (TiO_2 NPs) are one of the most widely used types of nanoparticles in the world, with great application in food additives, medical treatments, water purification, cosmetics, sunscreens, paints, and coatings. Since 1969, the European Union (EU) has approved the application of food-grade titanium dioxide (E171) as a colorant in the food industry [1, 2]. Because of its high refractive index and uniform dispersion, E171 is used as a lightening and brightening agent in foods, including confectionery (chocolate, sweets, and chewing gum), white sauces and icing. The US Food and Drug Administration (FDA) has also approved the use of titanium dioxide as food additive, merely limiting the amount to not exceeding 1% of the total food mass. This is consistent with the China Food and Drug Administration (CFDA) standards (GB 2760-2014). On the basis of the expert review of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1970, the EU did not establish an acceptable daily intake (ADI) for E171.

Recent studies have shown that up to 36% of particles in E171 are nanoparticles [2]. It was reported that nanoparticles can be absorbed more easily and excreted more difficultly than traditional coarse particles [3]. In addition, previous studies showed that TiO_2 NPs could penetrate the blood-brain barrier and placental barrier to accumulate in the central nervous system and fetus, and affect target organs [4]. With the rapid development of nanotechnology and the widespread application of TiO_2 NPs, assessment of the toxicity and safety of TiO_2 NPs has attracted much attention, but there is still considerable controversy [5]. In 2016, the European Food Safety Authority (EFSA) re-evaluated the safety of E171, concluding that the food additive E171 was not genotoxic and had a no observed adverse effect level (NOAEL) of 2,250 mg/kg body weight in rats. When calculating the mass of the nanoparticles contained in E171 at the NOAEL level with the upper mass concentration limit in E171 (3.2%) mentioned in the report, the NOAEL of the TiO_2 NPs should be at least 72 mg/kg body weight [6]. However, this is in contradiction with some experiments in which significant genetic damage was observed in groups treated with TiO_2 NPs at doses of less than 72 mg/kg body weight [7, 8]. The researchers reported that oral administration of E171 (10 mg/kg) for 7 days increased pre-cancerous colonic lesions in the rats [9], and E171 (5, 10, and 50 $\mu g/cm^2$) increased the micronucleus frequency (1.9–3.6-fold) of the HCT116 cell line in vitro [1]. This makes the debate about the safety of TiO_2 in foods more intense.

In EU 872/2012, it was specified that if nanotechnology was applied to the food additive, EFSA should be noticed, and then a new approval and a new product label for the modifications is required [10]. However, there is no regulation to specify the mass limitation of nanoscale particles as an additive in foods. The US National Institute for Occupational Safety and Health (NIOSH) has supported this distinction by setting two separate occupational exposure limits for fine TiO_2 particles and ultrafine TiO_2 (< 100 nm). Thus, traditional knowledge on the safety of occupational and environmental exposure to TiO_2 was challenged. Meanwhile, as TiO_2 NPs was classified as a Class 2B carcinogen by the International Agency for Research on Cancer in 2006, further attention should be paid to the genotoxicity and carcinogenicity of TiO_2 NPs. As such, re-evaluation of the safety of TiO_2 NPs is warranted, especially for dietary exposure closely related to consumers.

In this review, we focused on the toxicity of TiO_2 NPs associated with ROS generation. Research on the ROS-mediated toxicity of TiO_2 NPs in vitro or in vivo (via oral exposure) and the underlying mechanisms were summarized so as to provide insights for reducing their health risk.

