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Research article

Titanium dioxide nanoparticles Disrupt ultrastructure and function of Rat thyroid tissue via oxidative stress

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ABSTRACT

Nano-TiO_2 is widely used in various fields such as industry, daily necessities, food and medicine. Previous studies have shown that it can enter mammalian tissues through the digestive tract or respiratory tract and have effects on various organs and systems. However, the effect of nano-TiO_2 on the mammalian thyroid gland has not been reported. In this study, we fed SD rats with rutile nano-TiO_2 at a dose of 5 mg/kg body weight for 3 weeks, and then examined the thyroid histology and thyroid function of the rats. In vitro experiments were conducted to determine the effects of nano-TiO_2 on the viability, apoptosis, inflammatory factors, antioxidant enzymes, and oxidative stress of human thyroid follicular epithelial cells. Histological evidence showed abnormal morphology of rat thyroid follicles and organelle damage in follicular epithelial cells. Nano-TiO_2 caused a decrease in the level of sodium/iodide symporter (NIS), an increase in the level of apoptotic protein cleaved-caspase 3, and an increase in the levels of pro-inflammatory factors IL-1β and TNF-α in rat thyroid tissue. Nano-TiO_2 also resulted in increased serum FT4 and TPO-Ab levels. In in vitro experiments, nano-TiO_2 reduced the viability of human thyroid follicular cells, downregulated the levels and activities of antioxidant enzymes CAT, GPX1 and SOD, and increased the levels of ROS and MDA caused by oxidative stress. These results indicate that nano-TiO_2 damages the structure and function of thyroid follicular epithelial cells through oxidative stress. Long-term exposure to nano-TiO_2 could be a potential risk factor for thyroid dysfunction.

1. Introduction

Nanometer titanium dioxide (nano-TiO_2), also called titanium dioxide nanoparticles (TiO_2-NPs), is widely used in cosmetics, medicine, food, and chemistry. It has ultra-micro size, ultraviolet absorption, and highly efficient photocatalytic activity, which is

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closely related to its biological effect. Nano-TiO_2 is mainly divided into two kinds of crystals: Anatase and Rutile. Rutile has a strong ultraviolet ray shielding effect and is the most abundant natural form of TiO_2. It is widely used in sunscreens, functional fibers, high-grade plastics, etc. [1], and is often used in toxicological research [2–4]. Anatase nano-TiO_2 has strong photocatalytic activity. Due to its strong sterilization ability under the action of ultraviolet light, it is widely used in wastewater treatment, air purification, catalysts, and so on.

These extensive applications result in nano-TiO_2 being taken up by organisms through skin contact, respiratory inhalation, and oral administration [5–7], and affecting a variety of tissues and organs, including the respiratory [8,9], circulatory [10], digestive, urinary, immune [11], reproductive [12–15], and nervous systems [9,16–18]. Since TiO_2 NPs have been found to be genotoxic, they are classified as potential carcinogens [19,20].

The endocrine system may also be affected by Nano-TiO_2 [21,22]. Nano-TiO_2 can enter the body through the gastrointestinal tract via oral food or drugs [10], or through the skin via the application of personal care products such as sunscreen [23], so it can affect the thyroid gland. Previous studies have reported that nano-TiO_2 affects the thyroid function of zebrafish through water [17,18,24]. However, there are no observational reports on its effects on the thyroid gland in mammals.

In this study, we investigated the structural and functional changes in the rat thyroid gland exposed to TiO_2 and explored the mechanism. After continuous oral administration of rutile nano-TiO_2 to rats, their thyroid microstructure and ultrastructure were observed by histological sections, and the levels of thyroid-related hormones and autoantibodies were measured by serological tests. In addition, the effect of nano-TiO_2 on the cell activity, apoptosis level, antioxidant enzyme system and oxidative stress level of human thyroid follicular epithelial cells was tested by in vitro experiments.

2. Materials and methods

2.1. Animal model

The procedures for the animal experiments were followed the recommendations of the 8th edition of the Guide for the Care and Use of Laboratory Animals of the NIH and the Animal Management Rules of China and were ethically approved by the Laboratory Animal Care and Use Committee of Southwest Medical University (No. 2019083). Healthy adult male Sprague-Dawley rats weighing approximately 250 g were obtained from the Laboratory Animal Center of Southwest Medical University. The experimental animals were divided into two groups, namely the control group and the TiO_2 oral exposure group, with 12 animals in each group.

