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Ultrastructural evaluation of oocyte envelopes of zebrafish (*Danio rerio*) (Hamilton, 1822) after TiO2 nanoparticle exposure

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Abstract: Titanium dioxide (TiO_2) is one of the most widely used nanoparticles, and aquatic organisms are especially exposed to it. To examine reproductive toxicology, zebrafish were exposed to different concentrations (1, 2 and 4 mg/L) of TiO_2 nanoparticles. The ultrastructure of the theca cell, zona radiata structure and follicular epithelium were examined in detail by transmission electron microscopy (TEM). No abnormalities were observed in the control group; however, degeneration of pore and microvilli structures of the zona radiata, vacuolization in the ooplasm, mitochondrial swelling and mitotic catastrophe (the mechanism for eliminating mitosis-incompetent cells in eukaryotes) were detected in the exposure groups. These results indicate that TiO_2 nanoparticle exposure causes paraptotic-type cell death in zebrafish oocytes, follicular and theca cells. In light of the observed histopathological changes, it was concluded that TiO_2 exposure inhibited oogenesis and the reproductive capability in zebrafish.

Key words: titanium dioxide (TiO_2) nanoparticle; ultrastructure; histopathology; oocyte; zebrafish

INTRODUCTION

Nanoparticles are known to be a potential hazard in terms of environmental health. There are uncertainties about the half-life, bioavailability and behavior of nanoparticles in aquatic environments. Nanoparticles can enter the body via the oral route, by breathing, by dermal penetration, and are then distributed in different tissues. After the nanoparticles enter the body, their biological distribution varies, depending on particle size and surface functionality [1]. Many nanoparticles are metal-based, such as nanosilver, gold nanoparticles, iron oxide, silicon dioxide and titanium dioxide (TiO_2). Metal-based nanoparticles have been extensively used in the pharmaceutical industry, medicine and military applications. Nanoparticles affect proteins and enzymes in mammalian cells and lead to the production of reactive oxygen species (ROS) [2]

TiO_2 is widely used in many products and applications, it is found in nature and is derived from titanium oxide. TiO_2 nanoparticles are generally described as non-toxic. Such a perception may have been formed by short-term studies of TiO_2 nanoparticles with cells and some organisms (epidermal and reproductive cells in pigs and zebrafish) [3,4]. TiO_2 is often used to reflect and diffuse light in sunscreens. It is also used in color-cosmetics and personal care products to achieve whiteness, increase product opacity and reduce transparency in some nutrients, detergents and in most medicines, including vitamins [4-6].

The zebrafish is an important vertebrate model and there are many advantages to working with this fish species. Because of its reproductive capacity, easy rearing and transparent embryos, it is frequently used in scientific studies. It has been also used as a vertebrate toxicity model and as an ecotoxicological test species to determine the effects of chemicals on fish survival, growth and reproduction.

Despite its obvious importance, studies on the reproductive toxicology of nanoparticles are limited [1,7]. This study was carried out with the aim of determining the effects of TiO_2 on the reproductive toxicology of zebrafish. In this study, the effect of TiO_2

on zebrafish ovary vitelline membranes and the fine structure of follicular cells was examined using transmission electron microscopy.

MATERIALS AND METHODS

Experimental Design

Adult zebrafish individuals were obtained from Sakarya University Aquaculture Lab., Esentepe, Turkey. The fish were raised in dechlorinated tap water and maintained as follows: 14 h light/10 h dark photoperiod, 28.5±1°C and 7.0±0.5 pH. They were fed with Artemia sp. and Tetra© Pro Energy (Tetra Werke, Germany) twice a day.

Exposure

The TiO_2 nanoparticles were obtained from Sigma Aldrich (CAS number 13463- 67-7), and 150-nm particle size TiO_2 nanoparticles were used. Zebrafish individuals were divided into four groups (n=15; one control and 3 experimental groups exposed to 1, 2 and 4 mg/L TiO_2). For investigating the effects of TiO_2, the fish were anesthetized with ice water and ovary tissues were dissected after 5 days of the exposure.