2. OXIDATIVE STRESS INDUCED BY TiO_2 NPs

Excessive free radicals and ROS could destroy redox balance and induce inflammation and mitochondrial dysfunction, leading to cellular apoptosis. Shukla et al. demonstrated that TiO_2 NPs have a tendency to generate free hydroxyl radicals leading to oxidative stress, genotoxicity, and ultimately cell death in different cell lines [11, 12]. Subsequently, they also observed a dose-dependent increase in the generation of ROS (e.g., superoxide and H_2O_2) in the liver of mice exposed to TiO_2 NPs orally for 14 consecutive days, leading to cellular stress [13]. A significant dose-dependent decrease in reduced glutathione (GSH) levels also suggested increased oxidative stress in the liver, which may lead to hepatic injury. Indeed, oxidative DNA damage caused by oxidative stress con-

sequently initiated the expression of apoptotic proteins resulting in hepatic injury.

The ability of TiO_2 to produce ROS was reported to be closely related to the particle size, and the smaller the size, the stronger the ability. Xiong et al. found that compared with bulk TiO_2, the quantities of hydroxyl radicals in the TiO_2 NPs suspensions were much higher [14]. Hamzeh et al. also found that TiO_2 NPs decreased cell viability in Chinese hamster lung fibroblast cells through ROS generation in a mass-based concentration- and size-dependent manner [15]. In addition, the photocatalytic activity of TiO_2 NPs was considered to be the main basis for ROS production [16]. TiO_2 NPs significantly exerted an oxidative effect on cells upon exposure to solar or ultraviolet (UV) irradiation [17]. However, recent studies also found obvious effects of TiO_2 NPs under dark conditions on sensitive organisms such as *Physarum polycephalum* or *Escherichia coli* [18, 19]. TiO_2 NPs induced oxidative stress in macroplasmodium of *Physarum polycephalum* under dark conditions, evidenced by the increased levels of ROS, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and total soluble phenols (TSP) [19].

3. LIPID PEROXIDATION INDUCED BY TiO_2 NPs

The generation of free radicals and ROS can lead to the occurrence of lipid peroxidation, which in turn would induce autophagy and apoptosis of cells. It had been shown that ROS generated by TiO_2 NPs could induce lipid peroxidation by directly oxidizing the biological membranes or molecules [20]. A dose-dependent significant increase in the levels of malondialdehyde (MDA) was observed in TiO_2 NPs-treated mouse liver cells. MDA is a typical biomarker of lipid peroxidation. Several types of damage including oxidation of sulfhydryl groups, formation of disulfides, peptide fragmentation, and modification of prosthetic groups or metal clusters are documented due to lipid peroxidation [13]. Meanwhile, the MDA content of zebrafish gills exposed to TiO_2 NPs in illumination and dark was 217.2% and 174.3% of controls, respectively [14]. The level of hydroxyl radicals ascended with the increase of MDA, indicating that the occurrence of lipid peroxidation may be partly due to the generation of hydroxyl radicals.

Increased lipid peroxidation may be closely related to cell autophagy, apoptosis, and necrosis. As the autolysosomes derived from autophagy fail to repair peroxide damage, an advanced process of apoptosis or necrosis will be activated [21, 22]. Hussain et al. found that lysosomal membrane damage and lipid peroxidation might contribute to TiO_2 NPs -induced cell death [23]. Increased autophagy and apoptosis were also observed in cells exposure to other nanoparticles such as Ag or SiO_2 [24]. Previous study had indicated that TiO_2 NPs (5–150 mg/kg) induced apoptosis in the mouse splenocytes via mitochondria-mediated pathway and ROS accumulation [25]. A significant increase in cytochrome c, Bax, and caspase-3/-9 expression, and a significant decrease in Bcl-2 expression were detected in the TiO_2 NPs-treated mouse spleen. Park et al. and Shi et al. also demonstrated a TiO_2 NPs-induced, dose- and time-dependent decrease in cell viability, caspase-3 activation, and DNA condensation indicative of apoptosis in BEAS-2B bronchial epithelial cells, along with ROS production, GSH depletion, and increased heme oxygenase-1 [26, 27].

