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Silver nanoparticles induce oocyte maturation in zebrafish (*Danio rerio*)

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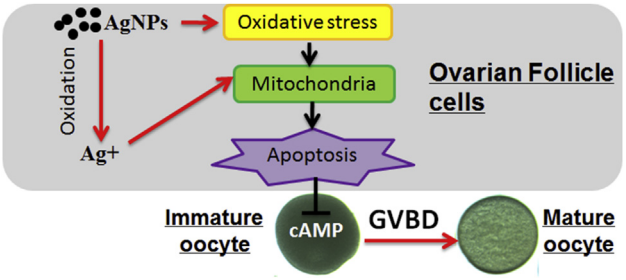
HIGHLIGHTS

- Both AgNPs and $AgNO_3$ unexpectedly induced zebrafish oocyte maturation *in vitro*.
- Both AgNPs and $AgNO_3$ reduced total cAMP concentration in zebrafish ovarian follicles.
- Both AgNPs and $AgNO_3$ induced apoptosis in the follicle cells surrounding the oocyte.
- H_2O_2 induced oocytes maturation by induction of apoptosis in the follicle cells.
- AgNP-induced apoptosis of the follicle cells may mainly due to the oxidative stress.

GRAPHICAL ABSTRACT



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ABSTRACT

Public concern regarding silver nanoparticles (AgNPs) in the environment has been increasing since they can cause adverse effects in some aquatic species. However, few data are actually available on the effects of AgNPs on the germ cells. In the present study, we used the zebrafish ovarian follicle as a model to assess the potentially adverse effects of AgNPs on oocyte maturation (germinal vesicle breakdown, GVBD) *in vitro*. Similar to the maturation inducing hormone (17α, 20β-dihydroxy-4-pregnen-3-one), AgNPs induced GVBD, and reduced the total cyclic adenosine monophosphate (cAMP) concentration in zebrafish ovarian follicles. The results from transmission electron microscope observation and Hoechst 33342 staining clearly indicated that AgNPs induced apoptosis in ovarian follicle cells surrounding the oocyte. Similar to AgNPs, $AgNO_3$ also induced GVBD, decreased cAMP concentration and induced apoptosis of ovarian follicle cells. However, the results from gene expression analysis showed that transcript levels of oxidative stress related genes were more sensitive to AgNPs than $AgNO_3$. Further more, H_2O_2 has an ability to induce zebrafish oocytes maturation by induction of apoptosis in ovarian follicle cells. Taken together, the results from our study indicated that oxidative stress appeared to be one

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of important mechanisms in AgNP induced apoptosis in ovarian follicle cells, which further triggered the GVBD.

1. Introduction

Nanomaterials are increasingly produced and utilized in a wide range of commercial products worldwide because of their novel and unique physicochemical properties that differ substantially from bulk materials of the same composition (Ahamed et al., 2010). Among various nanomaterials, silver nanoparticles (AgNPs), which have good antimicrobial efficacy against bacteria, viruses and other eukaryotic micro-organisms, are utilized in a wide variety of consumer products ranging from disinfecting medical devices and home appliances to water treatment (Poitras et al., 2015). Release of AgNPs into aquatic environments, has raised concerns on the potential adverse effects of AgNPs on human health and the environment (Benn and Westerhoff, 2008).

Aquatic organisms are susceptible to waterborne nanoparticles. Studies show that AgNPs are toxic to aquatic organisms including zebrafish (Yeo and Kang, 2008; Griffitt et al., 2009), perch (Bilberg et al., 2010), brown trout (Scown et al., 2010), *Ceriodaphnia* (Gao et al., 2009), *Daphnia pulex* (Griffitt et al., 2008) and algae (*Thalassiosira weissflogii*) (Miao et al., 2009). In addition to aquatic organisms, AgNPs may also pose a risk to human health. Conventional silver metals and silver salts, which have been used hitherto in silver-impregnated dressings and pharmaceuticals, are not thought to be toxic to the immune, cardiovascular, nervous or reproductive systems when they are used in reasonable amounts (Drake and Hazelwood, 2005). However, it is reported that AgNPs are highly toxic to various cultured mammalian cells (Braydich-Stolle et al., 2005; Hussain et al., 2006; Wen et al., 2007; Kawata et al., 2009), which indicates a potential toxicity of AgNPs to humans at the cellular and molecular level. Despite substantial information on the toxicity of AgNPs, studies concerning their potential effects on reproductive physiology are comparatively scarce, especially in gametogenesis which plays a vital role in ontogenesis (Gandolfi and Brevini, 2010). A study using a mouse spermatogonial stem cell line demonstrates a concentration-dependent cytotoxicity of AgNPs, whereas the corresponding soluble salts have no significant effect (Braydich-Stolle et al., 2005). One recent study shows that prepubertal male Wistar rat exposure to AgNPs causes alterations in adult sperm parameters (Mathias et al., 2015). Another study using an *in vitro* assay for porcine gametes shows that alloy nanoparticles with 80% silver molar fraction and pure AgNPs inhibit cumulus-oocyte maturation which implies that released Ag^+ is responsible for the observed toxicity (Tiedemann et al., 2014). Although AgNPs have been shown to have adverse effects on gametogenesis, there is still a knowledge gap in the evaluation of their reproductive toxicity, especially in vertebrate oogenesis. In addition, molecular mechanisms for the toxicity of AgNPs have not yet been reported.

Zebrafish (*Danio rerio*), a genetic and developmental model, is widely used to evaluate the toxic potential of environmental contaminants (Segner, 2009). As in other vertebrates, the principal events responsible for the enormous growth of teleost oocytes are due essentially to the accumulation of yolk proteins within their cytoplasm (Lubzens et al., 2010). After the oocyte completes its growth, it becomes ready for maturation (the resumption of meiosis), which is accompanied by several maturation processes, e.g. breakdown of the germinal vesicle (GVBD) (Nagahama and Yamashita, 2008). Another obvious character during the maturation process is that the oocyte becomes transparent as a result of disassembly of the internal crystalline structures (Nagahama and Yamashita, 2008). The maturation-inducing hormone, 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), induces oocyte maturation *in vitro* in most fishes (Nagahama and Yamashita, 2008). Therefore, our study aimed to assess the potential adverse effects of AgNPs during oocyte maturation *in vitro*. We further evaluated whether the toxicity of AgNPs is related to release of Ag^+ from the AgNPs, or specific effects of AgNPs. The molecular mechanisms whereby AgNPs and Ag^+ affect zebrafish oocyte maturation were also studied.