2.2. Nano titanium dioxide particle suspension

Rutile TiO_2 nanoparticles (CAS 1317-80-2) were purchased from Sigma Aldrich (St. Louis, MO, USA). According to the physical property data provided by the manufacturer, the molecular weight of the nanoparticles is 79.87 and the particle size is less than or equal to 25 nm. According to previous characterization studies, the NPs are composed of spherical and rod-shaped particles, present singly or in loosely arranged clusters, with sizes ranging from 21 to 31 nm (25.074 ± 3.593 nm), and are stable in suspension [2]. A 0.5 % hydroxypropylmethylcellulose (HPMC) solution was prepared using HPMC (Rhawn, Shanghai, China) and distilled water. HPMC facilitates the uniform dispersion of nano-TiO_2 in water. Nano-TiO_2 was dissolved in HPMC solution to form a suspension with a concentration of 4 mg/ml. The rats received 5 mg/kg body weight per day of nano-TiO_2 by gavage for 21 days. Rats in the control group received the same amount of 5 % HPMC solution (Fig. 1).

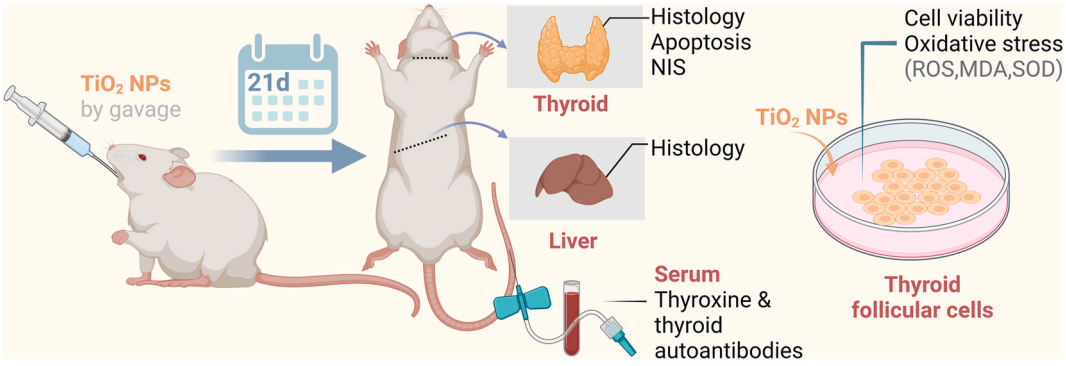

Fig. 1. Experimental procedure. TiO_2 NPs suspended in a 0.5 % HPMC solution were administered to rats by gavage at a dose of 5 mg/kg daily. After 21 days, histologic changes in the thyroid gland, expression of sodium/iodine transporter (NIS), level of thyroid cell apoptosis, level of pro-inflammatory factors in the thyroid gland, and serum levels of thyroxine, thyroid-stimulating hormone, and thyroid autoantibodies were determined. In vitro experiments, TiO_2 NPs were added to the culture medium of the thyroid follicular cell line Nthy-ori 3-1 cell, and cell viability, levels of antioxidant enzyme system, and marker of oxidative stress were detected by enzyme-linked immunosorbent assay.

2.3. Microstructural and ultrastructural observations of the thyroid gland in rats

Rats were euthanized by cervical dislocation after anesthesia with 5 % isoflurane oxygen. Arterial blood was collected by abdominal aortic puncture, and both thyroid glands were removed. At the same time, the rat liver was also removed for histological examination. Fresh thyroid tissue was fixed in 4 % paraformaldehyde. After dehydration through a graded series of alcohols, immersion in xylene, and embedding in paraffin, the tissues were sectioned at 4 μm. Sections were stained with hematoxylin-eosin and examined and photographed under a light microscope (Eclipse Ci-L, Nikon). Liver tissue was processed in the same manner for histological examination. The fresh thyroid gland was trimmed to a size of 1mm × 1mm × 1 mm and then fixed in a solution of 2.5 % glutaraldehyde and 1 % osmic acid. After alcohol dehydration, it was immersed in acetone solution, embedded in epoxy resin (SPI-Pon™ 812), and cut into 60- to 80-nm-thick tissue sections. The sections were stained with 2 % uranyl acetate saturated alcohol solution and 2.6 % lead citrate solution and viewed and photographed under a transmission electron microscope (HT7700, Hitachi).

2.4. Cell culture

The human thyroid cell line (Nthy-ori 3-1) was purchased from Biospecies Co, Ltd (Guangzhou, China). Nthy cells were cultured in RPMI 1640 complete medium containing 10 % fetal bovine serum and seeded at $1x10^5$ in a 96-well plate. After the cells reached 70 % confluence, nano-TiO_2 was added to the culture medium at concentrations of 0 μg/ml, 15 μg/ml, 30 μg/ml, and 60 μg/ml for 48 h.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Free triiodothyronine (FT3), free thyroxine (FT4), triiodothyronine (T3), thyroxine (T4), thyroid stimulating hormone (TSH) and thyroperoxidase antibody (TPO-Ab) in rat arterial blood were determined by ELISA. The test kits for FT3, FT4, and TSH were purchased from Elabscience (Wuhan, China). T3, T4 and TPO-Ab test kits were purchased from ELISAkits (Shanghai, China). IL-1β and TNF-α levels in the rat thyroid, cell viability, reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase 1 (GPX1), oxidized glutathione (GSSG) and glutathione (GSH) levels in Nthy cells were also determined by ELISA (Elabscience, Wuhan, China). The ELISA procedures were performed according to the manufacturer's instructions. The optical density (OD value) of each sample was measured at a wavelength of 450 nm using an automated absorbance

