Received: 4 October 2017 | Revised: 7 November 2017 | Accepted: 20 November 2017

DOI: 10.1002/jbt.22015

WILEY

Evaluation of the interaction between proliferation, oxidant–antioxidant status, Wnt pathway, and apoptosis in zebrafish embryos exposed to silver nanoparticles used in textile industry

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Abstract

Antimicrobial textile products are developing rapidly as an important part of functional textiles. Silver nanoparticles (AgNPs) are nanotechnology products with antimicrobial properties. However, exposure to nanoparticles in daily life is an important issue for public health, still being updated. Aim was to evaluate the effects of AgNPs on the development of zebrafish embryos focusing on Wnt pathway, proliferation, oxidant–antioxidant status, and apoptosis. The expressions of *ccnd1* and *gsk3β* were determined by RT-PCR, whereas β-catenin and proliferative cell antigen (PCNA) expressions were determined immunohistochemically. Lipid peroxidation, superoxide dismutase, and glutathione-S-transferase activities were determined spectrophotometrically. Apoptosis was determined using acridine orange staining. Oxidant status, apoptosis, immunohistochemical PCNA, and β catenin staining increased, whereas *ccnd1* and antioxidant enzyme activities decreased in AgNPs-exposed embryos in a dose-dependent manner. Our results indicate the interaction of possible mechanisms that may be responsible for the toxic effects of AgNPs in zebrafish embryos.

KEYWORDS

apoptosis, oxidant–antioxidant status, silver nanoparticles, Wnt pathway, zebrafish embryo

1 | INTRODUCTION

Functional textiles are one of the most important points that textile sector has reached by today. Antimicrobial textile products are developing rapidly as an important part of functional textiles. Accordingly it has become increasingly common to apply antimicrobial finishing treatment to textile materials, to protect both the user and the textile product against the microorganism, especially in medical and household textile products.[1,2] Silver nanoparticles (AgNPs) are nanotechnology products known especially for their antimicrobial properties. Silver interacts with the thiol groups of the bacterial protein and binds to their molecules and terminates the metabolic activity of the cell. As a result, it completely destroys the microorganism.[3] On the other hand, exposure to nanoparticles in daily life is an important issue for public health, which is still being updated.

As an evolutionarily conserved pathway, Wnt/β-catenin signaling pathway plays a major role in many processes including cell proliferation during development.[4] *ccnd1* is a proto-oncogene and an important regulator of G1 to S phase progression acts as a major transcriptional target of β-catenin/Wnt signaling.[5] *Ccnd1* binds cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) and forms active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein.[6] *Ccnd1* has been reported to be important for the development and progression of several cancers including breast, oesophagus, bladder, and lung cancers.[7,8] Induction of *Ccnd1* degradation has been suggested as a therapeutic intervention.[9] Glycogen synthase kinase 3β (GSK3β) has been reported to phosphorylate *Ccnd1* and induce its rapid turnover. Recent findings have questioned the role of GSK3β in mediating *Ccnd1* degradation.[10] β-Catenin, a multifunction protein

acting as a transcriptional co-regulator and an adaptor protein for intracellular adhesion, is regulated by the Wnt signaling and abnormal expression of β-catenin causes various diseases including cancer.[11]

Oxidative stress is related with the pathogenesis of various disorders and oxygen radical formation by redox cycling has been suggested to be a critical event in toxic effects of many compounds if the protective mechanisms of cells are disrupted. Increased reactive oxygen species (ROS) can lead oxidative stress and cause oxidative damage that may affect viability and severity of inflammation.[12]

Zebrafish embryos are used as alternative models to reduce the number of animal experiments and associated costs within the scope of the "3Rs" principle—replacement, reduction, and refinement of animal experiments. In recent years, zebrafish embryo has gained its popularity because of its external development, maintenance advantages, small size, high fecundity, fast development, and optical transparency.[13]

Here in this study, we aimed to evaluate the effects of AgNPs on the development of zebrafish embryo focusing on Wnt pathway, proliferation, oxidant–antioxidant status, and apoptosis. In order to evaluate the possible mechansims underlying the effects, the expressions of *Ccnd1*, *gsk3β* , β-catenin, and proliferative cell antigen (PCNA), which is an indicator of cell proliferation, were determined. Lipid peroxidation (LPO), superoxide dismutase (SOD), and glutathione-S-transferase (GST) activities and *in vivo* cell death were also evaluated in AgNPs-exposed zebrafish embryos.