2. Materials and methods

2.1. Preparation and characterization of AgNP suspensions

Based on the method of (Choi et al., 2010b), AgNPs were synthesized from $AgNO_3$ by appropriate mixing with $NaBH_4$ and polyvinyl alcohol (PVA). In brief, 5 mL of 14 mM $NaBH_4$ was added into 90 mL of 0.06% (wt) PVA solution. Afterwards, 5 mL of 14 mM $AgNO_3$ was injected slowly at a rate of one drop per second at room temperature, and mixed immediately using a magnetic stirrer at 700 rpm in the dark. The synthesized AgNPs were then concentrated and purified with centrifugal ultrafiltration (Millipore, Amicon Ultra-15 3 K, USA), and rinsed twice with Milli-Q water (Millipore, 18.2 M cm, USA). The characterization of the synthesized AgNPs was determined as described by (Sun et al., 2013). The morphology and particle size of these AgNPs were determined using transmission electron microscopy (TEM, Hitachi H-7650, Japan) and analyzed with Image J (http://imagej.nih.gov/ij/), as shown in Supplemental Fig. S1, and its size ranged from 30 to 55 nm. The AgNP stock suspensions were quantified using inductively coupled plasma optical emission spectrometry (PerkinElmer Optima 7000 DV, USA) after nitric acid digestion, and the concentration was 120 ± 7 μg/mL. All test suspensions were prepared by dilution of the ultrasonically dispersed AgNPs (10 min at 4 °C) in Cortland medium to the desired concentration and then added to the culture medium. In order to exclude potential effects from PVA, follicle enclosed oocytes were also cultured in Cortland medium containing PVA at a concentration the same as that in AgNP stock suspensions. In order to distinguish whether possible effects are caused by the nanoparticle as such or by Ag^+ ions released from the nanoparticles, oocytes were also co-incubated with silver nitrate ($AgNO_3$). In addition, the released Ag^+ from AgNPs in the Cortland medium was evaluated via inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx, USA) after ultrafiltration using Amicon centrifugal ultrafilter devices (Millipore 3 kDa, USA) according to the previous study (Liu and Hurt, 2010).

2.2. Zebrafish stock

The experimental fish were Tüebingen strain, which were housed in the zebrafish facility (ESSEN, China), maintained under constant temperature (28 °C), photoperiod (14 L:10D, lights on at 08:00), salinity (conductivity 500–1200 μS), and pH (7.2–7.6). The fish were fed three times per day with commercial tropical fish food (Otohime B2, Reed Mariculture, CA, USA), using standard

conditions for this species (Westerfield, 2000). Experimental protocols were approved by the Institutional Animal Care and Use Committee of Xiamen University.

2.3. Isolation of ovarian follicles

Gravid female zebrafish were euthanized in melting ice and humanely sacrificed by severing the spinal cord following deep anesthetization with 0.01% tricaine methanesulfonate (Sigma-Aldrich, China). The ovaries were removed and washed several times in Cortland medium (pH 7.7) containing (weight in g/L): NaCl, 7.25; KCl, 0.38; $CaCl_2 \cdot 2H_2O$, 0.26; $NaHCO_3$, 1.0; Na_2HPO_4, 0.46; $MgCl_2 \cdot 6H_2O$, 0.23; $MgSO_4 \cdot 7H_2O$, 0.23; bovine serum albumen, 1; Hepes, 5.20; penicillin, 0.03; and streptomycin, 0.05 (Pang and Ge, 2002). Individual ovarian follicles enclosing fully-grown immature oocytes ($\varnothing$ = 550–650 μm) were carefully separated using fine forceps and blades without damaging ovarian follicle cell layers. The diameters of the ovarian follicles were measured with an ocular micrometer under a dissecting microscope. Appropriately sized and healthy oocytes were selected and pooled for each experiment. The selected oocytes were pre-incubated in culture medium at 28 °C for 2 h. After pre-incubation, only oocytes showing normal morphology were randomly transferred to 24-well plates (NEST, USA) for further exposure treatments.

2.4. Assay of in vitro ovarian follicle incubation and oocyte maturation

To examine the effect of AgNPs or $AgNO_3$ on oocyte maturation, groups of 30 ovarian follicles were placed in each well and incubated with 2 mL Cortland medium containing different reagents. All incubation was conducted in triplicate for 4 h at 28 °C. To assess the maturation processes, germinal vesicles were examined under a binocular microscope after placing the follicles in clearing solution, or the GVBD was assessed by scoring the oocytes that became transparent (Pang and Ge, 2002).

2.5. Cyclic adenosine monophosphate (cAMP) measurement

In order to examine the effect of AgNPs or $AgNO_3$ on the cAMP concentration of ovarian follicles, the follicles were treated with 30 μg/mL AgNPs or 10 μg/mL $AgNO_3$ for 2 h. Control and DHP (10 ng/mL) treated groups were also examined at the same time. Groups of 50–60 ovarian follicles were placed in each well. Samples were collected and analyzed with a cAMP EIA kit, purchased from the Caymen Chemical Company (Ann Arbor, MI), following the manufacturer's instructions.

2.6. TEM

For TEM investigation, ovarian follicles treated with 30 μg/mL AgNPs or 10 μg/mL $AgNO_3$ for 2 h were carefully collected and immediately fixed (for 1 h at 4 °C) using 2.5% glutaraldehyde (phosphate buffered preparation). After fixation, the tissue was rinsed three times (15 min each time) with 0.1 M phosphate buffer, post-fixed with 1% OsO4 fixative for 2–3 h, dehydrated through an ethanol series, and embedded via propylene oxide in Taab epoxy resin (Taab Ltd, Aldermaston, UK). Thereafter, 60–80 nm thick sections of the fixed tissue were cut and double-stained with 3% uranyl acetate, followed by lead citrate, and then examined using TEM (JEM2100HC, JEOL Ltd, Tokyo, Japan).

2.7. Hoechst 33342 staining

The changes of nuclear chromatin morphology are indicative of apoptosis and can be detected using fluorescent microscopy with Hoechst 33342 (1 μg/mL) fluorescent staining. In addition to control and DHP (10 ng/mL) treatments, ovarian follicles were treated with 10 and 30 μg/mL AgNPs or 10 μg/mL $AgNO_3$. The follicles were collected after 1, 2, 3 and 4 h treatment, and incubated with Hoechst 33342 in 2 mL buffer containing 10 mM Hepes/KOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$ for 10 min at 4 °C. Morphological changes of the nuclear chromatin were observed under a fluorescence microscope (M165 FC, Leica Microsystems, Germany).