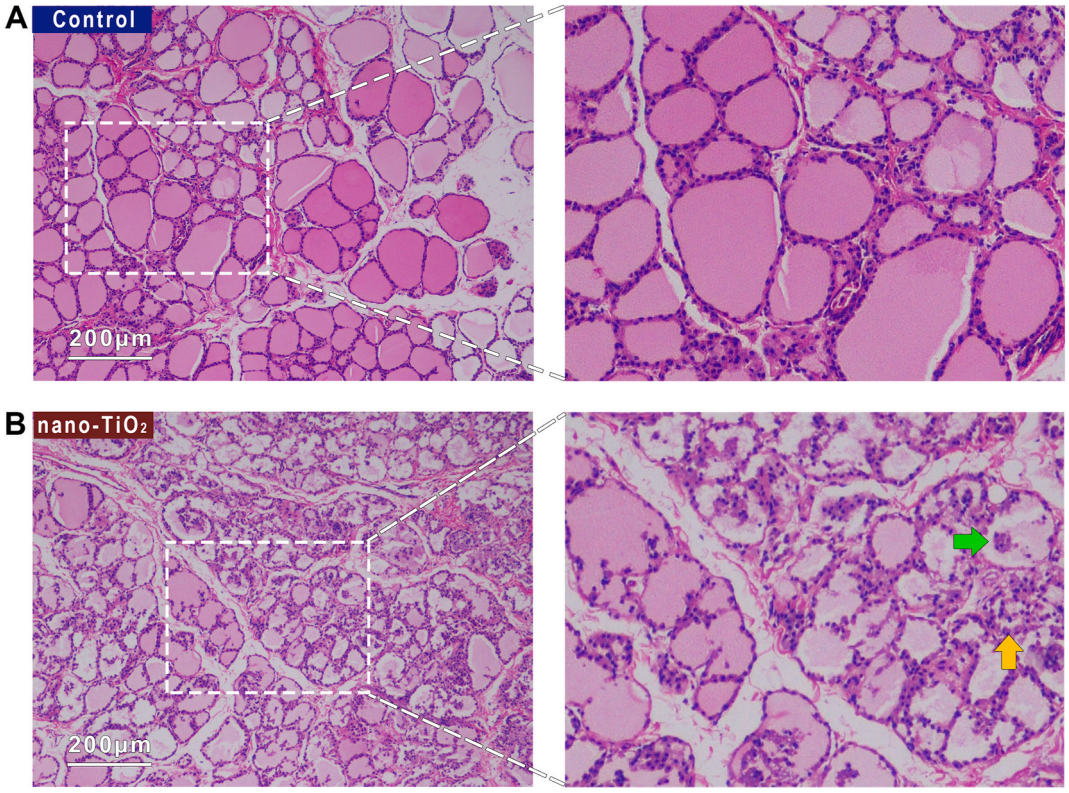

Fig. 2. Uptake of nano-TiO_2 caused histological changes in rat thyroid. A. Normal rat thyroid follicles and stroma. **B.** Thyroid gland of rats fed with nano-TiO_2 for 21 days. The mass of follicular epithelial cells had detached from the follicular wall and was floating in the colloid (green arrow). Inflammatory cells infiltrated the follicular stroma (yellow arrow). n = 5. Scale bar = 200 μm.

microplate reader (iMark 680, Bio-Rad Laboratories, CA, USA).

2.6. Western blot

Fresh thyroid tissue was ground to powder under precooling with liquid nitrogen, and RIPA lysis solution was used for protein lysis and extraction. Proteins were separated by electrophoresis in a 10 % polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Anti-sodium/iodide symporter (NIS) antibody (Absin, Shanghai, China) and anti-caspase-3 antibody (Absin, Shanghai, China) were used for protein identification. GAPDH was used as an internal reference. The Molecular Imager Gel Doc XR system (Bio-Rad Laboratories, CA, USA) was used to image the PVDF membrane, and ImageJ (National Institutes of Health, USA) was used for image analysis.

