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Exposure of Zebrafish (*Danio rerio*) to Titanium Dioxide Nanoparticle Causes Paraptosis: Evaluation of Ovarian Follicle Ultrastructure

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Authors' Contribution
NDY designed the experiments and supervised the work. CA and BÖ performed light microscopic studies. TK performed electron microscopic studied. CA wrote the article.

Key words
Nanoparticle, Cell death, Titanium dioxide, *Danio rerio*, Ovarian follicle paraptosis.

ABSTRACT

Titanium dioxide (TiO_2) is widely used nanoparticle all over the world. In this study, we have investigated the histopathological effect on zebrafish ovaries after exposure to TiO_2 nanoparticles. Adult zebrafish individuals were exposed to 1, 2 and 4 mg/L TiO_2 for 5 days, and then their ovaries were evaluated using light and transmission electron microscopy. Numerous degenerated follicles with cytoplasmic vacuolization, mitotic catastrophe in mitochondria , chromatin condensation, mitochondrial vesiculation and dispersion at ooplasm were observed. In mitochondria, mitotic catastrophe , vesiculation, swelling and loss of organization of cristae were detected. Here we showed that TiO_2 exposure trigger paraptotic type cell death in zebrafish ovary.

INTRODUCTION

Titanium dioxide (TiO_2) is one of the most commonly used nanoparticles. They are used in a range of products including sun screen, cosmetics, coatings, paint, plastics, papers, inks, medicine, medicines, pharmaceuticals, food products, toothpaste and building materials (Wolf *et al*., 2003; Kaida *et al*., 2004; Aitken *et al*., 2006; Wang *et al*., 2007). United States Environmental Protection Agency (USEPA) estimated the annual production of TiO_2 nanoparticles (nano-TiO_2) to be 2000 metric tons in around 2005, with 65% of this production used in products such as cosmetics and sunscreen lotions (USEPA, 2009). TiO_2 is generally not considered to be obvious toxic (Park *et al*., 2006). It is photocatalytic and has the potential to produce cumulative cellular damage (Bar-Ilan *et al*., 2013). It has been explored for use in water treatment to destroy chemicals such as polychlorinated biphenyls (PCBs), pesticides, and other complex organic contaminants. The conventional sized TiO_2 is considered to be physiologically inert and poses little risk to humans, and can be used as food additives (colorants). However, when TiO_2 is made at the nanoscale (particle size <100 nm), its biological and environmental effects deserve our emerging attention (Chen *et al*., 2011). It is inevitable for TiO_2 NPs to aggregate in water due to its strong interparticle self absorption properties. Dispersed NPs which resulted in toxic effects on the growth of zebrafish (Ispas *et al*., 2009). Aquatic organisms can also exposed nanoparticles via food chain. Zhu *et al*. (2010) provide that nanoscale TiO_2 particles ($nTiO_2$) can transfer from *Daphnia magna* to *Danio rerio* by dietary exposure.

A new type programmed cell death, paraptosis has been recently identified. Paraptosis, type III programmed cell death is morphlogically different from apoptosis and necrosis. Paraptosis, *has been characterized by chromatin condensation, cytoplasmic vacuolization,* numerous small vacuoles created in the cytoplasm, widened perinuclear space, and mitochondrial vesiculation and mitochondrial swelling (Sperandio *et al*., 2000; Danaila *et al*., 2013). Differently from apoptosis, features such as membrane blebbing and chromatin condensation aren't seen in paraptosis. Also, this non-apoptotic programmed cell death doesn't involve apoptotic markers such as caspase activation, apoptotic body formation (Sperandio *et al*., 2000). In paraptosis like programmed cell death mitotic catastrophe is seen (Bröker *et al*., 2005; Caruso *et al*., 2011).