2 | MATERIAL AND METHODS

2.1 | Chemicals tested

AgNPs were purchased in solution from (1000 ppm) Nanokar Ltd. (Istanbul, Turkey). They were all analytical grade with the highest purity available.

2.2 | Characterization of AgNPs

The micromorphology of AgNPs was analyzed using a Scanning Electron Microscope (SEM, Zeiss EVO HD15; Germany) using copper tapes. The AgNP solution was diluted in 1:10 ratio for SEM analyses.

2.3 | Maintenance of zebrafish

Wild-type AB/AB Strain zebrafish was originally obtained as a gift from Dr. Fuss from Boğaziçi University (Istanbul, Turkey) and subsequently bred and maintained in apparently disease-free conditions. Animals were kept in an aquarium rack system (Zebtec, Tecniplast, Italy) at 27 $\pm$ 1 °C under a light/dark cycle of 14/10 h. Animals were fed twice a day with commercial flake fish food complemented with live Artemia. All experiments were performed using reverse osmosis water supplemented with 0.018 mg/L Instant Ocean^{TM} salt. Fertilized embryos were collected following natural spawnings, cultured, and staged by developmental time and morphological criteria as described previously.[14]

2.4 | Embryo exposure

Range-finding experiments were applied initially to find out the lethal concentration that cause 50% mortality (LC50) in the zebrafish embryos and environmentally relevant concentrations of silver that affect development were determined as 0.5, 1, and 2 μg/mL. For the embryo toxicity tests, stock solutions of AgNPs were made up in ultrapure water, and sonicated for 1 h to ensure dispersal of the particles. Exposure groups were prepared as 0.5, 1, and 2 μg/mL. Each exposure group had 20 embryos and embryos were exposed to AgNPs in well plates for 72 h post fertilization (hpf). Embryo medium was used as the blank control. Each group was considered as three replicates. All exposure solutions were replaced with fresh solutions each day. At the end of the exposure period, the embryos were washed several times with water and allowed to develop until 120 hpf. Developmental parameters were monitored and documented daily. Embryonic mortality and hatching rate were evaluated every 24 h. The hatching rate is a ratio of hatching embryos to the living embryos in each well. During the exposure period (4–72 hpf), the images of malformations were captured under a stereomicroscope (Zeiss Discovery V8) and the percentage of abnormal embryos was counted every 24 h. The images of malformations were captured and differences were observed and noted. Accordingly individual malformations and abnormalities, such as axial malformations, pericardial edema, and yolc sac edema were listed. Delays in development were confirmed by comparing with the control embryos. Embryo staging was conducted as explained before,[14] and the pectoral fin, yolk sac, anal pore, and swim bladder were used as the indicators of development.

2.5 | Expression analyses

2.5.1 | Whole mount immunohistochemistry

The zebrafish embryos were fixed for whole-mount immunohistochemistry for the expression of proliferative cell nuclear antigen (PCNA) using the modified method.[15] Embryos were dechorionated in pronase (2.0 mg/mL, in E3 medium; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) for 3–5 min and rinsed five times in E3 medium. They were incubated for 1 h in 4% paraformaldehyde. Then they were washed four times for 5 min in PBSTx and incubated for 5 min in graded methanols. After washing, they were incubated for protein block and peroxidase block for 1 h in room temperature. Diluted primary antibodies Anti-PCNA antibody (Abcam; ab29; 1:500; USA) was added and the embryos were incubated overnight. The next day they were washed and incubated for 2 h with seconder antibody. After washing were incubated in DAB substrate for 10–30 min. After washing with PBSTx, the sections were imaged with a stereomicroscope (Zeiss Discovery V8; Germany).

