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Exposing Zebrafish to Silver Nanoparticles during Caudal Fin Regeneration Disrupts Caudal Fin Growth and p53 Signaling

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Abstract

Zebrafish were exposed to commercial silver nanoparticles (~10-20 nm) at 0.4 and 4 ppm during cadual fin regeneration. The silver was in the Ag^+ ionic form. Fin regeneration was slow in the group exposed to the lower concentration. The cadual fin, gill, and muscle were assayed after 48 hours and subjected to histological analysis. In all tissues sampled, fish exposed to nanoparticles exhibited infiltration, large mitochondria with empty matrices, and accumulation of nano-sized silver in blood vessels. The results suggested mitochondrial damage and induction of inflammation. Microarray analysis of RNA from young zebrafish (52 hours post-fertilization) that were exposed to nanometer-sized silver particles, showed alteration in expression of the p53 gene pathway related to apoptosis. Gene expression changes in the nanoparticle-treated zebrafish led to phenotypic changes, reflecting increased apoptosis.

Keywords: Silver nanoparticles, Biological toxicity, Caudal fin regeneration, p53, Zebrafish

Nanometer-sized silver materials are now used in multiple applications, including fabric, cosmetics, toothpastes and in washing machines[1,2]. The major commercial property of nano-silver materials is their antibacterial effect[3,4]; however, there is little information regarding the effects of silver nano-material in everyday human use. Concerns about the toxicity of nanometer-sized material have been raised. Exposure of zebrafish to commercial nanometer-sized silver significantly affects expression of the gene for Selenoprotein N[5], although little is known about how the nanoparticles enter the cell. Research about the accumulation and excretion of nano-sized silver materials in living organisms is needed to assess their risks and to determine concentration and effluent standards.

Vertebrate regeneration is useful system for investigating the accumulation and excretion of potentially harmful substances such as nanometer-sized materials. Zebrafish are amenable to genetic analysis and regenerate an impressive array of structures, including spinal cord, optic nerve, heart, and fins[6-8]. The fin in particular, is an excellent model organ for studying regeneration. First, fins have a simple architecture, consisting of several segmented, bony fin rays composed of concave, facing hemirays that surround connective tissue, nerves and blood vessels[9]. Second, surgery is simple. Finally, regeneration is rapid and reliable, with most structures replaced within 1-2 weeks[10].

Therefore, we investigated the permeation, accumulation and excretion of silver nanometer-sized material in several organs using zebrafish fin regeneration. Gene expression was also investigated with microarrays, in order to investigate the effects caused by nanometer-sized silver on zebrafish embryogenesis.

Characteristics of Commercial Nanometer-Sized Silver Material

SEM and TEM images of the nanometer-sized silver material used in this study are shown Figures 1A and B. The silver particles, supported by Ti (Figure 1B) are approximately 10-20 nm in size (Figure 1B) and consist of Ag_3O, Ag_4H and titanium oxide, similar to the XRD analysis pattern (Figure 1C). The supporting material was shown to be titanium oxide.

The Effects of Nanometer-Sized Silver on Regeneration of The Zebrafish Fin

Regeneration of amputated caudal fins that were exposed to nano-sized silver at 0.4 or 4 ppm is shown as whole mounts at 2, 10, 24 and 36 days postamputation in Figure 2A. The groups exposed to nano-sized silver at 0.4 and 4 ppm showed clear defects in fin regeneration by 10 days postamputation. Regeneration was slowest in the lower concentration group