In the current study, the toxic effects of TiO_2 nanoparticles (<150 nm particle size) were evaluated with

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light and transmission electron microscopy at zebrafish ovary tissue. Zebrafish is a well known vertebrate model for reproduction and development studies. The zebrafish's hardiness makes them excellent stress test subjects, as they can survive fairly severe environmental changes without succumbing, surviving long enough to show developmental defects.

MATERIALS AND METHODS

Experimental design

Adult, one year old, zebrafish individuals were obtained from Sakarya University Aquaculture Lab., Esentepe, Turkey were raised in a computer-controlled incubation chamber at 14 h light/10 h dark photoperiod, 28.5 ± 1°C temperature, 7.0 ± 0.5 pH and 61% humidity. They were fed with *Artemia* sp. TetraMin© Hauptfutter (Tetra Werke, Germany) twice a day. After one week of adaptation period zebrafish were divided into four groups (n=15), one control and 3 experimental groups for 1, 2 and 4 mg/L TiO_2 treatment. For investigating the effects of TiO_2, fishes were anesthesized with ice water and ovary tissues were dissected after 5 day of the exposure.

Titanium dioxide nanoparticle (<150 nm particle size) was obtained from Sigma Aldrich CAS number 13463-67-7.

Histological sgtudies

The ovaries were fixed in neutral formaldehyde for 24 h. After fixation, tissues were dehydrated in ascending concentrations of ethanol, equilibrated in xylene. The tissues were then embedded in paraffin wax and cut into 5-7 µm sections on a Leica microtome. The sections were mounted on glass slides and stained with hematoxylin and eosin before examination under a Olympus light microscope.

Electron microscopy

For transmission electron microscopy, ovarian tissues were fixed by immersion in a solution containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 4 h. The ovaries were fixed further overnight at 4°C using 2% glutaraldeahyde in 0.1 M phosphate buffer at pH 7.4. After an additional fixation with 1% OsO_4 and pre-embedding staining with 1% uranyl acetate, ovaries were dehydrated and embedded in Embed 812 resin. The sectioning was performed using a Leica Ultracut ultramicrotome. Thick sections were stained with tolouidine blue and visualized in a Olympus light microscope to select the area of interest. There after, thin sections were collected and counterstained with 1% uranyl acetate and lead citrate and examined with Jeol transmission electron microscope.