4. PROTEIN PEROXIDATION INDUCED BY TiO_2 NPs

A wide variety of pathways could be activated by oxidizing amino acid residues of proteins, including cell signaling proteins (NF-κB, MAPK, Keap1-Nrf2-ARE, and PI3K-Akt), ion channels and transporters (Ca^{2+} channels and mitochondrial permeability transition pore), and the protein kinase and the ubiquitination/proteasome system [28, 29]. It has been shown that peroxidation of signaling proteins induced by ROS accumulation from TiO_2 NPs can disrupt ion channels and transporters and cause cellular dysfunction and apoptosis [30]. Additionally, ROS can influence various ion channels and transporters, including voltage-gated Ca^{2+} channels, mitochondrial Ca^{2+} uniporter, Ca^{2+} release-activated Ca^{2+} (CRAC), channels, as well as P2X, IP3R/RyR, and voltage-gated K^+ channels. It was reported that TiO_2 NPs-induced oscillations in the intracellular calcium concentration could be attributed to the oxidation of certain redox sensitive residues in the mitochondrial channels such as the mitochondrial permeability transition pore (MPTP) [31]. The activation of those residues can prolong the MPTP openings and release

a ROS and Ca^{2+} burst. The phenomenon that ROS trigger MPTP-related ROS release has been termed "ROS-induced ROS release" (RIRR) [31].

5. INFLAMMATORY RESPONSE INDUCED BY TiO_2 NPs

Evidence accumulated over the past two decades has pointed to significant connections between inflammation and oxidative stress, with both processes contributing to fuel the other one, thereby establishing a vicious cycle able to perpetuate and propagate the inflammatory response [32]. With sub-chronic intragastric administration of TiO_2 NPs (2.5–10 mg/kg) in mice, a significant increase in NF-κB, TNF-α, IL-1β/-6, and IFN-α and a significant decrease in I-κB were observed in the heart of mice. It indicated that the low-dose and long-term exposure to TiO_2 NPs can cause a cardiac inflammatory response in mice [33]. Another long-term study indicated that the alteration of Th2 factor expression may be involved in the control of hepatic inflammation induced by chronic TiO_2 NPs toxicity [34]. The study found a significantly upregulated expression of the inflammation-related genes including IL-4, IL-5, IL-12, IFN-γ, GATA3, GATA4, T-bet, STAT3, STAT6, Eotaxin, MCP-1, and MIP-2 in the liver of mice after oral gavage of TiO_2 NPs for 6 months. Renal inflammation was also reported in mice after a chronic gavage administration of TiO_2 NPs (1.25–5 mg/kg) for 9 months [35]. It was observed that pathways in the MAPKs family, including JUN, p38, and ERKs pathways were activated in the kidney [35]. In addition, the same researchers also found thymic inflammation in mice following exposure to TiO_2 NPs [36]. These studies together suggested that chronic exposure to TiO_2 NPs may disrupt immune cell homeostasis and induce systemic inflammatory response.

6. MITOCHONDRIAL DYSFUNCTION INDUCED BY TiO_2 NPs

It is well known that endogenous cellular ROS are mainly generated in the process of mitochondrial oxidative phosphorylation. Increased ROS production is generally associated with mitochondrial dysfunction. Many studies have pointed out that TiO_2 NPs could induce mitochondrial dysfunction in vitro and in vivo [37–39]. After incubation of mitochondria isolated from rat lung tissue with TiO_2 NPs, significant decreases in NADH levels and mitochondrial transmembrane potential ($\triangle\Psi m$) were observed [37]. In addition, the generation of ROS after exposure to TiO_2 NPs was found to be mainly (46% out of 46.5%) caused by dysfunction of mitochondrial respiratory complexes [37]. Another in vitro experiment on the BV2 cell line confirmed that TiO_2 NPs could interfere with respiratory chain complexes, and that a dysfunction of the respiratory complex could increase the generation of ROS [39]. The exogenous ROS can oxidize and consecutively open the MPTP mitochondrial ion channel. The consecutive opening of MPTP will alter the permeability of mitochondria, causing mitochondrial swelling which may interfere with mitochondrial electron transport chain (ETC) function. Damage to or interference with ETC (such as complexes I and III) may increase the production and release of endogenous ROS, which may in turn exacerbate mitochondrial dysfunction. This is the way that RIRR functions, and might be the primary pathways for oxidative stress induced by TiO_2 NPs [31]. TiO_2 NPs also produced morphological changes, damage of mitochondria, and an increase in mitochondrial membrane potential in rat and human glial cells (C6 and U373) [38]. Meanwhile, Shi et al. found that TiO_2 NPs cause apoptosis in BEAS-2B cells through the caspase 8/t-Bid-independent mitochondrial pathway [27]. Mitochondrial dysfunction may be able to serve as a sensitive biomarker for the toxicity of TiO_2 NPs [40]. It could be detected even in the hippocampus of offspring after maternal exposure to TiO_2 NPs in rats [41].