Preparation of samples and transmission electron microscopy (TEM)

Tissues were immediately fixed by immersion in 2.5% glutaraldehyde in a phosphate buffer for 24 h at 4°C. The specimens were washed in phosphate buffer for 1-3 h. After an additional fixation with 1% OsO_4 and pre-embedding staining with 1% uranyl acetate, the ovaries were dehydrated and embedded in Embed 812 resin. Tissues were stained with toluidine blue after they were sectioned using a Leica microtome. Semithin sections were examined under the light microscope to select the area of interest. Ultrathin sections were mounted on copper grids. Thin sections (0.5-0.7 μm) were counterstained with 1% uranyl acetate and lead citrate. They were examined under a transmission electron microscope (Jeol Jem, 1011) and photographs were taken.

RESULTS

Control group

Based on the examination of samples from the control group, the follicular membrane was comprised of two layers of the primary oocyte. The first layer was the oocyte neighboring follicle cell and the second layer was the theca cell. The follicular epithelium was based on the basal lamina. The follicle and theca cells were similar, with a flat nucleus, wide mitochondria and vesicular structure. Short microvilli between the follicular cell apex and the oocyte membrane were detected. An amorphous electron dense structure was observed between the microvilli and the neighboring oolemma (Fig. 1A). In the cortical alveolar phase, the follicular cell was flat-shaped. The follicular cells stood by the basal lamina. The theca layer was observed in the upper region of the basal lamina. While the cortical alveoli were forming, a thickening of the oocyte membrane was observed (Fig. 1B). At the initial stage of vitellogenesis, the theca cells did not change but the follicular cells expanded. The zona radiata of the vitellogenic oocyte had thickened and microvilli had cavities or porosities (Fig. 1C). The zona radiata was porous or had small channels through which microvilli extended and reached maximum thickness in the mature oocyte. We also detected that the intercellular space of the follicular epithelial cell contains low electron-dense material. These are thought to be vitellogenic protein precursors transported into the oocyte (Fig. 1D).

Exposure groups

In the experimental group treated with 1 mg/L TiO_2, deterioration of the integrity of the follicular cell cytoplasm, electron-dense regions in the cell nucleus, degeneration in the structure of basal lamina between the theca and follicular cells and structural deterioration in the theca cell were observed in primary oocytes (Fig. 2A). At this stage, the formation of edema in the follicular cell and formation of vacuolar spaces in the ooplasm were also observed (Fig. 2B). The follicle cells showed degeneration in the cortical alveolus stage. Openings were seen in the basal lamina. Structural integrity was not observed in the theca cell (Fig. 2C). Vacuolization was observed in the

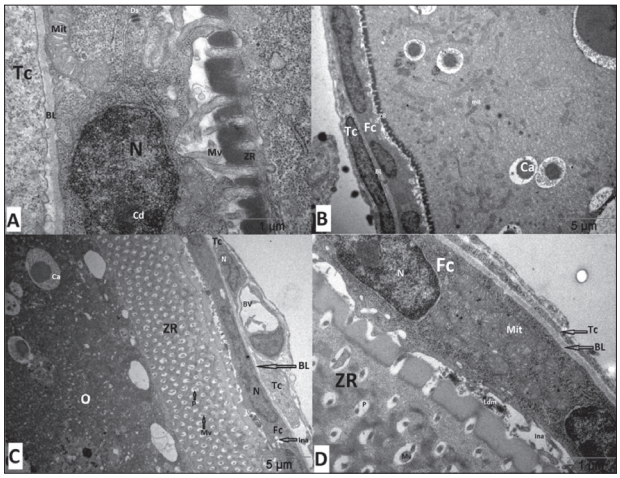

Fig. 1. Different stages of oocyte in the control group. **A** – Primary oocyte. **B** – Cortical alveolus stage oocyte. **C** – Vitellogenic oocyte. **D** – Mature oocyte. Tc: theca cell; Fc: follicular cell; BL: basal lamina; O: ooplasm; Ca: cortical alveoli; Mit: mitochondria; Ds: desmosome; N: nucleus; Cd: dense chromatin; Mv: microvilli; P: pore; ZR: zona radiate; BV: blood vessel; Ina: intercellular area; Ldm: low electron dense material; Ina: intercellular area.