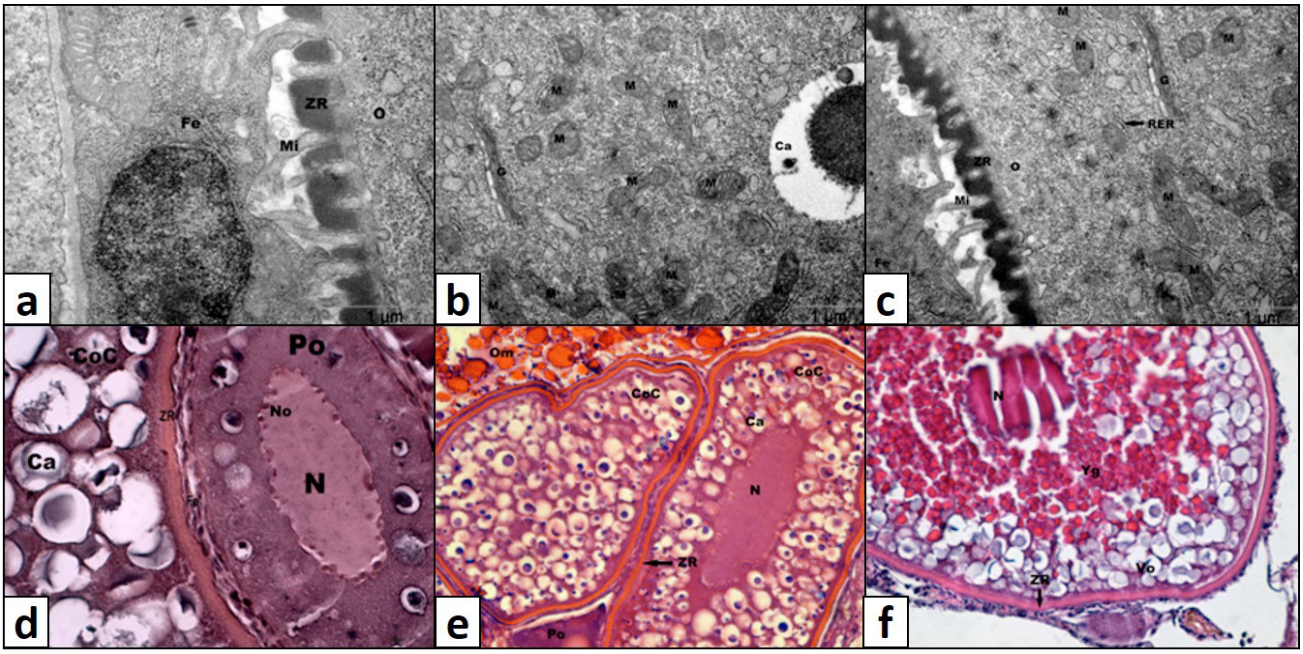

Fig. 1. Hıstological and electron micrographic structure of ovary of control group; a,b,c, Electron micrograph of follicles; d,e,f, light micrograph of follicules. Fe, follicular epithelium. Mi, microvilli; ZR, zona radiata; O, ooplasm; M, mitochondrium; G, golgi; RER, rough endoplasmic reticulum; Po, primary oocyte; Ca, cortical alveoli; N, nucleus; No, nucleolus; CoC, cortical alveolus stage; Vo, vitellogenic oocyte; Om, mature oocyte; Yg, yolk granule. Magnification: d, x100; e, x20; f, x40.

RESULTS

Zebrafish ovary is an extremely dynamic organ in which the follicles undergo asynchronous development. The development of zebrafish oocytes is divided into four stages, based on morphological features (Koç *et al*., 2008).

Control group

In control group, normal ovarian histology was observed. All development stages were monitored. In primary growth phase multiple nucleoli were observed at the germinal vesicle of oocytes (Fig. 1d). In cortical alveoli stage, oocytes were identified due to growing cortical alveoli surrounding the nucleus (Figs. 1b, 1d, 1e). In this stage, increase in size of the oocytes were seen. Zona radiata structure began to emerge. Zona radiata and follicular epithelium structures were clearly monitored (Figs. 1a, 1c, 1d). During vitellogenic stage, the oocytes began to increase in size, due to accumulation of yolk. Zona radiata structure was thicker (Fig. 1f). In mature oocytes, the nucleus was dissolved and the ooplasm which consists of yolk bodies were also monitored (Fig. 1e).