2.8. Gene expression analysis

In addition to the control and DHP (10 ng/mL) treatments, groups of 30 ovarian follicles were treated with AgNPs (10 μg/mL) or $AgNO_3$ (1 μg/mL) for 2 h. Total RNA was extracted from the ovarian follicle samples using RNAzol reagent (MRC, Cincinnati, OH, USA). The same amount of total RNA (2.0 μg) was used for synthesis of the first strand cDNAs using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and following the manufacturer's instructions. The relative expression levels of *caspase3*, *caspase9, gst2 (glutathione S transferase pi 2), hsp70 (heat shock protein 70), mt2 (metallothionein 2)* and house-keeping-gene *ß-actin* were determined using real-time quantitative PCR (qPCR) and gene-specific primers (shown in Supplemental, Table 1S), which have been examined for their specificity and amplification efficiency on serial dilutions of respective target gene plasmid DNA (1×10^2–1×10^7 copies/μL). The qPCR was performed in a 20 μL reaction on a 7500 FAST real-time PCR detection system (Applied Biosystems, USA) using default setting. Copies of *β-actin*, which showed no significant difference among different stages (data not shown), were used as an internal control. The relative mRNA levels of the target genes were determined using the comparative Ct method (Schmittgen and Livak, 2008).

2.9. Statistical analysis

All data were presented as means ± standard error of the mean. Student's t-test was conducted to determine any significant differences between two groups. The analyses were performed using the GraphPad Prism 5 software package (GraphPad Software, San Diego, CA).

3. Results

3.1. Effects of AgNPs and Ag^+ on oocyte maturation

Neither AgNPs nor Ag^+ inhibited DHP-induced GVBD (data not shown), but similar to DHP, both significantly induced GVBD (Fig. 1). As control, PVA had no effects on the ovarian follicles. At a concentration of 1 μg/mL, AgNPs had some effects on GVBD, but 1 μg/mL $AgNO_3$ exhibited a significant induction effect on GVBD. However, at high dosage groups (30 and 50 μg/mL), AgNPs showed a stronger induction effect on oocyte maturation than $AgNO_3$ (Fig. 2A). Besides, some ovarian follicles exhibited abnormal morphology in response to AgNPs or $AgNO_3$ (as shown in Supplemental Fig. S2). Significant increases of abnormal rates in response to AgNPs or $AgNO_3$ required at least 30 μg/mL or 1 μg/mL (Fig. 2B). Moreover, at the same dosage, $AgNO_3$ induced higher rates of abnormal oocytes than AgNPs. These results indicated that $AgNO_3$ was more cytotoxic than AgNPs to the oocyte. It is worth noting that, when 1 μg/mL AgNPs were spiked into the culture medium, the released Ag^+ (data not shown) was below method detection limit (0.5 ng/mL). When 10 and 30 μg/mL AgNPs were spiked into the culture medium, concentration of dissolved silver had a sharp rise in a short time (8–15 ng/mL), and then gradually

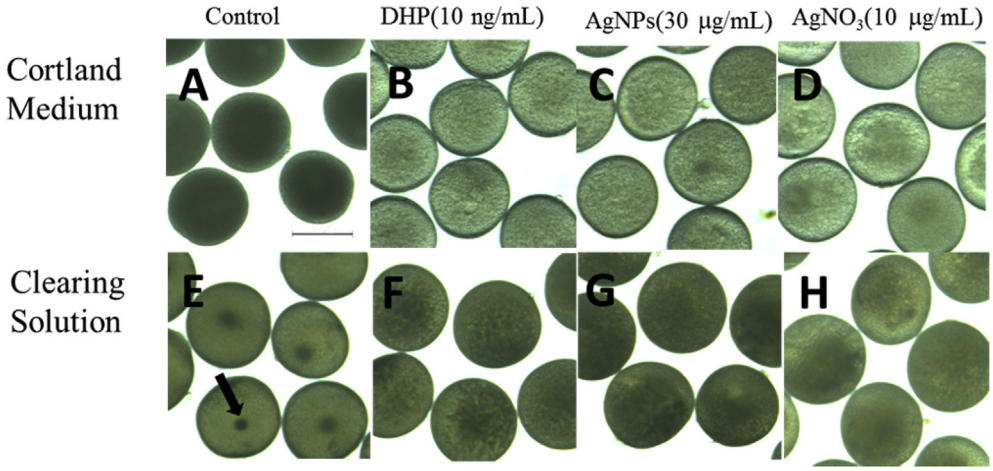

Fig. 1. Silver nanoparticles (AgNPs) or $AgNO_3$ induce oocyte maturation in zebrafish. Ovarian follicles enclosing fully-grown immature oocytes remain opaque in the control group (A) whereas they become transparent after the exposure of DHP (B), AgNPs (C) or $AgNO_3$ (D). After clearing solution treatment, germinal vesicles are observed at the center of oocytes in the control group (E), whereas they disappear after the treatment of DHP (F), AgNPs (G) or $AgNO_3$ (H). The arrow indicates the germinal vesicle. Scale bar: 500 μm.

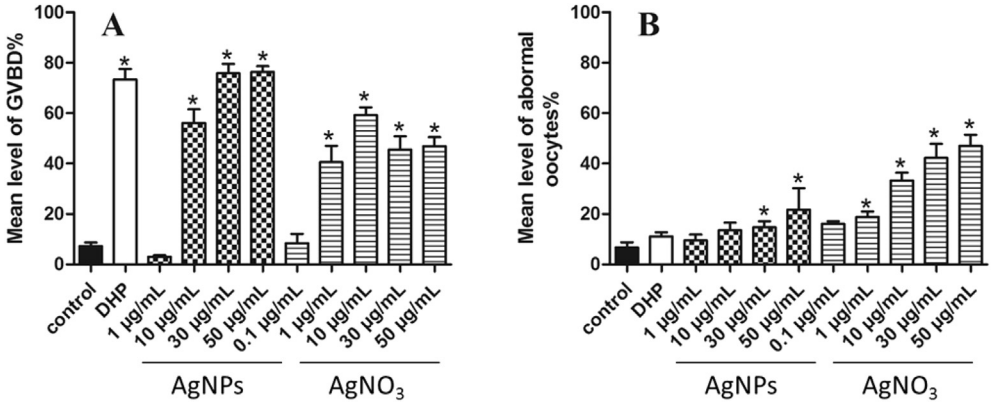

Fig. 2. Silver nanoparticles (AgNPs) or $AgNO_3$ induce oocyte maturation and abnormal development of zebrafish oocytes. Ovarian follicles enclosing fully-grown immature oocytes were incubated with Cortland medium only (control), the medium containing DHP (10 ng/mL), or various concentrations of AgNPs or $AgNO_3$. Each treatment group contains at least 30 oocytes. Percentages of oocytes undergoing germinal vesicle breakdown, or those with abnormal appearance were determined after a 4 h exposure. Experiments were repeated six times, and the data are expressed as average of six replicates (mean ± SE, n = 6). Bars marked with asterisk are significantly different from the control group ($p < 0.05$, Student's *t*-test).