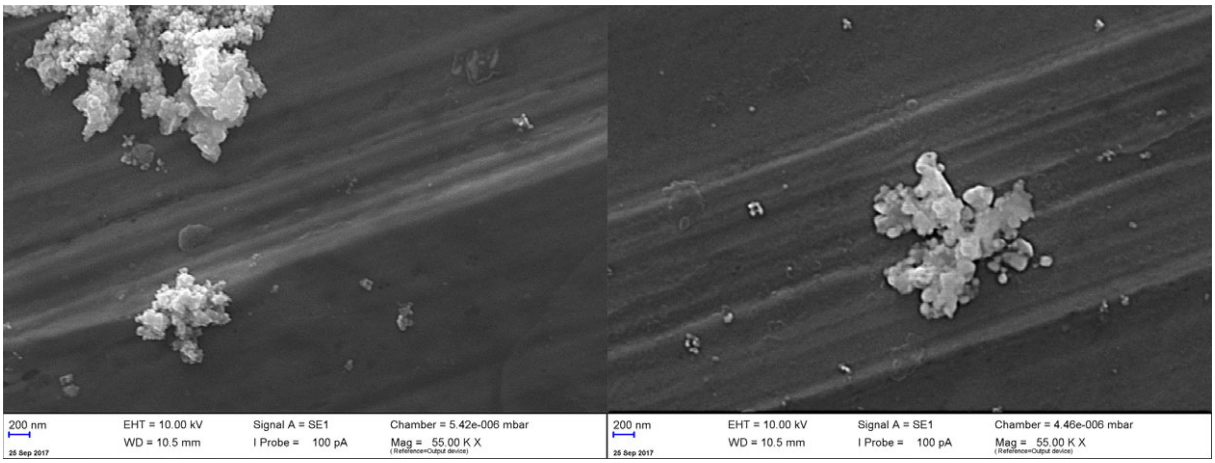

FIGURE 1 SEM micrographs of silver nanoparticles

2.5.2 | Reverse transcription (cDNA synthesis) and quantitative real-time PCR

RNA was isolated from the embryos using Rneasy Mini Kit and Qiacube (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 1 μg of total RNA using RT^2 Profiler PCR Arrays (Qiagen, Hilden, Germany). PCRs were performed using the DNA Master SYBR Green kit (Qiagen, Hilden, Germany). The expressions of *ccnd1* and *gsk3β* were evaluated by quantitative RT-PCR using the Qiagen Rotor Gene-Q Light Cycler instrument. All of the Real-Time PCR primer products were obtained from (Qiagen, Hilden, Germany). β actin was used as the house keeping gene. Relative transcript levels were calculated by using the ΔΔCT method by normalizing the values with the house keeping gene.[16]

2.6 | *In vivo* cell death assay

Cellular death was detected in living embryos by using acridine orange (AO) staining, which is a nucleic acid selective metachromatic dye that connects with DNA and RNA by intercalation or electrostatic attractions. AO does not stain normal cells but selectively stains necrotic or late apoptotic cells with disturbed plasma membrane permeability. For this method, living embryos were immersed in 5 μg/mL AO for 10 min at room temperature and then they were washed with E3 medium. Embryos were anesthetized with tricaine for 3 min before examination and they were visualized and imaged for less than 1 min. Apoptotic cells were identified with a fluorescence microscope (Zeiss V16 Axio Zoom microscope with 546 nm filter).

2.7 | Biochemical assays

For the biochemical assays 100 zebrafish embryos at 72 hpf were used. They were pooled and homogenized in 1 mL PBS, followed by centrifuging briefly. The supernatant was used for the determination of LPO levels, SOD, and GST activities.

2.7.1 | Determination of LPO

Malondialdehyde (MDA) level was determined in embryo homogenates as thiobarbituric acid reactive substances by the method of Yagi.[17] The extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used and LPO was expressed in terms of MDA equivalents as nmol MDA/mg protein.