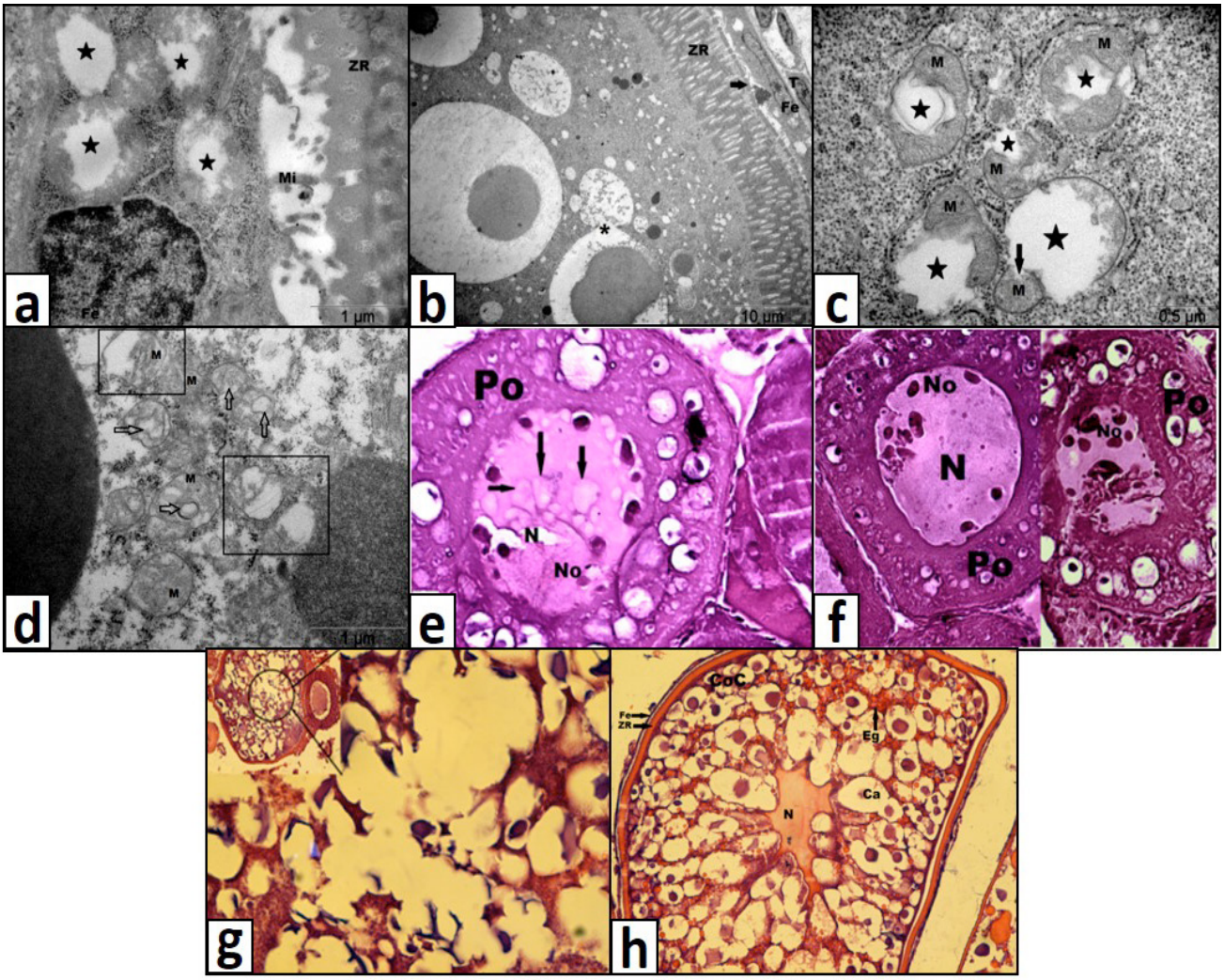

Fig. 2. Hıstological and electron micrographic structure of ovary of 1 mg/L TiO_2 exposed group; a, degeneration at microvili structure and mitochondrial deterioration (star) at follicular epithelium cells; b, cytoplasmic vacuolization and unification (asterix) of cortical alveoli; c, mitochondrial deterioration (star); d, mitochondrial swelling (square) and loss of organization of cristae (arrow) at mitochondrium; e, cytoplasmic vacuolization (black arrow) at primary oocytes; f, loss of nucleolus organization at primary oocyte; g, severe cytoplasmic vacuolization and degeneration at cortical alveoli structure; h, Eosinophilic granules accumulation at cortical alveolus stage ZR, zona radiata; Mi, microvilli; Fe, follicular epitheium; T, teca cells; M, mitochondrium,;N, nucleus; No, nucleolus; Ca, cortical alveoli; Eg, eosinophilic granule. Magnification: e, f,g, x100; h x40.