reached stable (Fig. S3). Because the highest GVBD rates were obtained at 30 μg/mL AgNPs or 10 μg/mL $AgNO_3$, the subsequent experiments were performed at these concentrations unless otherwise indicated.

3.2. AgNPs or Ag^+ reduced cAMP concentration in oocytes

It is well established that a high concentration of cAMP in fish oocytes is essential for maintaining meiotic arrest, and treatment with maturation inducing hormone causes a decrease in adenylyl cyclase activity (Pace and Thomas, 2005). Therefore, to determine whether AgNPs or $AgNO_3$ may act via the same mechanism to induce oocyte maturation, we further investigated the effects of AgNPs and $AgNO_3$ on cAMP concentration in treated oocytes. As expected, oocytes treated with DHP (10 ng/mL) caused a significant decrease in cAMP production compared to the control. Similarly, oocytes treated with 30 μg/mL AgNPs, or 10 μg/mL $AgNO_3$ also resulted in a significant decrease in cAMP concentration (Fig. 3). These results clearly indicated that AgNPs or $AgNO_3$ induced oocyte maturation in the zebrafish by decreasing the cAMP concentration.

3.3. Effects of AgNPs or Ag^+ on ultrastructure of ovarian follicle cells

In vertebrates, cAMP generated in ovarian follicle cells can enter the oocyte via heterologous gap junctions connecting follicle cells and the oocyte. Therefore, we further determined whether AgNPs or $AgNO_3$ had cytotoxic effects on the follicle cells. We first examined the ultrastructure of the ovarian follicle. As shown in Fig. 4A, the zebrafish oocytes as in other vertebrates are surrounded by ovarian follicle cells layers that include two major cell layers, an outer thecal cell layer and an inner granulosa cell layer. However, after exposure to AgNPs (30 μg/mL) and $AgNO_3$ (10 μg/mL), both thecal and granulosa cells revealed irregular cell morphology, and exhibited disorganization in the cytoplasm, nuclear condensation and fragmentation (Fig. 4B and C). In addition, the follicle cells of the $AgNO_3$ treatment group showed acute vacuolation, and the mitochondria swelled but had an intact inner mitochondrial membrane (Fig. 4D). These results suggested that ovarian follicle cells underwent apoptosis.

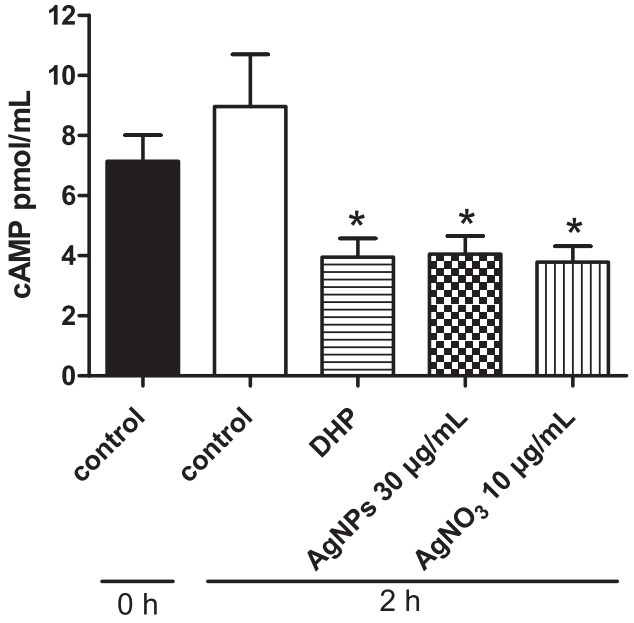

Fig. 3. Silver nanoparticles (AgNPs) or $AgNO_3$ inhibit cAMP production in zebrafish ovarian follicles. The concentrations of cAMP were determined from a pooled extraction of 50–60 ovarian follicles enclosing fully-grown immature oocytes that were incubated with Cortland medium (control), the medium containing DHP (10 ng/mL), AgNPs (30 μg/mL), or $AgNO_3$ (10 μg/mL) for 2 h. The experiment was repeated six times and data was expressed as the average of six replicates (mean ± SE, n = 6). Bars marked with asterisk are significantly different from the control group ($p < 0.05$, Student's *t*-test).

3.4. Effects of AgNPs and Ag^+ on induction of apoptosis in ovarian follicle cells

In order to further confirm that both AgNPs and $AgNO_3$ induced apoptosis in ovarian follicle cells, we used Hoechst 33342 to reveal that ovarian follicle cells surrounding the oocyte had undergone apoptosis. Within 3 h exposure in both control and DHP (10 ng/mL) groups, most of the follicle cells appeared to be intact and of oval shape and their nuclei were stained with a weak blue fluorescence, and only a few of the follicles showed apoptosis features, such as a spherical bead shape and bright blue fluorescence (Fig. 5A and B). In contrast, a vast majority of the follicle cells underwent apoptosis after 3 h exposure to AgNPs at 10 μg/mL (Fig. 5C), 1 h exposure to AgNPs at 30 μg/mL (Fig. 5D), and 1 h exposure to $AgNO_3$ at 10 μg/mL (Fig. 5E).