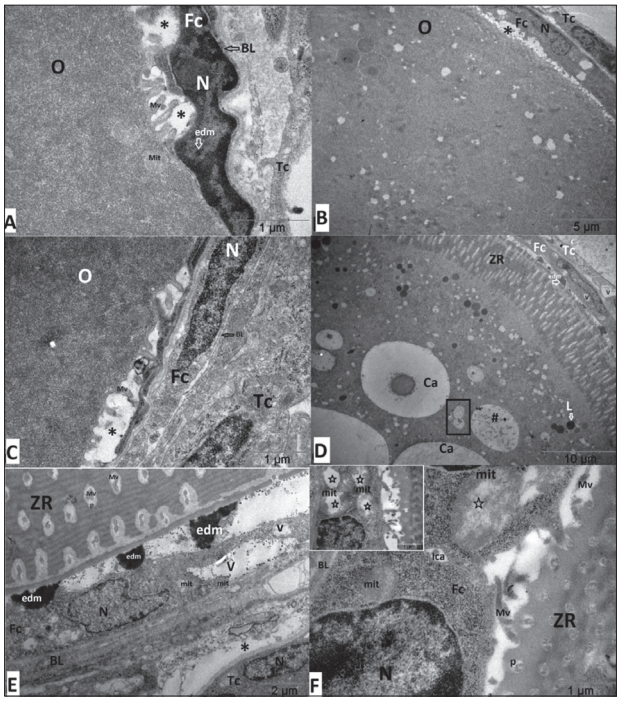

Fig. 2. One mg/L TiO_2 exposure group. Swelling at mitochondria, electron-dense materials, vacuolization at ooplasm and follicular cell, degeneration and coalescence at cortical alveoli were detected. **A-B** – Primary oocyte. **C** – cortical alveolus stage oocyte. **D** – Vitellogenic oocyte. **E-F** – Late vitellogenic oocyte. Tc: theca cell; Fc: follicular cell; BL: basal lamina; O: ooplasm; Ca: cortical alveoli; Mit: mitochondria; N: nucleus; Mv: microvilli; P: pore; ZR: zona radiate; Ica: intercellular area; L: lipid droplet; edm: electron-dense material; V: vacuolization; # – degeneration and drainage in cortical alveoli; rectangle: coalescence of cortical alveoli; * – edema (opening); star: swelling in mitochondrial cristae.

ooplasm in the early vitellogenic stage. The increase in cytoplasmic vacuolization was thought to be a sign of paraptotic cell death. Degeneration in the cortical alveoli within the ooplasm and drainage in the cortical alveoli were detected. Coalescence was observed in the drained cortical alveoli. Vacuolization was seen in the follicular and theca epithelium (Fig. 2D). In the late vitellogenic phase, electron-dense structures were observed in the perivitelline space between the zona radiata and the follicular epithelial cell. Deterioration of microvilli structures was noted. There was expansion in the intercellular space between the follicular epithelial cell and the theca cell (Fig. 2E). The structural integrity of the mitochondria in the follicle epidermis was found to be impaired. Swelling in mitochondrial cristae was detected (Fig. 2F).