1mg/L TiO_2 exposed group

Degeneration and structural changes at mitochondria in both follicular epithelium and ooplasm were monitored (Fig. 2a, 2c, 2d). Deterioration of the integrity of mitochondria was observed (Fig. 2a, 2c, 2d). In mitochondria, swelling and loss of organization of cristae were detected (Fig. 2d). Deterioration at microvilli structure of zona radiata were also established (Fig. 2a). Numerous cytoplasmic vacuoles in ooplasm of the follicles have been identified (Fig. 2b, 2e). In follicles, cytoplasmic vacuolization which is one of the sign of paraptotic cell death were observed (Fig. 2b, 2g). In cortical alveolus stage, unification at cortical alveoli were monitored (Fig. 2b, 2g) and eosinophilic granues were detected (Fig. 2h). Nucleolus organization were lost at primary oocytes (Fig. 2f).

2 mg/L TiO_2 exposed group

Besides cytoplasmic vacuolization, opening between zona radiata and follicular epithelium were monitored (Fig. 3a). Chromatin condensation at follicular epithelium (Fig. 3a) and degenerated microvilli were also detected (Fig. 3b). Mitochondrial swelling (Fig. 3c) and loss of organization of cristae were observed (Fig. 3c, 3d). Oocytes were begining to shrink (Fig. 3e, 3h). In cortical alveolus stage, vacuolization were severe (Fig. 3f) and many vacuoles were monitored under zona radiata layer (Fig. 3e). In vitellogenic oocyte, unification at cortical alveoli were detected (Fig. 3g).