3.5. Effects of AgNPs and Ag^+ exposure on gene expression in ovarian follicles

To elucidate the molecular mechanisms of AgNPs (10 μg/mL) and $AgNO_3$ (1 μg/mL) induced apoptosis of follicle cells, the expression of several candidate genes relevant to oxidative stress (*gst2, hsp70* and *mt2*) and apoptosis (*caspase3*, *caspase9)* were determined using qPCR. The results showed that DHP had no effects on the expression of all tested genes. The AgNPs and $AgNO_3$ significantly induced the upregulation of transcript levels of *caspase9* and *caspase3*, respectively (Fig. 6A and B). A significant increase in *gst2* transcript level was observed only in the AgNPs treatment group (Fig. 6C). The most sensitive gene to silver was *hsp70*, the expression of which increased ~5 and ~300 fold in response to AgNPs and $AgNO_3$ (Fig. 6D). Similarly, the expression levels of *mt2* transcripts increased significantly in response to AgNPs or $AgNO_3$ treatment (Fig. 6E).

3.6. H_2O_2 induced oocytes maturation by induction of apoptosis in ovarian follicle cells

To confirm that the oxidative stress appeared to be an important mechanism in AgNPs inducing apoptosis of ovarian follicle cells, we further examine the effects of H_2O_2 on zebrafish oocytes maturation. The results showed that H_2O_2 exhibited a significant induction effect on the oocytes maturation, although the GVBD rates of H_2O_2 groups were significantly lower than that of DHP, AgNPs and $AgNO_3$ groups (Fig. 7A). Besides, Hoechst 33342 staining revealed that ovarian follicle cells surrounding the oocyte had undergone apoptosis (Fig. 7B, C, D).

4. Discussion

The use of engineered nanoparticles has risen exponentially over the last decade. However, our understanding concerning the possible adverse effects of nanoparticles is still incomplete. The potential disrupting effects of environmental contaminants on germ cells have received significant attention, especially regarding the possible disrupting effects which may not only affect the person or animal directly exposed to the environmental contaminants but can also be passed on to the following generations (Diamanti-Kandarakis et al., 2009). However, toxicological testing of nanoparticles on germ cells has so far been frequently neglected.

In fish, fully grown oocytes are arrested at the prophase of the first meiotic division. When such oocytes are cultured in medium supplemented with DHP, oocyte maturation (also named as GVBD) is induced in *vitro* in a wide variety of teleosts (Nagahama, 1994). Using this standardized *in vitro* system, in a small pilot study, we examined whether AgNPs would affect the process of GVBD induced by DHP. After 4 h co-incubation with AgNPs (50 μg/mL) and DHP (10 ng/mL), the results showed that AgNPs has no inhibitive effects on zebrafish oocyte maturation (data not shown). However, we observed a significant increase of oocyte maturation rate in the AgNPs treatment group.

So far, few studies have been published concerning the impact of nanoparticles on ovarian follicle. Hou et al. (2009). examined the effect of titanium dioxide nanoparticles on ovarian follicle development and oocyte maturation of rats. Hsieh et al. (2009). studied the influence of CdSe-core quantum dots on oocyte maturation, fertilization, and subsequent pre- and post-implantation development in the mouse. One recent study investigated the reprotoxicity of gold, silver and gold–silver alloy nanoparticles on porcine cumulus-oocyte-complexes (Tiedemann et al., 2014). These studies report inhibitive effects of the respective nanoparticles on oocyte maturation, which are different from the results observed in our study. In order to explain this result, we examined the effects of AgNPs on the cAMP concentrations in the ovarian follicle, because a decrease of cAMP concentrations in oocytes of vertebrates is required for the resumption of meiosis (Dekel and Beers, 1978; Pace and Thomas, 2005; Nagahama and Yamashita, 2008). Our results showed that, similar to DHP, AgNPs also induced a reduction of total cAMP concentrations in zebrafish ovarian follicles, which likely led to meiosis resumption and oocyte maturation. Furthermore, results from TEM and Hoechst 33342 staining suggested that AgNPs induced apoptosis of ovarian follicle cells. Previous studies in zebrafish and carp (*Cyprinus carpio*) have shown that removal of ovarian follicle layer led to a striking increase in spontaneous GVBD (Pang and Thomas, 2010; Majumder et al., 2015). In teleost, the inhibitory influence of ovarian follicle cells on GVBD is mediated, at least partly, through increases in cAMP concentrations via estrogen produced by the ovarian follicle cells (Pang et al., 2008; Pang and Thomas, 2010; Majumder et al., 2015). It is possible that apoptosis of the ovarian follicle cells induced by the AgNPs resulted in