2.7.2 | Determination of SOD activity

The method based on the ability of SOD to increase the effect of riboflavin-sensitized photo-oxidation of o-dianisidine was used to determine SOD activities in embryo homogenates. The absorbance of the product was measured in 460 nm by a spectrophotometer. The net absorbance was calculated by measuring absorbances at 0 and 8th minutes of illumination. The results were expressed as U/mg protein. [18]

2.7.3 | GST activity

The activity of GST was determined based on the spectrophotometric evaluation of the absorbance at 340 nm of the product formed by GSH and 1-chloro-2,4-dinitro-benzenin conjugation.[19]

2.8 | Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). All data were expressed as mean ± S.E. One-way ANOVA followed by post-hoc Dunn's Multiple Comparison Test was used to analyze the differences between the groups using Graph Pad 6, A P value of ≤0.05 was considered as significant.

3 | RESULTS

3.1 | SEM results

To analyze the morphology of AgNPs, SEM is considered as an important technique.[19] Figure 1 shows the SEM graphs of the AgNPs. SEM graphs reveal the sphere structures of the AgNPs with an average size of 30–60 nm.

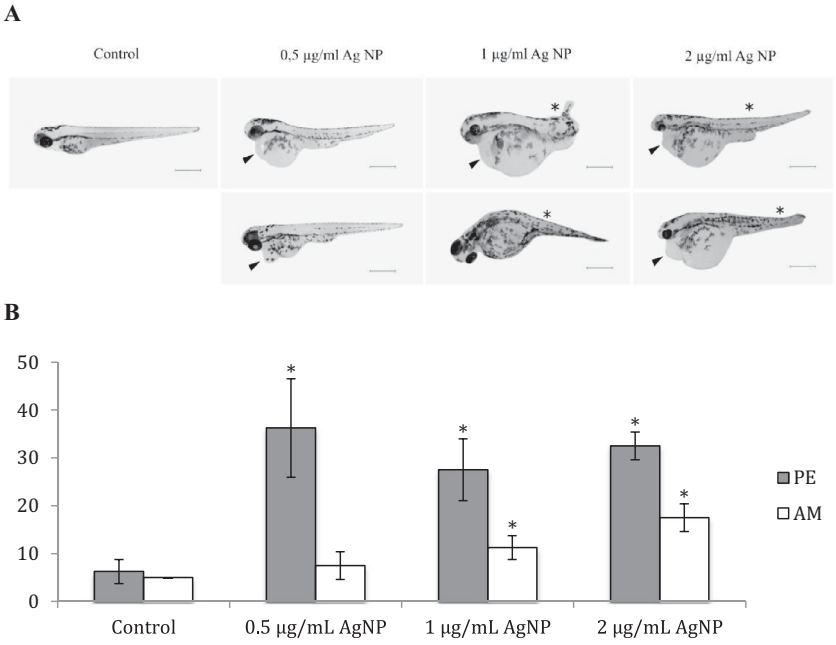

FIGURE 2 (A) Individual morphologic abnormalities observed in zebrafish embryos exposed to AgNPs on 72 hpf; arrow head, pericardial edema; star, axial malformation; scale bar: 500 μm. (B) Percentage of individual morphologic abnormalities occurring in zebrafish embryos exposed to AgNP on 72 hpf. Data are expressed as means ± S.D. from four independent experiments, $n = 20$ (*$P < 0.05$ compared with the control group). AM, axial malformations; PE, pericardial edema