In the experimental group treated with 2 mg/L TiO_2, the integrity of the follicular epithelium was impaired (Fig. 3A). In addition, a partial opening between the zona radiata and the follicle epithelium was detected (Fig. 3A, 3B). There is a regional gap in the basal lamina between the follicle epithelium and the theca cell (Fig. 3B). Vacuolization in the ooplasm and degeneration in the cortical alveoli were detected in the cortical alveolus stage oocyte. The structural integrity of microvilli was impaired. Vacuolization in the follicular epithelium was also detected (Fig. 3C). Vacuolization in the ooplasm was remarkable in the vitellogenic oocyte. There was an opening between the zona radiata and the follicular cell, and hollow vesicular structures were detected. Large vacuole formations (vacuolization) were seen in the follicular cell. Chromatin condensation in the nucleus of the follicular epithelium was detected (Fig. 3D). At this stage, myelin-like structures were observed in the perivitelline space. Hypertrophy of the mitochondria in the follicular epithelium with deteriorating structural integrity was detected. The opening of the zona radiata pores and deterioration of the structural integrity of the microvilli were displayed (Fig. 3E). Degeneration of the cytoplasmic integrity, chromatin condensation in the nucleus and the deterioration of mitochondrial structures were observed in the theca cell (Fig. 3F).

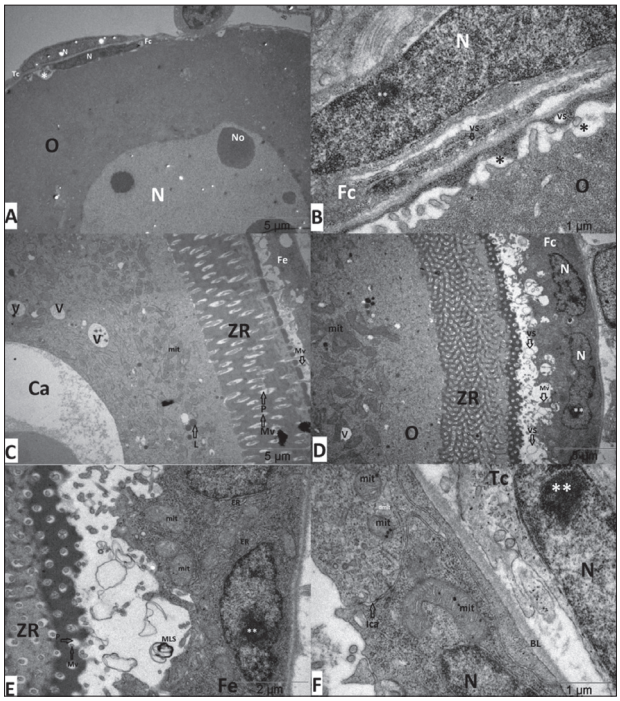

Fig 3. Two mg/L TiO_2 exposure group. Vacuolization at ooplasm and follicular cell, chromatin condensation at follicular and theca cell, degenerated microvilli, myelin like structures and hollow vesicle structures were observed. **A-B** – Primary oocyte. **C** – Cortical alveolus stage oocyte. **D-E-F** – Vitellogenic oocyte. Tc: theca cell; Fc: follicular cell; Fe: follicular epithelium; BL: basal lamina; O: ooplasm; Ca: cortical alveoli; Mit: mitochondria; N: nucleus; No: nucleolus; Mv: microvilli; P: pore; ZR: zona radiate; Ica: intercellular area; L: lipid droplet; ER: endoplasmic reticulum; V: vacuolization; dmit: degenerated mitochondria; MLS: myelin-like structure; vs: vesicle. * – edema (opening), ** – chromatin condensation.