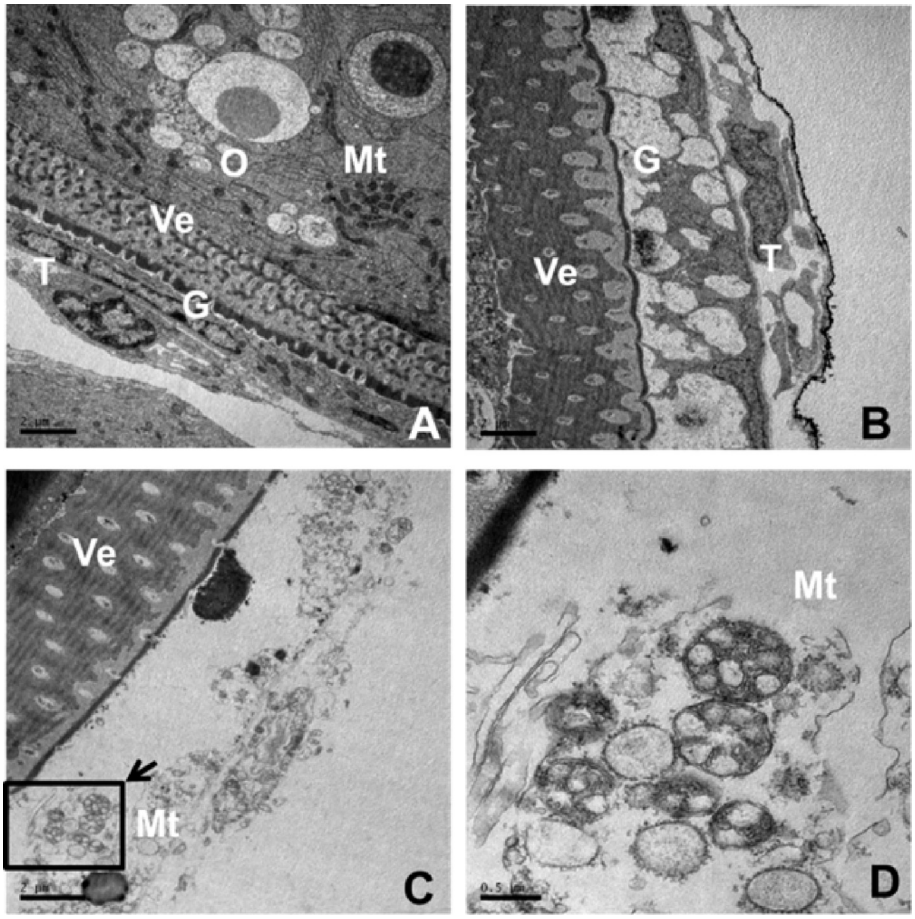

Fig. 4. Effects of silver nanoparticles (AgNPs) or $AgNO_3$ on ultrastructure of ovarian follicle cells. Ovarian follicles enclosing fully-grown immature oocytes were incubated with Cortland medium containing designed reagents for 2 h. (A), control group showing normal appearance of ovarian follicle cells (Scale bar, 2 μm); (B) AgNPs (30 μg/mL) and (C) $AgNO_3$ (10 μg/mL) treated group showing ovarian follicle cells have irregular cell morphology, acute vacuolation, nuclear condensation and fragmentation (Scale bar, 2 μm); (D) a high magnification image of the area indicated in C showing a novel mitochondrial swelling with intact inner mitochondrial membrane (Scale bar, 0.5 μm). G: granulosa, Mt: mitochondria, O: oocyte, T: thecal cell, Ve: vitelline envelope.

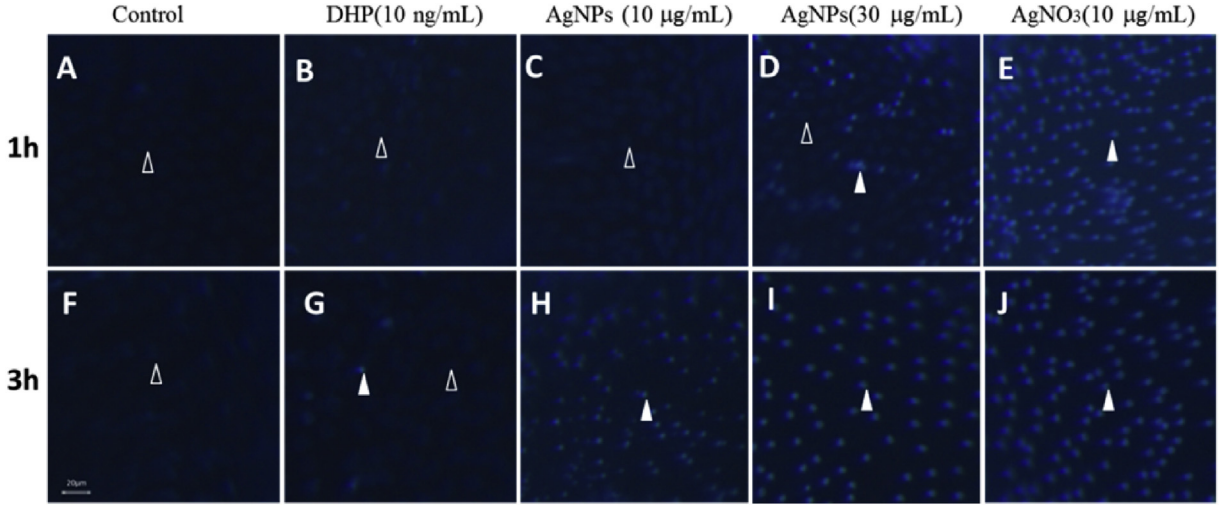

Fig. 5. Effects of silver nanoparticles (AgNPs) or $AgNO_3$ on apoptosis in ovarian follicle cells. Ovarian follicles enclosing fully-grown immature oocytes were incubated with Cortland medium (control, panel A,F); or medium containing DHP (10 ng/mL, panel B,G); AgNPs (10 μg/mL, panel C,H); AgNPs (30 μg/mL, panel D,I); and $AgNO_3$ (10 μg/mL, panel E,J) that were collected 1 or 3 h following treatment. After Hoechst 33342 staining, the representative fluorescence images were taken to showing the follicle cells layer surrounding the oocytes. Open arrowhead indicated the intact follicle cells with oval shape and their nuclei were stained with a weak blue fluorescence; Filled arrowhead indicated the follicle cells exhibit apoptosis features such as spherical bead shape and bright blue fluorescence when treated with AgNPs or $AgNO_3$. Scale bar, 20 μm.