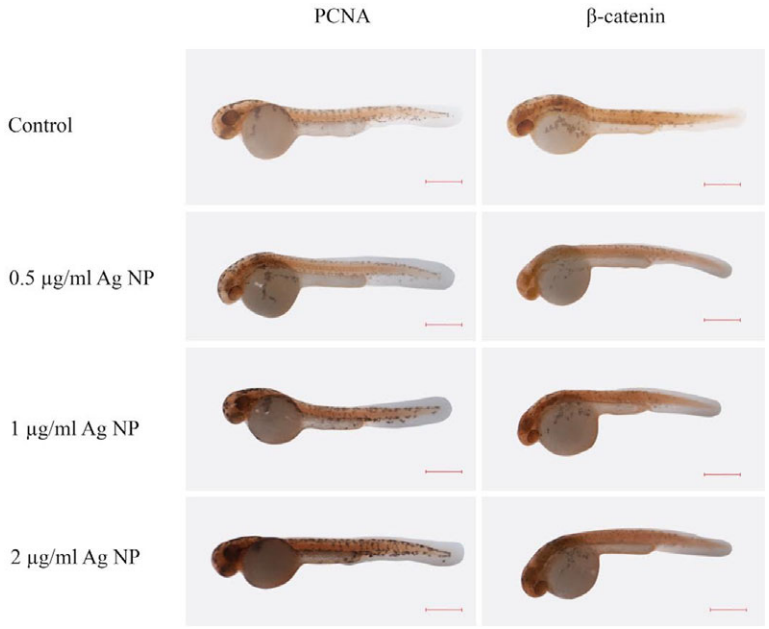

FIGURE 3 Immunohistochemical PCNA and β-catenin staining of the 48 hpf zebrafish embryos in the control group and 0.5, 1, and 2 μg/mL AgNP-exposed embryos

3.2 | Morphological abnormalities of the embryos exposed to AgNPs

The main abnormalities observed in zebrafish embryos exposed to AgNPs on 72 hpf were pericardial edema and axial malformations. The malformations are given in Figure 2A as representative images and the percentages of the malformations are given in Figure 2B. Exposure to 0.5 μg/mL AgNP led to the highest percentage pericardial edema and the highest percentage of axial malformations was observed in the 2 μg/mL AgNP. Microcephaly that is characterized by a significant reduction in brain volume was also observed in two of the 2 μg/mL AgNP-exposed embryos.

3.3 | Immunohistochemical analysis results

Representative immunohistochemistry images of zebrafish embryos showing PCNA, and β-Catenin expressions are given in Figure 3. A weak staining was observed in the control group for PCNA, however,

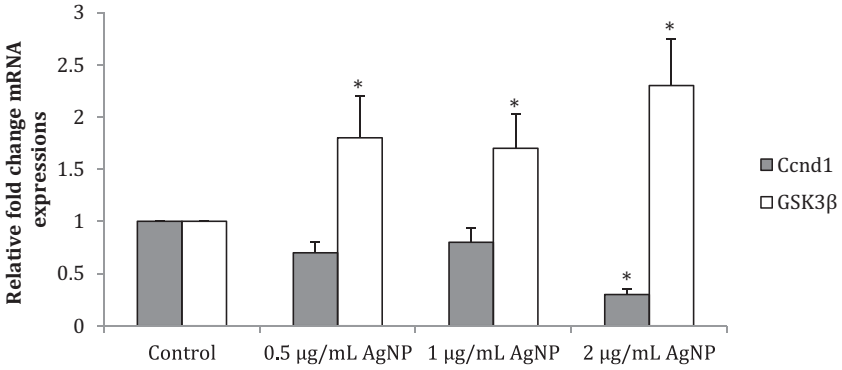

FIGURE 4 Bar graph presentation of fold change of *ccnd1 and gsk3β* transcript quantified by RT-PCR. All RT-PCR results are normalized to *β*-actin, the house keeping gene and expressed as change from their respective controls. The average values were obtained from three experiments. Data presented are mean ± S.D. Significant difference is indicated by asterisk, and *P* value < 0.05 was considered as significant