2.7. Statistical analysis

Data are presented as the mean ± standard deviation (SD). Prism 8 (GraphPad, San Diego, CA, USA) was used for data aggregation, statistical analysis and statistical graph generation. Differences between the two groups were compared by Student's t-test. Differences between multiple groups were compared by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Nano-TiO_2 ingestion caused changes in the microstructure and ultrastructure of the rat thyroid gland

SD rats were fed 5 mg/kg nano-TiO_2 for 21 days. The rats were euthanized and the thyroid glands were harvested and weighed. It was found that the thyroid weight of the rats in the nano-TiO_2-fed group increased slightly, but there was no statistical difference

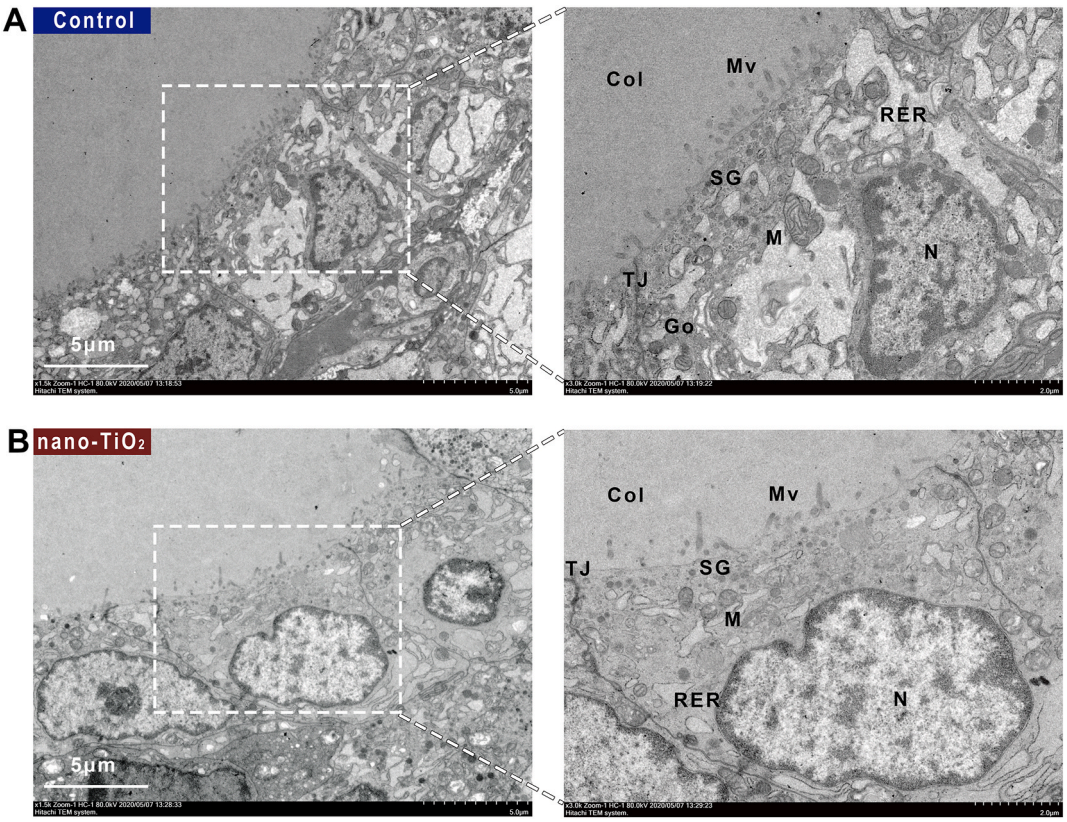

Fig. 3. Ultrastructural changes in the rat thyroid gland induced by nano-TiO_2 uptake (transmission electron microscopy). A. Ultrastructure of normal rat thyroid follicular cells. The intracellular membrane structures were clear and complete, and the intercellular tight junction was dense. **B.** Thyroid gland of rats fed with nano-TiO_2 for 21 days. Microvilli of follicular epithelial cells were small and sparse. Membranous structures such as mitochondria were blurred. n = 5. Scale bars = 5 μm.
Abbreviations: Col: colloid, Mv: microvilli, TJ: tight junction, N: nucleus, M: mitochondria, RER: rough endoplasmic reticulum, Ly: lysosome, SG: secretory granule Go: Golgi apparatus.

(Fig. S1). Hematoxylin-eosin staining of rat thyroid tissue sections showed that thyroid follicles were small and irregularly shaped in rats fed nano-TiO_2 (Fig. 2 A, B). The epithelial cells of thyroid follicles were shed into the follicular cavity (Fig. 2 B, green arrow). Inflammatory cells infiltrated the follicular stroma (Fig. 2 B, yellow arrow). The ultrastructure of the rat thyroid was examined by transmission electron microscopy (Fig. 3 A, B). It was found that the number of microvilli (Mv) at the apical tip of the rat thyroid follicular epithelial cells was reduced in the nano-TiO_2 feeding group and the distribution was sparse (Fig. 3 B). The number of mitochondria (M) was reduced and the shape was unclear. Other membranous organelles such as rough endoplasmic reticulum (RER), Golgi apparatus (Go), and secretory granules (SG) were also indistinct.