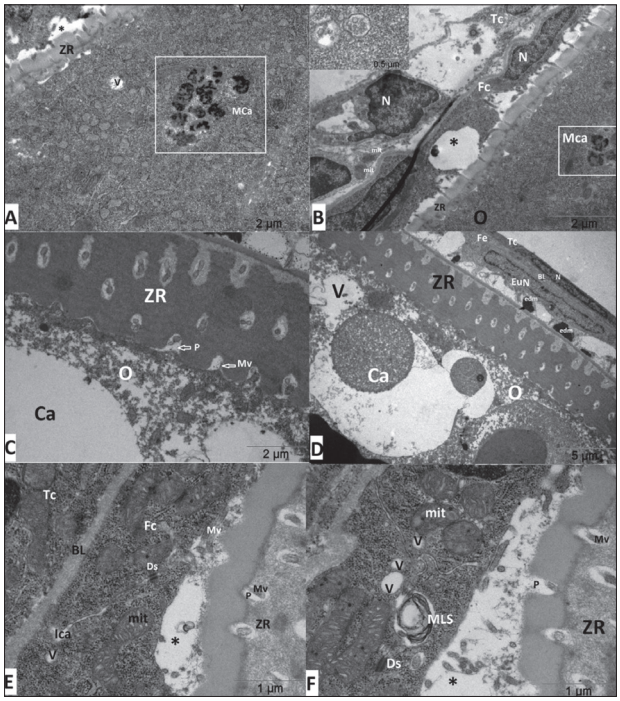

Fig. 4. Four mg/L TiO_2 exposure group. Severe vacuolization at follicular cell, degeneration of pore and microvilli structures in zona radiata, myelin like structure, mitotic catastrophe and dispersion at ooplasm were detected. A-B) Primary oocyte, C-D) Cortical alveolus stage oocyte E-F) Vitellogenic oocyte. Tc: theca cell, Fc: follicular cell, Fe: follicular epithelium O: ooplasm, Ca: cortical alveoli, mit: mitochondria, N: nucleus, EuN: euchromatic nucleus, Mv: microvilli, P: pore, ZR: zona radiata, BL: basal lamina, Ica: intercellular area, L: lipid droplet, V: vacuolization, MCa: mitotic catastrophe, edm: electron-dense material, Ds: desmosome, MLS: myelin-like structure. *edema (opening).

Mitotic catastrophe, a significant sign of paraptotic cell death, was observed in the ooplasm of the primary oocytes in the experimental group treated with 4 mg/L TiO_2 (Fig. 4A). A significant opening at the perivitelline space was observed between the zona radiata and the follicular epithelium. There was loss of contact between some neighboring follicular cells. Chromatin condensation was seen in the nucleus of the follicular epithelium. Degeneration of the integrity of theca cell, vacuolization in the cytoplasm, degeneration of mitochondria, increased chromatin-dense areas in the nucleus and impaired nuclear morphology were detected (Fig. 4B). Distortion of the pore structure and the microvilli of the zona radiata in the cortical alveoli of cortical alveolus stage oocyte was observed. Thawing and distortion of the ooplasm (Fig. 4C) and structural deterioration and fusion were observed. Vacuolization was observed in the ooplasm. Electron-dense structures were observed in the perivitelline space. euchromatic nuclei were detected in the follicular epithelium (Fig. 4D). Deterioration of zona radiata microvilli structures was observed in vitellogenic oocyte stage. The desmosome, which connects two adjacent follicular epithelia, was noted. Hypertrophy and deterioration of the structural integrity of mitochondrial cristae were detected in the follicular epithelial cell. Deterioration of the structural integrity of mitochondria in the theca cell was observed. (Fig. 4E). In addition, vacuolization and myelin-like structures were observed in the follicular epithelium (Fig. 4F).

DISCUSSION

Nanoparticles have different shapes, size and structure as mono- or multielements. Thanks to nanotechnology,

various products have acquired new functions and features, such as conductivity, high durability, corrosion protection, water and dirt retention, scratch resistance. The use of nanoparticles in electronics, cosmetics, food, sporting goods and the pharmaceutical industry has become widespread in recent years. The result of this widespread use is an accumulation of nanoparticles in the environment. Toxicology studies have shown that nanomaterials can produce side effects on the central nervous system, the immune system and the lungs [8-11]. In recent years, zebrafish has been used to evaluate reproductive toxicity [12,13], and in this study, TiO_2-treated zebrafish served as a model to investigate reproductive toxicology in female gametes.