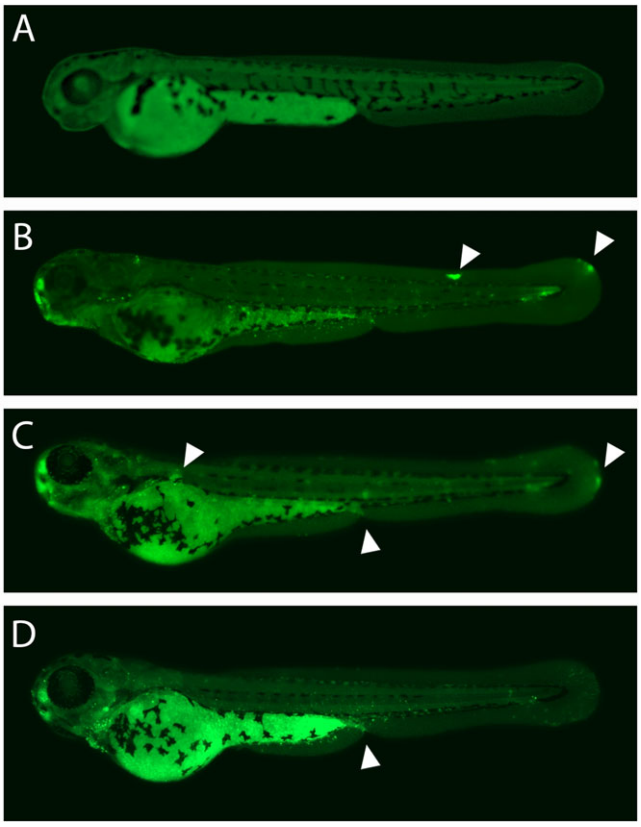

FIGURE 5 Representative images of *in vivo* embryo cell death determined by using acridin orange staining at 72 h in the control and AgNP exposed embryos. Images were detected by fluorescence microscope. Arrow heads indicate apoptotic cells. (A) Control group, (B) 0.5 μg/mL AgNP, (C) 1 μg/mL AgNP, (D) 2 μg/mL AgNP exposed embryo

AgNP-exposed groups appeared to have more intense PCNA staining in a dose-dependent manner. The expression of PCNA was evaluated as a marker for proliferation and an intense staining in neural tube, eye, brain, neural tube, and notochord. Immunohistochemical *β*-catenin expressions were also more intense in the exposure groups being particularly pronounced in neural tube and notochord in 2 μg/mL AgNP-exposed group (Figure 3).

3.4 | RT-PCR analysis results

The expressions of *ccnd1* and *gsk3β* are given as fold change of transcript quantified by RT-PCR. RT-PCR results were normalized to *β-actin*, the house keeping gene and expressed as change from their respective controls. The average values obtained from three experiments are given in Figure 4. Increased *gsk3β* expressions were found in the AgNP-exposed embryos. On the other hand, *ccnd1* expression decreased significantly in the 2 μg/mL AgNP-exposed embryos. No significant change was observed in *ccnd1* expression in 0.5 and 1 μg/mL AgNP-exposed groups.

3.5 | *In vivo* cell death assay results

In vivo cell death results of AO staining are presented as representative images of whole embryo cell death determined by using AO staining at 72 h in the control and AgNP-exposed embryos (Figure 5). Apoptotic cells were observed in the head, pericardium, and tail regions of the exposure groups, especially in the 0.5 and 2 μg/mL AgNP groups.

3.6 | Biochemical results

MDA levels as an index of LPO increased significantly in all exposure groups when compared with the control group ($P < 0.05$). SOD activity decreased in all AgNP exposure groups whereas GST activity decreased only in the 2 μg/mL AgNP-exposed group (Table 1).

4 | DISCUSSION

We demonstrated that exposure to AgNPs caused malformations as pericardial edema and axial defects in zebrafish embryos, led to apoptosis, increased LPO, decreased antioxidant activity, changed the expressions of PCNA, *β*-catenin, *ccnd1*, and *gsk3β*.

Previous studies have shown that Ag ion and AgNP are toxic to fish.[20,21] In our experiments 48-h and 72-h LC50 values of AgNP were approximately 5 μg/mL. Different LC50 values have been reported for AgNPs depending on the sizes of the nanoparticles and the

TABLE 1 Malondialdehyde levels, superoxide dismutase, and glutathione-S-transferase activities of the control and AgNP exposure groups

	Control	0.5 μg/mL AgNP	1 μg/mL AgNP	2 μg/mL AgNP
MDA (nmol/mg pr)	0.79 ± 0.09	1.39 ± 0.16*	1.41 ± 0.17*	1.68 ± 0.13*
SOD (U/ mg pr)	1.17 ± 0.23	0.72 ± 0.09*	0.63 ± 0.11*	0.61 ± 0.11*
GST (U/mg pr)	0.059 ± 0.006	0.052 ± 0.008	0.053 ± 0.004	0.038 ± 0.004*