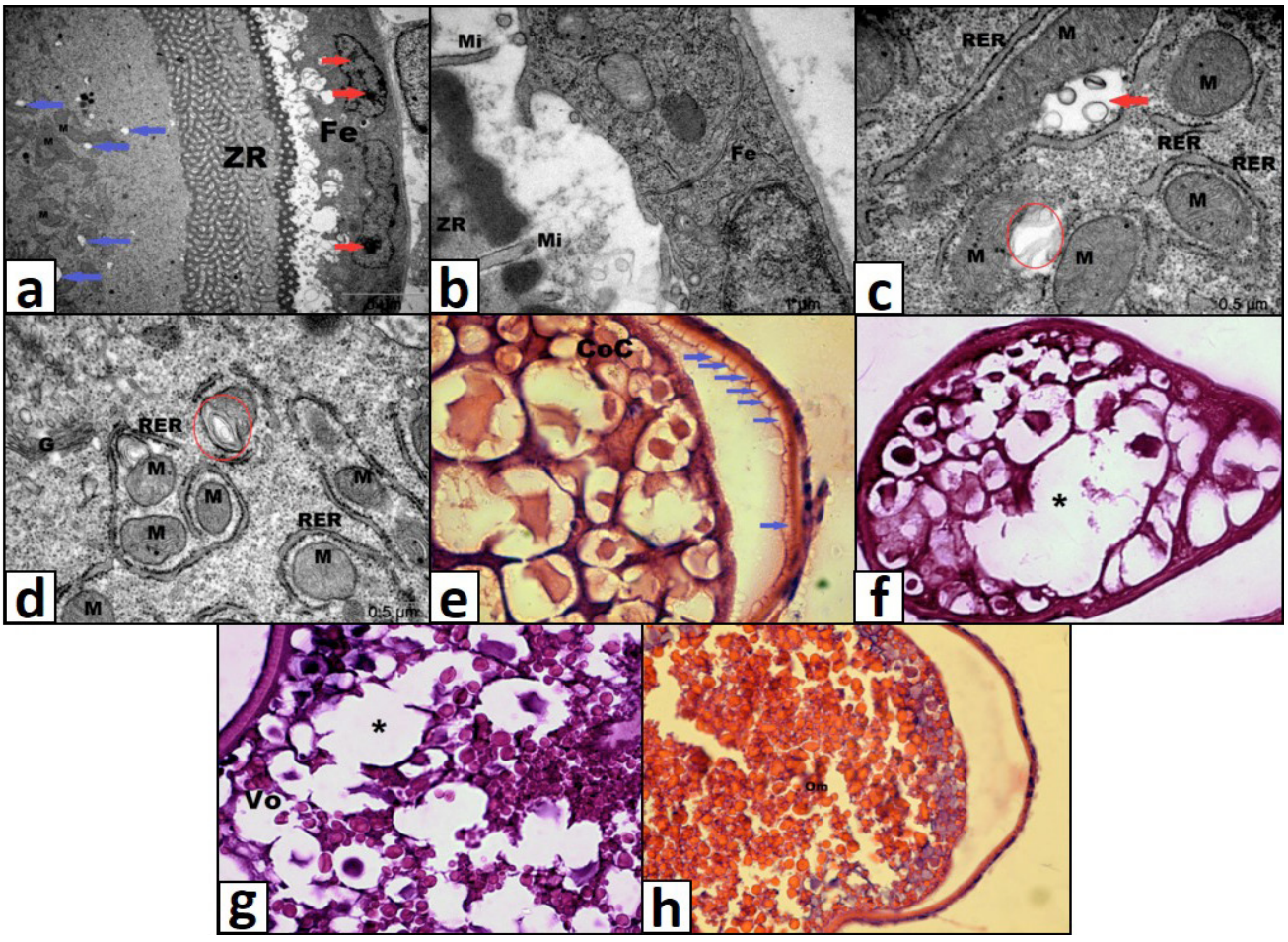

Fig. 3. Light microscopic and electron micrographic structure of ovary of 2 mg/L TiO_2 exposed group. A, chromatin condensation (red arrow) and cytoplasmic vacuolization (blue arrow); b, opening between zona radiata and follicular epithelium and degeneration at microvili structure; c, mitochondrial swelling (red arrow) and loss of organization of cristae (circle); d, loss of organization of cristae (circle); e, vacuolization under zona radiata (blue arrows); f, cytoplasmic vacuolization; g, unification of cortical alveoli (asterix); h, shrinking of mature oocyte. ZR, zona radiata; Fe, follicular epithelium; Mi, microvilli; M, mitochondria; G, golgi; RER, rough endoplasmic reticulum; CoC, cortical alveolus stage; Vo, vitellogenic oocyte; Om, mature oocyte. Magnification: e, f, g) x40, h) 20 x.