Wang et al. [3] reported that 0.1 mg/L of TiO_2 treatment damaged the reproduction in female zebrafish. TiO_2 exposure (0.1 mg L^{-1} and 1.0 mg L^{-1}) resulted in a decrease in egg production. It has been proposed that TiO_2 exposure can cause degenerations in folliculogenesis, and according to this hypothesis, as a result of TiO_2 exposure, there is an increase in primary follicles and a decrease in other developing follicles. Also, according to Wang [3], TiO_2 affects the ovaries by way of blood circulation and directly affects oocyte development by acting on this organ. Our results are consistent with this study. We observed a decrease in the number of developing follicles with dose increase. Increased concentrations of TiO_2 were thought to inhibit follicles, reduce follicular quality and inhibit egg production. However, when TiO_2 nanoparticles (0.1 mg L^{-1} and 1.0 mg L^{-1}) were applied short-term to zebrafish in a study by Ramsden et al. [12], only a slight change in oocyte development was observed in female gonads. In a study by Tsukue et al. [14], TiO_2 was observed in ovarian cells in treated mice. Also, TiO_2 reduced fertility and caused changes in sex hormones.

Recent studies have shown that nanoparticles cause DNA damage in cells of the female reproductive system. Zhu et al. [15] reported that TiO_2 nanoparticles exhibit toxic effects in Chinese hamster ovary cells, resulting in the breakage of DNA strands. Kim et al. [16] also reported that gold nanoparticles induce micronucleus formation and DNA breakage in Chinese hamster ovary cells. Although experimental results show that TiO_2 particles can pass through the placenta into fetal tissue, it is not yet clear whether or not they cause reproductive and developmental toxicities in humans exposed to TiO_2 particles. However, it has been shown that in different species, including zebrafish, TiO_2 exposure can inhibit hatching and cause malformations [3,17]. Park and Yeo [18] reported that TiO_2 nanoparticles and nanotubes at 1 mg/L caused asymmetric regeneration of zebrafish fins, and that exposure to 20 mg/L caused apoptosis in zebrafish embryos.

Komatsu et al. [19] investigated the effects of TiO_2 nanoparticles in the mouse testis Leydig cell line. TiO_2 nanoparticles have been reported to accumulate in Leydig cell cytoplasm. According to Federici et al. [20], TiO_2 application caused the destruction of trout gill tissues. Mucus secretion and gill pathology have been reported to be the result of toxicological effects on gill tissue. Pinheiro et al. [11] investigated the possible effects of TiO_2 nanoparticles on *Daphnia magna* and *Lemna minor*. Significant amounts of TiO_2 accumulation in *D. magna* exposed to nanoparticles, as well as high mortality, were observed after exposure to high TiO_2 concentrations (>10 mg/L). Morphological changes have been reported in *L. minor* exposed to TiO_2 nanoparticles at concentrations above 25 mg/L. It has been stated that the exposure of organisms to TiO_2 nanoparticles could alter the physiology and affect population levels, creating a risk for aquatic ecosystems [11]. Prasad et al. [21] revealed that TiO_2 nanoparticles cause breaks in single and double strands of DNA, damage chromosomes and are proinflammatory.

According to Dayal et al. [1], the accumulation of gold nanoparticles causes significant changes in zebrafish ovaries: in the mature and vitellogenic oocytes, the zona radiata was separated from the oocyte membrane, but not in the primary and cortical alveolus stage oocytes; atretic oocytes were seen in vitellogenic and mature oocyte stages; cell surface irregularities were observed. In our study, the zebrafish ovary exposed to TiO_2 nanoparticles exhibited degeneration of the follicular epithelium, and an opening between the zona radiata and the follicular epithelium of primary, cortical and vitellogenic oocytes were observed with the increase in exposure dose. Tiedemann et al. [22] investigated the response of oocytes to BSA-coated gold nanoparticles (particles with a wide range of uses, including biomedical applications, pulse lasers, etc.). This study was performed *in vitro* and the particles were assimilated in a large number of oocytes but had no effect on oocytes.