Results are given as mean ± S.D. $P < 0.05$ is regarded as statistically significant.
Replicate pools of 72 hpf zebrafish (n = 5, 100 individuals per pool) were used.
*Significantly different when compared with the control group, ANOVA Test followed by Tukey's Multiple Comparisons Test was used.

solvents used.[22–24] Duan et al. showed pericardial edema and tail malformation in embryos exposed to AgNPs.[25] These malformations have also been reported in embryos treated with titanium dioxide nanoparticles.[26,27]

Oxidative stress may induce apoptosis and has been related with nanoparticle induced toxicity.[12,28] AgNPs induced oxidative stress, DNA damage, and apoptosis in zebrafish liver tissues.[24] In our study, increased level of MDA, a byproduct of cellular LPO, indicates that AgNP induced oxyradicals in the embryos. In the presence of hydrogen peroxide and an acidic environment, dispersed AgNPs induce hydroxyl radicals. Inside the cell, hydrogen peroxide can accelerate the dissolution of AgNPs leading to a stronger oxidative stress.[29,30] Although interactions of antioxidant molecules with AgNPs may partly antagonize oxidative stimuli to reduce toxicity of the latter, excessive ROS may alter their structure, function and their antioxidant ability.[31,32] In our study GST and SOD activities decreased in AgNP-exposed groups. As Ag ions may catalyze ROS production in the presence of oxygen species, our results are in accordance with the studies that have suggested ROS and oxidative stress production by AgNPs.[33] We may suggest that the AgNP-induced (apoptotic) cell death was likely mediated by intracellular ROS and oxidative stress.[34]

AgNPs-exposed embryos appeared to have more intense PCNA staining. PCNA is essential for DNA replication and repair. PCNA expression is used as a cell proliferation marker.[35,36] Increased PCNA may be induced by growth factors or as a result of DNA damage in the absence of cell cycling.[35,37] Down-regulation of PCNA may indicate cell cycle arrest as PCNA is synthesized in early G1 and S phases.[34] AgNPs-induced S-phase arrest independent of ROS production, in a study where level of PCNA protein expression in AgNP-exposed A549 cells was measured.[34]

Ccnd1 mRNA expression decreased significantly in the 2 μg/mL AgNPs group consistent with intense immunohistochemical PCNA staining. Immunohistochemical β-catenin staining was also intense in the AgNP exposure groups. β-Catenin is a key downstream effector of the Wnt pathway and its stabilization leads to activation of signaling, promoting cell proliferation. Moreover, mRNA levels of *gsk3β* increased in AgNPs-exposed groups suggesting *gsk3β* involvement in proliferation and apoptosis induced by AgNPs. Gsk3β protein modulates cell signaling, growth metabolism and induces apoptosis in conditions including DNA damage, hypoxia, and endoplasmic reticulum stress.[38–40] AgNPs exposure may be a condition where Gsk3β protein induces apoptosis, by inhibiting prosurvival transcription factors, facilitating proapoptotic transcription factors like p53.[41,42] Since Ccnd1 degradation is mediated by phosphorylation by *gsk3β*, increased *gsk3β* expression may be the reason for decreased *ccnd1* expression observed in 2 μg/mL AgNPs group.[7]

This is the first study to evaluate the relation between cell proliferation, apoptosis, Wnt/ β-catenin pathway, and oxidant-antioxidant status in AgNPs-exposed zebrafish embryos. Further mechanisms in the crosstalk between these pathways to confirm their roles in the adverse effects induced by AgNPs are necessary.

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How to cite this article: Eryılmaz O, Ateş PS, Ünal İ, et al. Evaluation of the interaction between proliferation, oxidant-antioxidant status, Wnt pathway, and apoptosis in zebrafish embryos exposed to silver nanoparticles used in textile industry. *J Biochem Mol Toxicol*. 2018;32:e22015. https://doi.org/10.1002/jbt.22015