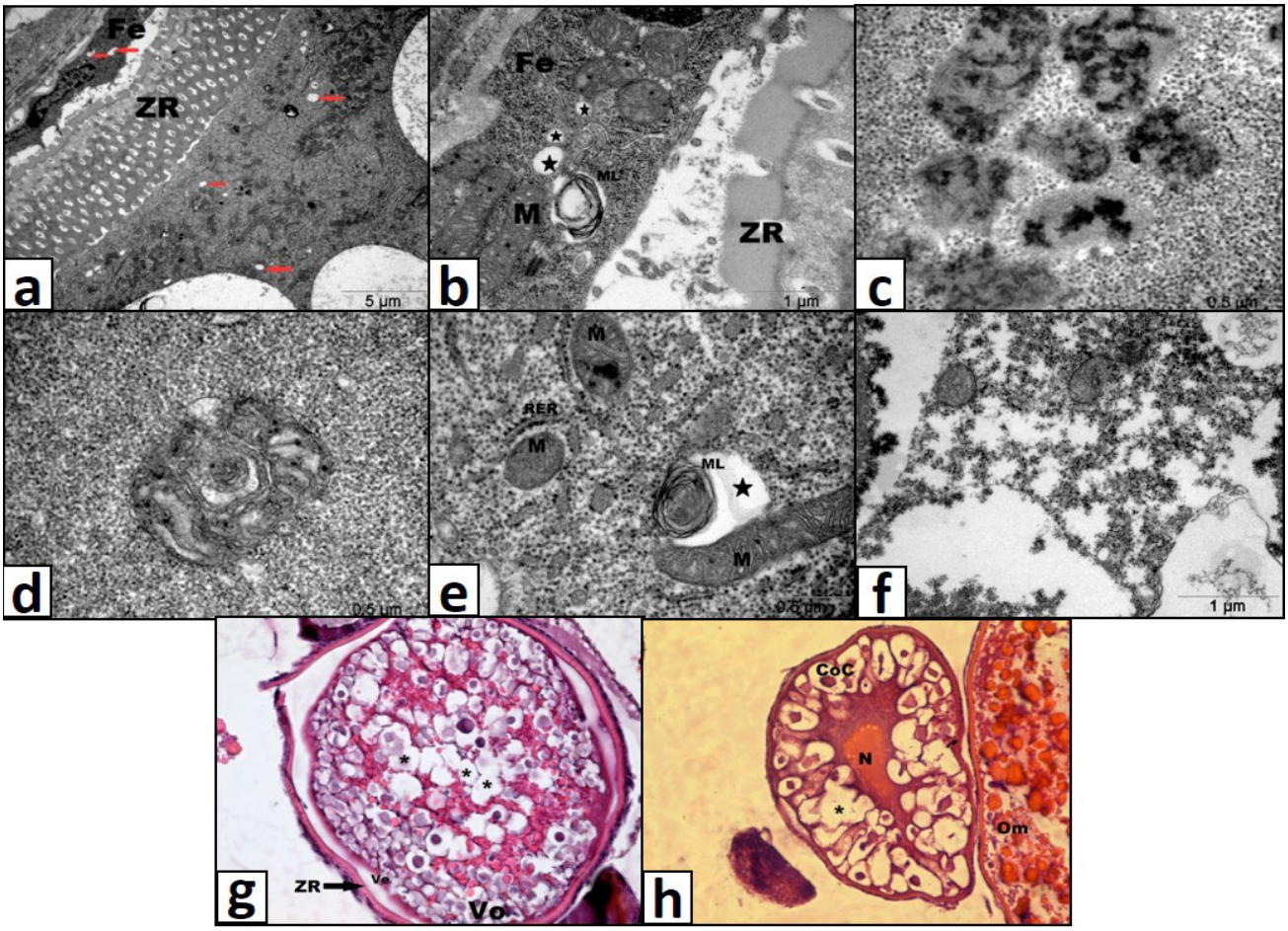

Fig. 4. Light microscopical histological and electron micrographic strucure of ovary of 4 mg/L TiO_2 exposed group. A, cytoplasmic vacuolization (red arrow) at follicular epithelium and ooplasm; b, multilamellar vesicle and cytoplasmic vacuolization (star); c, mitotic catastrophe; d, altered mitochondrium morphology and loss of cristae organization; e, cytoplasmic vacuolization (star) and multilamellar vesicle; f, dispersion at ooplasm; g, degenerated vitellogenic oocyte; h, unification of cortical alveoli (asterix). ZR, zona radiata; Fe, follicular epithelium; M, mitochondrium; ML, multilamellar vesicle; RER, rough endoplasmic reticulum; Ve, vitelline envelope; Vo, vitellogenic oocyte; N, nucleus; CoC, cortical alveolus stage; Om, mature oocyte. Magnification: g, h, x20.

4 mg/L TiO_2 exposed group

Degeneration and vacuolization at follicular epithelium were observed (Fig. 4a, 4b). Opening between zona radiata and follicular epithelium was also seen at this group too (Fig. 4a, 4b). Microvilli structure were degenerated (Fig. 4b). Multilamellar vesicle formation were detected at ooplasm and follicular epithelium cytoplasm (Fig. 4b, 4e). Cytoplasmic vacuolization were monitored at both follicular epithelium and ooplasm (Fig. 4a, 4b, 4e, 4g, 4h). Disrupted mitochondria were established. Mitotic catastrophe, the signature characteristic of paraptosis were monitored at mitochondria (Fig. 4c). Altered morphology and loss of organization of cristae were detected at mitochondrium (Fig. 4d). In cortical alveolus stage, dispersion at ooplasm were monitored (Fig. 4f). Unification at the cortical alveoli structure were detected at follicles (Fig. 4g, 4h).