7. GENETIC DAMAGE INDUCED BY TiO_2 NPs

Oxidative stress and inflammatory responses were originally thought to be the mechanism by which oral exposure to the TiO_2 NPs induced genetic toxicity. Trouiller et al. observed elevated expression of inflammatory cytokines such as TNF-α, IFN-γ, and IL-8 in the blood of mice after oral intake of TiO_2 NPs at 100 mg/kg for 5 days [8]. They suggested that the genotoxicity in mice induced by TiO_2 NPs may be mainly associated with the inflammation and/or oxidative stress, which was called a secondary geno-

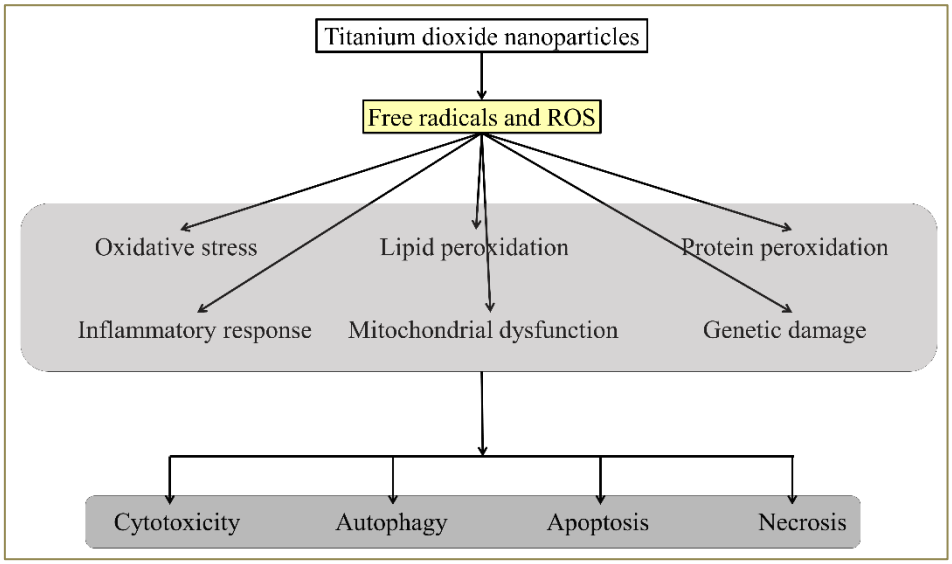

FIGURE 1. The role of free radicals and ROS in the toxicity of TiO_2 nanoparticles. As illustrated, formation of free radicals and ROS may be an upstream event leading to various forms of oxidative/inflammatory damage and consequent cell injury and death.

toxic mechanism. Afterwards, several publications confirmed that the genotoxicity of most nanomaterials is likely to be associated with indirect consequences of inflammation and generation of oxidative species by inflammatory cells (neutrophils and macrophages) [7, 42–48]. Studies in vitro have also shown that TiO_2 NPs can cause cytotoxicity and genotoxicity through oxidative stress in cells including human hepatocytes and epidermal cells [12, 49–51]. Our previous studies had found TiO_2 NPs could induce genetic toxicity both in vitro and in vivo via oral exposure [52], as well as obvious inflammatory response evidenced by significantly increased white blood cell count and serum concentrations of IL-1α, IL-4, and TNF in rats after daily oral exposure to TiO_2 NPs at 50 mg/kg for 90 days [53]. Inflammation induced by TiO_2 NPs produced obvious oxidative stress, which was evidenced by decreased level of GSH and increased levels of glutathione disulfide (GSSG) and MDA in the liver tissue of TiO_2 NPs-exposed rats.