3.2. Oral administration of Nano-TiO_2 inhibited the level of thyroid iodine uptake protein in rats and upregulated the levels of apoptosis-related protein and pro-inflammatory factors

Sodium Iodide Symporter (NIS) is an iodine uptake membrane protein of thyroid follicular cells. It plays an important role in iodine metabolism and regulation of thyroid function [25]. Nano-TiO_2 caused a decrease in NIS levels in rat thyroid tissue (Fig. 4A, Fig. S3). The level of apoptosis-associated protein, cleaved caspase-3, increased in the thyroid gland of rats treated with nano-TiO_2 (Fig. 4B, Fig. S3). It has been reported that the proinflammatory cytokines IL-1β and TNF-α can affect thyroid function by altering epithelial integrity [26]. Examination of rat thyroid tissue revealed that oral administration of nano-TiO_2 resulted in upregulation of IL-1β and TNF-α levels (Fig. 4C and D).

3.3. Nano-TiO_2 disrupted thyroid function in rats and increased thyroid autoantibody levels

Blood samples were collected from rats by puncture of the abdominal aorta. Free triiodothyronine (FT3), free thyroxine (FT4), triiodothyronine (T3), thyroxine (T4), thyrotropin (TSH), and thyroperoxidase antibody (TPO-Ab) were detected by enzyme-linked immunosorbent assay. The results showed that nano-TiO_2 intake increased serum FT4 and TPO-Ab in rats (Fig. 5. B and F). There was a slight increase in serum FT3 and T4, but it was not statistically significant (Fig. 5. A and D). Feeding nano-TiO_2 did not affect T3 and TSH in rats (Fig. 5. C and E).

3.4. Nano-TiO_2 inhibits the proliferation activity and antioxidant enzyme system of human thyroid follicular cells and causes oxidative stress

In the in vitro experiment, the effects of different concentrations of nano-TiO_2 on the activity, antioxidant enzyme system and oxidative stress level of human thyroid follicular cell line Nthy-ori 3-1 cells (Nthy cells) were tested. Nthy cell is derived from immortalized human thyroid follicular epithelium [27]. 0 μg/ml, 15 μg/ml, 30 μg/ml, or 60 μg/ml nano-TiO_2 was added to the culture medium of Nthy cells for 48 h, respectively. The Cell Counting Kit-8 assay showed that the viability of Nthy cells decreased at a concentration of 30 μg/ml and 60 μg/ml nano-TiO_2, (Fig. 6A).

The effects of nano-TiO_2 on several essential antioxidant enzymes in thyroid follicular cells, including catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD), were tested. The results showed that nano-TiO_2 inhibited the levels of CAT and GPX1 (Fig. 6B and C). Endogenous H2O2 in thyroid follicular cells is converted to H2O under the catalysis of GPXs, and this process is accompanied by the conversion of GSSG to GSH [28,29]. Nano-TiO_2 increased the ratio of GSSH/GHS in Nthy cells (Fig. 6D), which represents a decrease in the activity of GPXs.

Oxidative stress and repair-related markers, reactive oxygen species (ROS), malondialdehyde (MDA), and superoxide dismutase (SOD), were detected by enzyme-linked immunosorbent assay. It was found that in the presence of nano-TiO_2, the level of SOD, which is involved in the antioxidant response, decreased in the presence of 30 μg/ml and 60 μg/ml nano-TiO_2 (Fig. 6E). On the other hand, the level of ROS increased in Nthy cells. And the increase of ROS was positively correlated with the concentration of nano-TiO_2