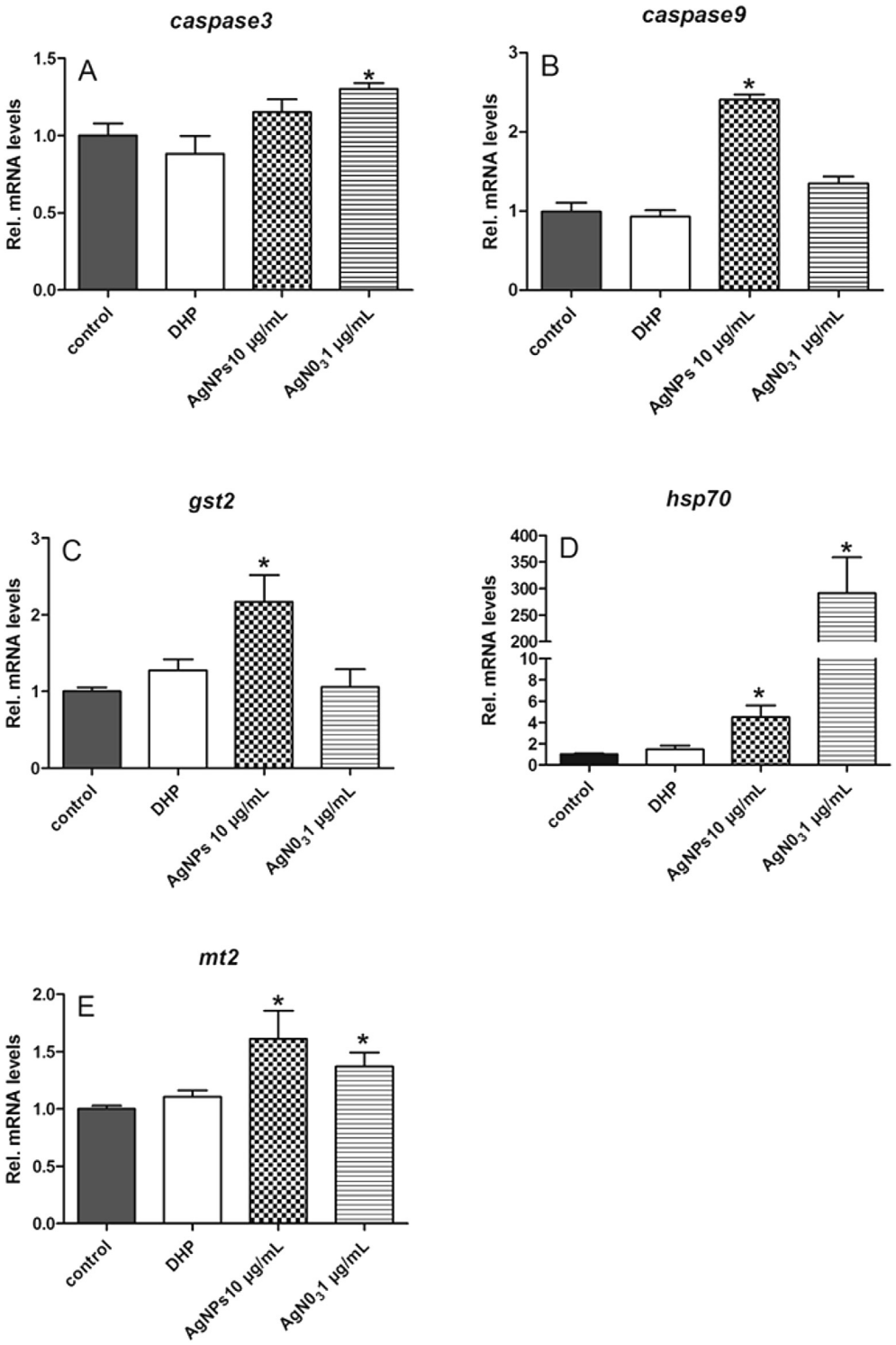

Fig. 6. Exposure to silver nanoparticles (AgNPs) and $AgNO_3$ affect gene expression in ovarian follicles. Total RNA was extracted from a group of 30 ovarian follicles enclosing fully-grown immature oocytes that was incubated with Cortland medium as a control, or the medium containing DHP (10 ng/mL), AgNPs (10 μg/mL), or $AgNO_3$ (1 μg/mL) for 2 h. Transcripts of *caspase3*, *caspase9*, *gst2*, *hsp70*, and *mt2* were determined using qPCR and normalized to an internal reference gene (*β-actin*). Data are expressed as the mean ± SE (n = 6) relative to respective transcript levels measured in the control group. Bars marked with an asterisk are significantly different from the control group ($p < 0.05$, Student's *t*-test).

termination of the transfer of cAMP from the follicle cells to the oocyte and production of estrogen, both of which result in a decrease of cAMP concentration in the oocyte that triggered the resumption of meiosis.

The exact mechanism by which AgNPs inflict damage to organisms is still a matter of debate. Although the toxicity seems to be driven by oxidation and inflammation, it is unclear whether silver in its nanoparticulate form is responsible for the toxic effects, as

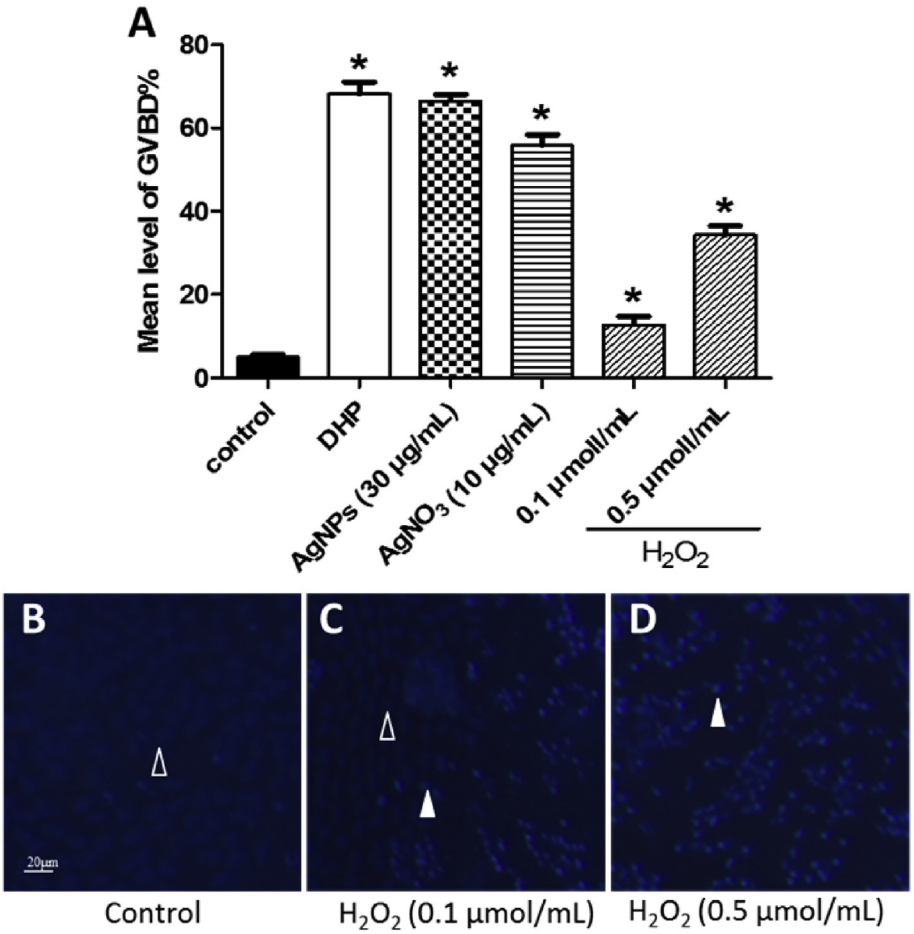

Fig. 7. Effects of H_2O_2 on zebrafish oocyte maturation and apoptosis in ovarian follicle cells. (A) Ovarian follicles enclosing fully-grown immature oocytes were incubated with Cortland medium only (control), the medium containing DHP (10 ng/mL), AgNPs (30 μg/mL) or $AgNO_3$ (10 μg/mL) or H_2O_2 (0.1 and 0.5 μmol/mL) for 4 h. Each treatment group contains at least 30 oocytes. Experiments were repeated six times, and the data are expressed as average of six replicates (mean ± SE, n = 6). Bars marked with asterisk are significantly different from the control group ($p < 0.05$, Student's *t*-test). (B-D) Ovarian follicles enclosing fully-grown immature oocytes were incubated with Cortland medium (control); or H_2O_2 (0.1 and 0.5 μ,ol/mL) for 3 h. After Hoechst 33342 staining, the representative fluorescence images were taken to showing the follicle cells layer surrounding the oocytes. Open arrowhead indicated the intact follicle cells with oval shape and their nuclei were stained with a weak blue fluorescence; Filled arrowhead indicated the follicle cells exhibit apoptosis features such as spherical bead shape and bright blue fluorescence when treated with H_2O_2. Scale bar, 20 μm.