Oxidative DNA damage is a major marker of genotoxicity induced by TiO_2 NPs. The DNA base pairs can be attacked by oxidants, of which guanine is the most sensitive and can be oxidized to 8-OHdG in most cases. It has been observed that TiO_2 NPs could induce increased 8-OHdG in the liver of C57BL/6Jp^{un}/p^{un} mice [8]. In addition, 8-OHdG can mimic T functionally in the syn conformation and form a stable 8-oxoG(syn)•A(anti) base pair. Thus, the absence of 8-OHdG repair before replication can lead a G-to-T transversion mutation [54, 55]. The mutant frequency of 6TGRHGPRT, which is an early indicator of potential carcinogenicity, showed a significant 2.98-fold increase after TiO_2 NPs exposure in mammalian lung fibroblasts cells (V79) [56]. TiO_2 NPs can interfere with the redox-sensitive base excision repair enzyme, or regulate the gene for the repair of basic excision and thereby cause accumulation of genetic damage [57]. In comparison with the nuclear genome, the mitochondrial genome is more likely to be oxidized by ROS without the protection of histone and nuclear envelope. It was also reported that TiO_2 NPs could induce single-strand breaks or double-strand cleavage mediated by ROS [58]. The Comet assay, which is often used to detect DNA double-strand breaks, was the most commonly used method for genotoxicity testing of nanomaterials and showed higher probability of positive results [59]. The formation of γ-H2AX foci, a biomarker of double-strand DNA breaks, also showed a significant dose-dependence positive relationship with TiO_2 NPs

concentration [6]. The increased micronucleus frequency, as an indicator of chromosome damage, was also reported. Increased micronucleus frequency was observed in the blood of marine fish *Trachinotus carolinus* 72 h after administration with TiO_2 NPs (1.5 or 3.0 μg/g body weight) via a single intraperitoneal injection [60]. In mice administered with TiO_2 NPs (40–1000 mg/kg body weight) by gavage for 7 days, a significant dose-dependent increase in the micronucleus frequency in the liver and kidney was observed [7].

8. CONCLUSION AND PERSPECTIVES

In conclusion, substantial evidence suggests a critical involvement of ROS in TiO_2 NPs-induced toxicity (**Figure 1**). Nevertheless, studies on the toxicity of TiO_2 NPs are still in their infancy. There are three areas that need further study. Firstly, the toxicity of TiO_2 NPs can be influenced by various of physical and chemical characters, which complicates the risk assessment of TiO_2 NPs. For example, the toxicity of TiO_2 NPs depends on crystalline form, size, duration of exposure, surface properties, dosage, route of administration, and surface modification. The different characteristics of particles among studies could lead to contradictory conclusions [4–6, 61]. Secondly, the origin and location of TiO_2 NPs- induced ROS were not yet clear. Therefore, the causal relationship between TiO_2 NPs-induced ROS and many biological effects remain to be clarified. A few studies have shown that the toxicity of TiO_2 NPs decreases significantly in case of co-administration with antioxidants. These related studies were helpful to clarify the causal relationship, but further studies are still needed. Lastly, current research on the toxicity of TiO_2 NPs has been limited to animal experiments in vivo or cell experiments in vitro. Whether the same results will be observed in humans is still unknown. The well-designed epidemiological study is recommended to evaluate the effect of TiO_2 NPs on health following a long-term, low-dose exposure.

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