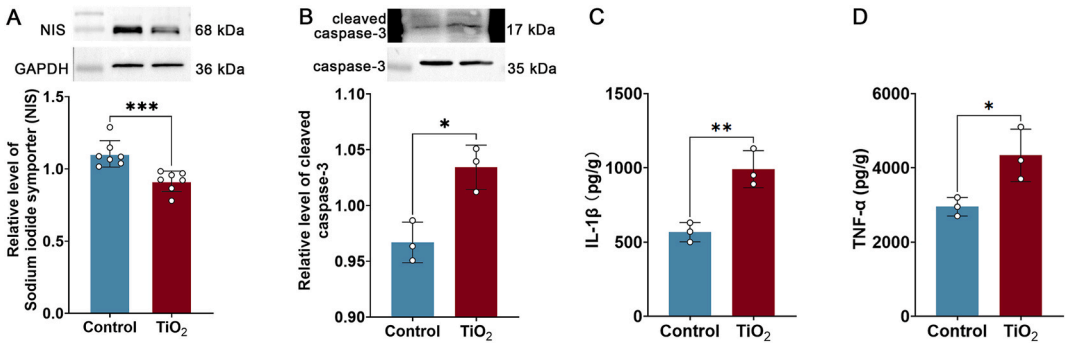

Fig. 4. Nano-TiO_2 downregulated thyroid iodine uptake protein and upregulated apoptosis and pro-inflammatory cytokine levels in rats. **A.** Nano-TiO_2 inhibited the sodium iodide symporter (NIS) in rat thyroid gland ($n = 7$). **B.** Nano-TiO_2 increased the level of cleaved caspase3 in rat thyroid gland ($n = 3$). **C.** Oral administration of Nano-TiO_2 upregulated IL-1β levels in rats (n = 3). **D.** Oral administration of Nano-TiO_2 upregulated TNF-α levels in rat thyroid (n = 3). Data are expressed as mean ± SEM. $^*P < 0.05$, $^{***}P < 0.001$.

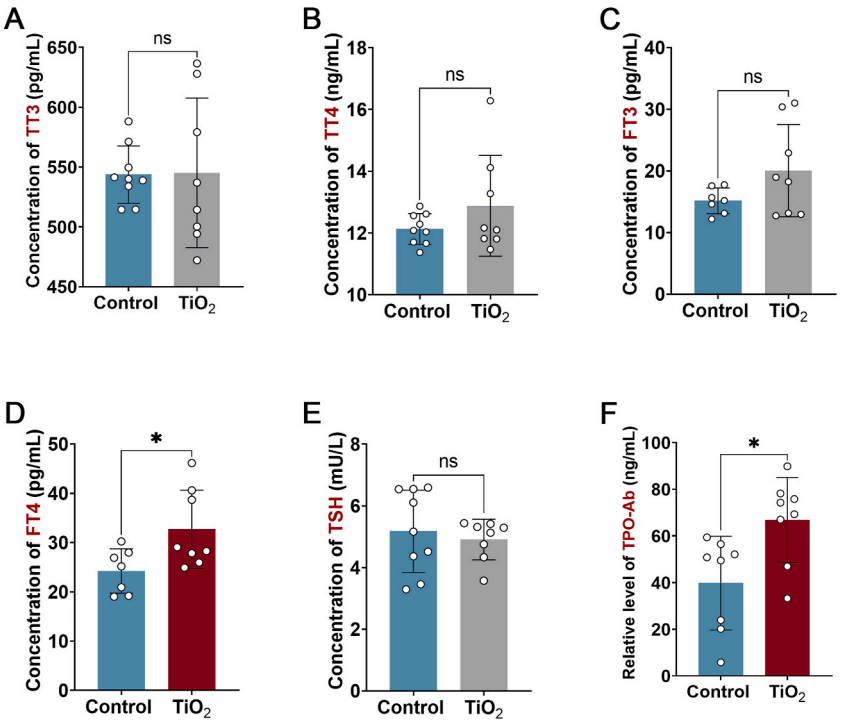

Fig. 5. Ingestion of nano-TiO_2 impaired thyroid function in rats. A-E. Nano-TiO_2 caused an increase in free thyroxine (FT4). It also caused a slight change in free triiodothyronine (FT3) and thyroxine (T4) levels without statistical significance. The levels of triiodothyronine (T3) and thyrotropin (TSH) were not affected. F. Ingestion of nano-TiO_2 resulted in an increase of thyroperoxidase antibodies (TPO-Ab) in the serum of rats. Data are expressed as mean ± SEM. $n = 8$, $^*P < 0.05$.

(Fig. 6F). At a concentration of 60 μg/ml nano-TiO_2, the level of MDA in Nthy cells also increased (Fig. 6G).

4. Discussion

Studies have shown that nano-TiO_2 can be transferred to various tissues and organs after ingestion through the gastrointestinal tract, and its distribution and toxicity are related to its diameter. In general, nano-TiO_2 with a larger diameter (80 nm) tends to accumulate in the liver, while those with a smaller diameter (25 nm) tends to disperse more widely and cause lesions in other organs [30,31]. Nano-TiO_2 showed hepatotoxicity in mouse experiments, accumulating in the liver and inducing hepatic lobular edema and hepatocyte necrosis [12,32,33]. In this study, it was also found that rats administered nano-TiO_2 orally had inflammatory cell infiltration in the hepatic lobule (Fig. S2, blue arrow), hepatocyte edema, and cell nucleus staining was paler than that of the control group (Fig. S2, orange arrow).