some studies claim, or whether they are caused solely by silver ions dissolving in the course of oxidation of the metal. A recent study in pigs implies that release of Ag^+ is responsible for the observed inhibition effects of AgNPs on cumulus-oocyte maturation (Tiedemann et al., 2014). Therefore, we also examined whether Ag^+ would induce zebrafish oocyte maturation. The results showed that, similar to AgNPs, $AgNO_3$ also induced oocyte maturation, decreased cAMP concentrations, and induced apoptosis in ovarian follicle cells. We observed that follicle cells treated with Ag^+ showed a novel mitochondrial swelling while the inner membrane was still partially visible. This novel ultrastructure has been reported in a study that examined the effect of Ag^+ isolated rat liver mitochondria, and suggests that Ag^+ promotes a nonclassical permeability transition and releases apoptogenic cytochrome c which triggers the apoptotic cascade (Almofti et al., 2003). In the meantime, our results indicated that silver ion release rate was about 1‰ in the culture medium containing AgNps, and $AgNO_3$ was more toxic than AgNPs to both follicle cells and oocytes. Taken together, although both AgNPs and $AgNO_3$ induced apoptosis in follicle cells, the molecular mechanisms induced by these two compounds might have been different. Therefore, gene expression analysis was performed in order to distinguish the impacts of AgNPs from Ag^+ ions (Griffitt et al., 2009; Poynton et al., 2012).

Caspases (cysteine-aspartic acid proteases) are activated during apoptosis in many cells and are known to play a vital role in both the initiation and execution of apoptosis. The upregulation of *caspase3* and *caspase9* in response to $AgNO_3$ and AgNPs, respectively, further proved that both AgNPs and $AgNO_3$ induced apoptosis in ovarian follicle cells. HSPs have been classified as stress response proteins owing to their induction by several types of cellular stress such as infection and inflammation (Bouwmeester et al., 2011). In addition, the HSPs may be involved in preventing cells from apoptosis (Li et al., 2009). In the present study, both AgNPs and $AgNO_3$ exposure induced up-regulation of *hsp*70, which was in agreement with the previous studies showing an up-regulation of HSP genes after AgNPs and Ag^+ ion exposure (small *hsps*, *hsp*40, *hsp*70, *hsp*90 and *hsp*110 family) (Foldbjerg et al., 2012). Therefore, *hsp70* is a sensitive biomarker of both AgNPs and $AgNO_3$ inducing cellular damage e.g. apoptosis. It is reported in several studies that genes from the metallothionein superfamily that are involved in metal binding and the response to metal exposure are up-regulated (Kawata et al., 2009; Lim et al., 2012; Ding et al., 2014; Eom et al., 2014). A study in zebrafish demonstrates that *mt* mRNA expression in liver increases after treatment with AgNPs (Choi et al., 2010a). As reported, results from our study showed that both AgNPs and $AgNO_3$ induced an upregulation of *mt2* transcript levels.

Because the cysteines of metallothioneins bind oxidant radicals such as superoxide and hydroxyl radicals (Kumari et al., 1998; Formigare et al., 2007), it is possible that, in the ovarian follicle, *mt2* is induced mainly as a response to oxidative stress. Therefore, we further analyzed the expression of glutathione S-transferases (*gst*), which catalyzes the conjugation of glutathione (GSH) with various electrophilic substances, and plays a role preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH (Ketterer et al., 1983). Results from our study showed that AgNPs, but not $AgNO_3$, induced the up-regulation of *gst2* transcript levels. The results from gene expression analyses suggested that AgNPs-induced apoptosis of ovarian follicle cells may mainly be due to the increase of oxidative stress. Previous studies in mammalian liver cells indicated that AgNPs and silver nanoclusters cause cytotoxicity by oxidative stress-induced apoptosis and damage to cellular components (Piao et al., 2011). Based on these information, we applied H_2O_2 to increase the oxidative stress of ovarian follicles. The results indicated that H_2O_2 had an ability to induce zebrafish oocytes maturation by induction of apoptosis in ovarian follicle cells. Thus, oxidative stress appeared to be one of mechanism in AgNPs inducing apoptosis of follicle cells.

It has been demonstrated that the bioaccumulation of AgNPs by marine medaka (*Oryzias melastigma*) from the waterborne phase was very low (Wang and Wang, 2014b). However, dietary exposure of AgNPs may also act as a potential uptake route of AgNPs in fish. Recent studies in zebrafish (Rahmani, et al., 2016) and marine medaka (Wang and Wang, 2014a) have provided evidence that AgNPs can transfer from brine shrimp *(Artemia salina)* nauplii to the fish by dietary exposure. Although the amount of assimilated Ag (from AgNPs contaminated food) is small, it still generated toxic effects (e.g., inhibition of Na^+/K^+-ATPase and SOD activity, reduction of total body length, and water content) on marine medaka. Although we can't provide direct evidence to approve the quality of zebrafish oocyte is damaged by AgNPs, study in mammals have clear demonstrated that ROS-induced mitochondrial damage has widespread impacts on multiple aspects of oocyte quality (Boudoures and Moley, 2015). Therefore, results from present study may suggest a potential environmental risky of trophic transfer of AgNPs into the fish, and AgNPs-generated free radicals further affect oocyte quality.

In summary, our results confirmed that *in vitro* maturation of fish oocytes represented a very sensitive system for the exploration of nanotoxicology in which even subtle effects could be visualized. To our knowledge, this is the first report of AgNPs and Ag^+ inducing the maturation of fish oocytes. The results of gene expression patterns suggested that oxidative stress appeared to be one of important mechanisms in AgNP-induced apoptosis in ovarian follicle cells. Further study is necessary to examine the quality of mature oocytes affected by AgNPs and Ag^+ using other animal models.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.12.016.

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