Suganthi et al. [23] investigated the effect of three different doses of ZnO nanoparticles (ZnO powder has an extremely wide range of application, including the production of plastic, rubber, lubricants, food, pigments, cement, ceramics, etc.) on different tissues (brain, intestine, muscle, gill, ovaries). At the lowest dose (30 mg/L), the degeneration of late vitellogenic oocytes in ovary tissue and the vacuolization in their cytoplasm were detected. Necrosis in oocytes, degeneration in the late vitellogenic oocytes and vitellogenic fluid accumulation in the ovarian parenchyma were observed after exposure to the medium dose (50 mg/L). At the highest examined dose (70 mg/L), degeneration and vacuolization of oocytes was observed. Despite the use of different substances, similar results were obtained in our study. Our results are compatible with the described vacuolization and degeneration of developing oocytes. Hanna et al. [9] investigated the physiological effects of ZnO nanoparticles on the mussel species, *Mytilus galloprovincialis*. Zn accumulation varied with mussel size and Zn concentration, and after 12 weeks of treatment, the rate of respiration increased with ZnO concentration. Another study conducted with ZnO nanoparticles has examined the subacute toxicity and accumulation of ZnO nanoparticles in carp (*Cyprinus carpio*) [10]. The authors found that after 30 days of exposure to 50 mg/L ZnO nanoparticles, significant accumulation in different tissues was detected. The authors also reported that exposure to 50 mg/L ZnO nanoparticles resulted in higher intracellular oxidative stress and more severe histological changes than exposure to ZnO_2 at the same concentration. The toxic mechanism of ZnO nanoparticles appears to be associated with increased intracellular stress.

Rather et al. [24] investigated the effect of chitosan (a linear polysaccharide with a broad use in medicine, agriculture and wine production) nanoparticles on carp reproduction. Histologic examinations revealed that empty follicular structures were formed within the ovary tissue. Chen et al. [25] reported that silver nanoparticles (used in biosensors, chemical sensors, cosmetics industry, medicine and the paint industry) induce oocyte maturation in zebrafish, promote apoptosis in follicular cells surrounding the oocyte, induce germinal vesicle breakdown, decrease cAMP concentration and cause apoptosis of ovarian follicle cells.

Paraptosis has been characterized by chromatin condensation, cytoplasmic vacuolization with many small vacuoles created in the cytoplasm, the widening of the perinuclear space, mitochondrial vesiculation and mitochondrial swelling [26,27]. Our findings are consistent with these results. We observed degeneration and edema of follicular cells, the appearance of openings in the basal lamina, disintegration and vacuolization of the ooplasm, follicular and theca cells and deterioration of pore and microvilli structures of the zona radiata. Two of the most important findings were mitochondrial swelling and mitotic catastrophe, which signify paraptotic cell death. Thus, this study shows that TiO_2 nanoparticle exposure causes paraptosis of oocytes that could affect the female reproductive function and process by affecting oogenesis and follicle maturation, leading to histopathological effects on oocyte cells in the zebrafish ovary. These results are conclusive evidence that TiO_2 can negatively affect reproduction in zebrafish.

CONCLUSION

Several studies have indicated that TiO_2 nanoparticles can cause deformations in different tissues of organisms. In this study, we analyzed the ultrastructure of the zona radiata, follicular and theca cells after TiO_2 exposure in zebrafish oocytes. Severe degeneration was detected in the microvilli and the pore structure of the zona radiata. Disintegration and chromatin condensation were observed in both follicular and theca cells. Mitochondrial structures were degenerated and the ooplasm underwent vacuolization. Mitotic catastrophe was detected. These findings suggest that TiO_2 seriously impacts the ultrastructure of follicular and theca cells and leads to paraptotic cell death in oocytes, follicular cells and theca cells. Our test system appears to be an effective tool for the detection of the impacts of nanoparticles, including TiO_2.

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Conflict of interest disclosure: The authors declare that there is no conflict of interest.

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