Nano-TiO_2, particularly rutile, is widely used in sunscreens, functional fibers and advanced plastics due to its strong ability to shield ultraviolet rays. We believe that the thyroid, as an endocrine organ with an abundant blood supply, has the potential to be exposed to nano-TiO_2. Previous evidence has shown that nano-TiO_2 can enter and distribute in the mammalian body through the digestive tract [12], but there have been no reports of its effects on the thyroid. This study found that oral administration of rutile nano-TiO_2 caused irregular follicular morphology, follicular epithelial cell desquamation, and inflammatory cell infiltration in the interfollicular matrix of rat thyroid. The ultrastructure of follicular epithelial cells showed a reduction in cell apical microvilli. The intracellular membrane structures such as mitochondria, rough endoplasmic reticulum, and Golgi apparatus had irregular shapes and blurred borders. The number of secretory granules was decreased. Biological detection showed that nano-TiO_2 caused a decrease in the level of NIS in rat thyroid gland, an increase in activated caspase-3, and an increase in inflammatory factors IL-1β and TNF-α. Serum FT4 (thyroxine) and TPO-Ab levels increased.

In vitro experiments showed that nano-TiO_2 reduced the survival rate of human thyroid follicular cells in a dose-dependent manner. Previous studies have shown that nanoparticles generate reactive oxygen species by interacting with cell membranes, causing oxidative stress to cells [34–38] and even apoptosis and necrosis [39–42]. The concentration of hydrogen peroxide in thyroid follicular cells is naturally high because hydrogen peroxide is necessary for thyroid cells to synthesize thyroid hormones. Under normal circumstances, thyroid follicular cells remove excess hydrogen peroxide through the powerful catalase (CAT) system, glutathione peroxidase (GPX) system, and peroxidase (Prx) system to prevent H2O2 from damaging the nuclear genome [43]. Therefore, we focused on detecting the effects of nano-TiO_2 on CAT, SOD, and GPX1 and their activities in thyroid follicular cells. The results showed that nano-TiO_2 significantly downregulated the levels of CAT, SOD, and GPX1 in a concentration-dependent manner and inhibited the

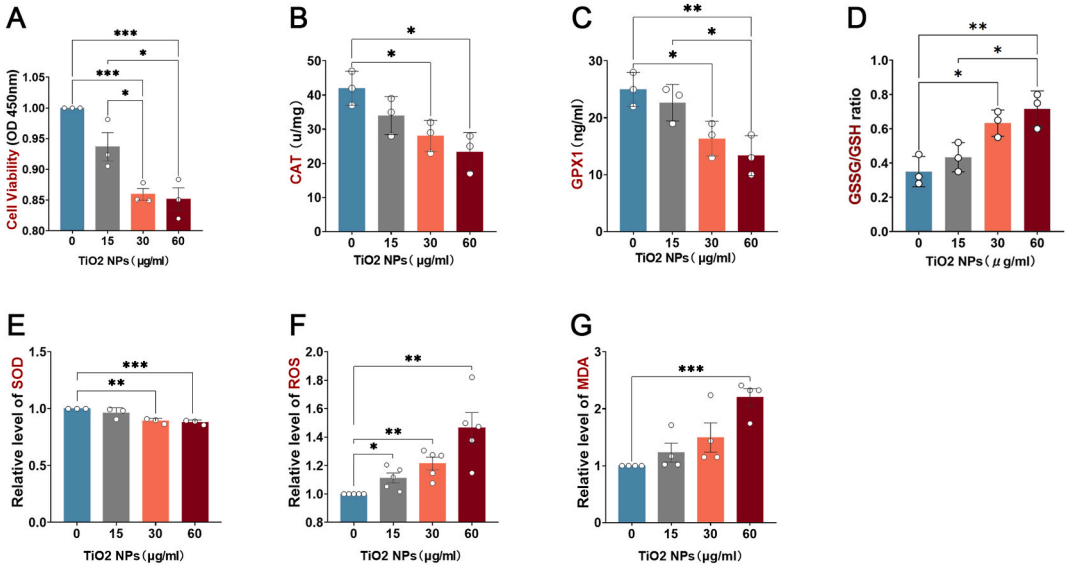

Fig. 6. Nano-TiO_2 inhibits the activity and antioxidant enzyme system of thyroid follicular cells, leading to oxidative stress. A. Human thyroid follicular epithelial cell line Nthy-ori 3-1 cells were cultured with nano-TiO_2 at concentrations of 0 μg/mL, 15 μg/mL, 30 μg/mL, and 60 μg/mL for 48 h. Cell viability was determined using the Cell Counting Kit-8 (CCK8). With the increase of nano-TiO_2 concentration, the viability of Nthy cells gradually decreased (n = 3). **B.** Nano-TiO_2 downregulated CAT (Catalase) level in thyroid follicular cells. **C.** Nano-TiO_2 downregulated GPX1 (glutathione peroxidase 1) level in thyroid follicular cells in a concentration-dependent manner. **D.** The GSSG/GSH ratio was increased in the presence of nano-TiO_2. **E.** The level of superoxide dismutase (SOD) in Nthy cells decreased when the concentration of nano-TiO_2 was 30 μg/ml and 60 μg/ml ($n = 3$). **F.** The level of reactive oxygen species (ROS) in Nthy cells increased with increasing concentration of nano-TiO_2 ($n = 5$). **G.** The level of malondialdehyde (MDA) increased significantly when the concentration of nano-TiO_2 was 60 μg/ml ($n = 4$). Data are expressed as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