DISCUSSION

Nanoparticles are potential aquatic pollutants and entering waterways. They also have risks human health via water and aquatic systems (Moore, 2006). Evaluation the toxicological effects of these materials has a great importance in aquatic organisms.

Nanoparticle exposure cause histopathological changes in tissues. Chen *et al*. (2011) evaluated the effects of TiO_2 nanoparticles on growth and some histological parameters of zebrafish after a long term exposure (2-6 months). They detected distinct nanoparticle accumulation

and morphological alternation at gill. They monitored a hyperplasia-like thickening of the primary lamellae in gill filaments.

Nanoparticle exposure aslo inhibit reproduction. In particular TiO_2 nanoparticles have been used in crop production, dietary supplements, food additives, food packaging components, medicine, toothpastes, sunscreens, cosmetics, and waste water treatment. This widespread use of TiO_2 nanoparticles has inevitably led to harmful biological responses in humans and animals (Zhao *et al*., 2014). Yön and Akbulut (2014) conducted a study about histopathological effects of bisphenol A on zebrafish ovary and they showed bisphenol A slowed down oogenesis in zebrafish. Similarly, Wang *et al*. (2011) investigated chronic exposure effects of TiO_2 nanoparticles on zebrafish reproduction. They used female individuals and provided that chronic exposure of zebrafish to 0.1 mg/L TiO_2, can significantly impair zebrafish female reproduction. They found 29.5% reduction in the cumulative number of zebrafish eggs after 13 weeks of $nTiO_2$ exposure. The distribution of follicular developmental stages was skewed by the TiO_2 treatment toward the immature stage, as evident by an increase in stage I follicles and some reduction in other stages, especially, stage IV follicles. Distinctively we investigated histological and ultrastructural effects of TiO_2 nanoparticle. We proved that TiO_2 nanoparticle exposure inhibit oogenesis via causing cell death, which is also corroborated by Wang *et al*. (2011).

Zhu *et al*. (2008) examined developmental and toxic effects of TiO_2 exposure for 96 h at 1-500 mg/L concentration on zebrafish embryo and larvae. They proved that neither $nanoTiO_2$ nor TiO_2 bulk showed any toxicity to zebrafish embryos and larvae. Toxic response may be different in embryo. This response may be due to three-layered acellular envelope, called chorion which protects embryo before hatching.

Many studies have shown that TiO_2 treatment cause cell death in many cells. Cell death in human bronchial epithelial cells induced by titanium dioxide exposure was proved by Chen *et al*. (2008). Nanoparticle aggregates also cause oxidative stress in zebrafish embryos. Faria *et al*. (2014) exhibited TiO_2 aggregates impaired embryo growth and generated oxidative stress in the absence of solar simulated radiation. Cell death can be trigger by oxidative stress formation. Disruption of antioxidant defense mechanisms and generation of reactive oxygen species by cytoplasmic degeneration and nuclear destruction were reported on hepatocytes treated by TiO_2 nanoparticles (Alarifi *et al*., 2013).

Several studies indicated that TiO_2 has direct effects on ovarian functions. In a study that investigation the effects of TiO_2 on rat ovarium showed mitochondrial swelling and loss of cristae, condensation of nuclear material and apoptosis (Wang *et al*., 2011). These results are consistent with our study.

CONCLUSION

In this study we found TiO_2 nanoparticle exposure inhibit oogenesis and cause cellular cell death in zebrafish. Paraptosis is a programmed cell death type which has been recently defined. There are few information about it and many the details haven't been illuminated. As far as known about paraptosis we can say that our data are exactly match feature of paraptosis and TiO_2 exposure cause paraptosis type cell death in zebrafish ovaries.

Statement of conflict of interest

Authors have declared no conflict of interest.

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