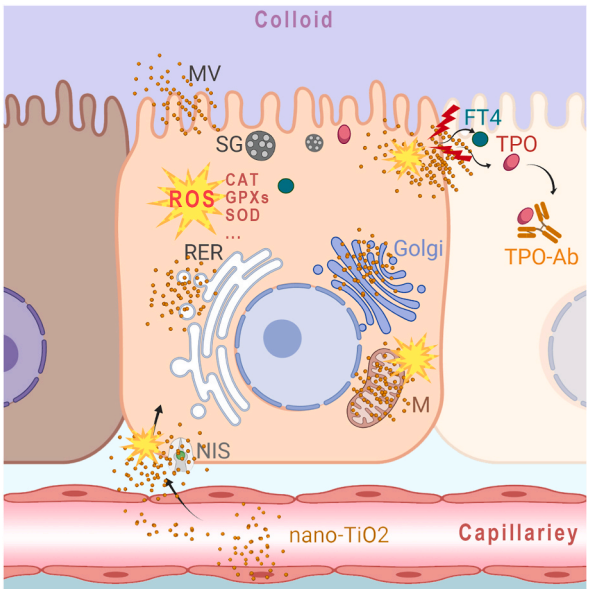

Fig. 7. The effect of nano-TiO_2 on thyroid follicular epithelial cells. Nano-TiO_2, which enters the body via the gastrointestinal tract, reaches thyroid follicular epithelial cells via the bloodstream. Rutile titanium dioxide nanoparticles cause oxidative stress to the membrane structure of thyroid follicular cells, the intracellular antioxidant enzyme systems (including CAT, GPXs and SOD, etc.) are inhibited, and the levels of oxidative stress markers (such as ROS and MDA) are increased. Oxidative stress causes damage to cell membranes, mitochondria, rough endoplasmic reticulum and microvilli. Intracellular FT4 and TPO are released outside the cell, resulting in increased serum FT4 and TPO-Ab.**Abbreviations**
ROS: reactive oxygen species, MDA: malondialdehyde, NIS: the sodium/iodide symporter, CAT: catalase, GPXs: glutathione peroxidase, SOD: superoxide dismutases, M: mitochondria, RER: rough endoplasmic reticulum, MV: microvilli, SG: secretory granule, Golgi: Golgi apparatus, FT4: free thyroxine, TPO: thyroid peroxidase, TPO-Ab: thyroid peroxidase antibody.

GSSG/GSH conversion rate. On the other hand, it upregulated the markers of oxidative stress, ROS and MDA. We speculate that oxidative stress is the cause of ultrastructural destruction of thyroid follicular epithelial cells, which in turn leads to cell dysfunction and cell death. Due to the destruction of follicular epithelial cells, thyroid autoantigens (such as TPO) are released into the peripheral blood, leading to an increase in thyroid autoantibody levels. As the follicular cavity collapses, thyroxine is released into the peripheral blood, resulting in an increase in serum FT4 (Fig. 7).

This study has several limitations. First, the mechanism by which nano-TiO_2 causes thyroid damage has not been fully elucidated. Second, we did not investigate potential protective agents of nanoparticles, such as antioxidants and mitochondrial/lysosomal protectants [44–48], for possible protection against nano-TiO_2-induced thyroid damage. However, despite these limitations, this study found that oral administration of nano-TiO_2 affects thyroid tissue structure and function, which is important for improving our understanding of endocrine disruptors in the environment. The hydrophilicity/hydrophobicity, surface charge, particle size, and surface modification of nanoparticles affect their interaction with organisms. Modification of these aspects could regulate the biotoxicity of nano-TiO_2 and even eliminate its toxicity.

5. Conclusions

This study demonstrates the effects of nano-TiO_2 exposure on thyroid structure and function in rodents. Long-term exposure to nano-TiO_2 may be a potential risk factor for thyroid dysfunction. Further studies should be conducted to clarify the thyroid toxicity of nano-TiO_2 and its mechanism.

Funding

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Data availability statement

All data presented in this study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Hong-Zhen Gong: Investigation. **Sha Li:** Investigation. **Fu-Yi Wang:** Investigation. **Ye Zhu:** Investigation. **Qi-Lan Jiang:** Investigation. **Xiao-Ling Zhu:** Investigation, Conceptualization. **Yang Zeng:** Validation, Project administration, Data curation. **Jun Jiang:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34722.

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