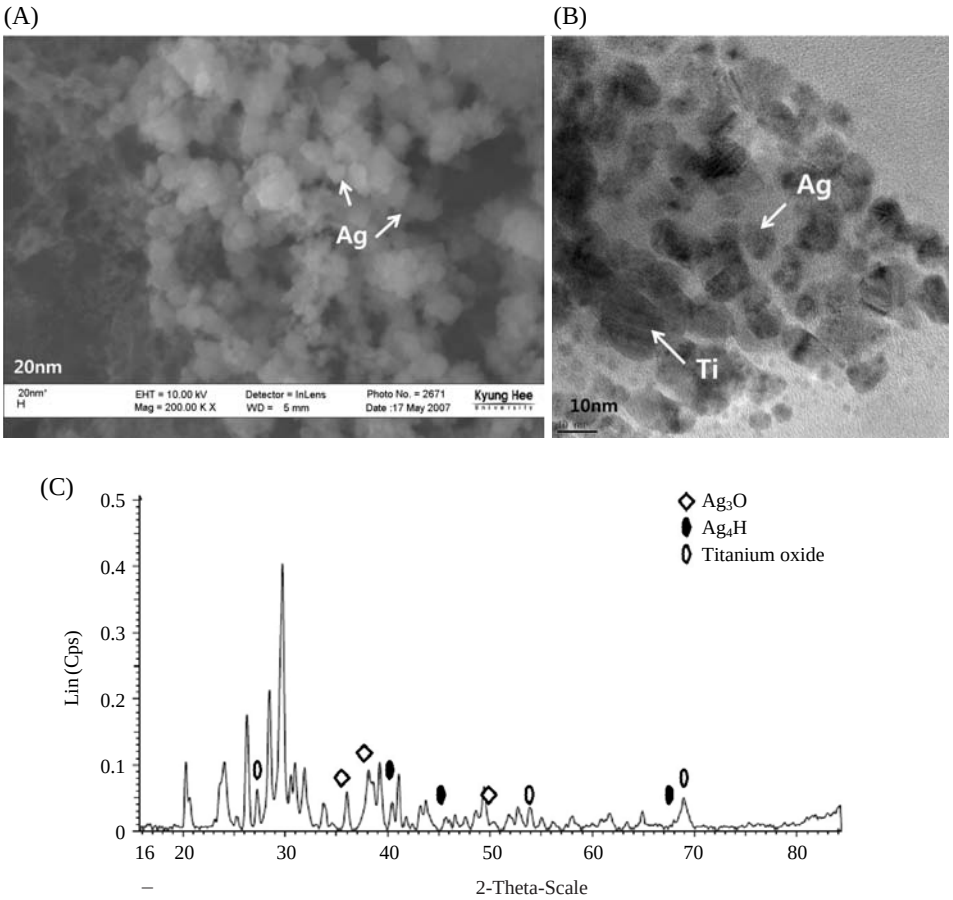

Figure 1. FE-SEM analysis of the nano-silver solution (A) and HRTEM images (B). The X-RD pattern is shown in (C). Several compounds (Ag_3O, Ag_4H and titanium oxide) are present in the nanometer-sized silver material.

(0.4 ppm). The control showed complete fin regeneration at 10 days (Figure 2B).

Histological Analysis of The Effects of Nano-Sized Particles on Zebrafish Tissues

TEM images of caudal fin tissue from zebrafish exposed to nano-sized silver are shown in Figure 3A and B. Nano-sized silver material penetrated all organelles, including the nucleus. In particular, large mitochondria with empty matrices were observed, along with accumulation of nano-sized silver in blood vessels. The situation was same in gill (Figure 3C) and muscle (Figure 3D).

Residual Silver Concentration in Tissues

After nano-sized silver exposure, the residual concentration of silver in tissues was analysed over 140 hours by Inductively-Coupled Plasma (ICP) spectrometry (Figure 4). After two hours post-exposure (hpe), the concentration of silver was high in muscle, and showed a continuous increase in intestine. After 100 hpe, residual silver could not be measured in muscle and testis.

Changes in The Gene Expression Profile

The gene expression profile of young zebrafish treated with nanoparticles was compared to untreated controls (Table 1). Five genes involved in apoptosis, coding for Tumor protein p53; bcl2-associated X protein; phosphatidylinositol glycan, class C; phosphatidylinositol glycan, class P; and insulin-like growth factor binding-protein 3, were upregulated 2.05 to 3.08 -fold, while one gene, coding for insulin-like growth factor 1, was significantly downregulated. Based on the expression profile changes that occurred after treatment with nanoparticles, a model pathway for apoptosis was generated (Figure 5).

Discussion

The present study focuses on the nanometer-sized silver ions used in commercial products (Figure 1). The ionic form potentially increases the antibacterial effect 11 and the nanometer size results in an increased surface area. These results suggest that nanometer-sized silver should be in the ionic form for use in

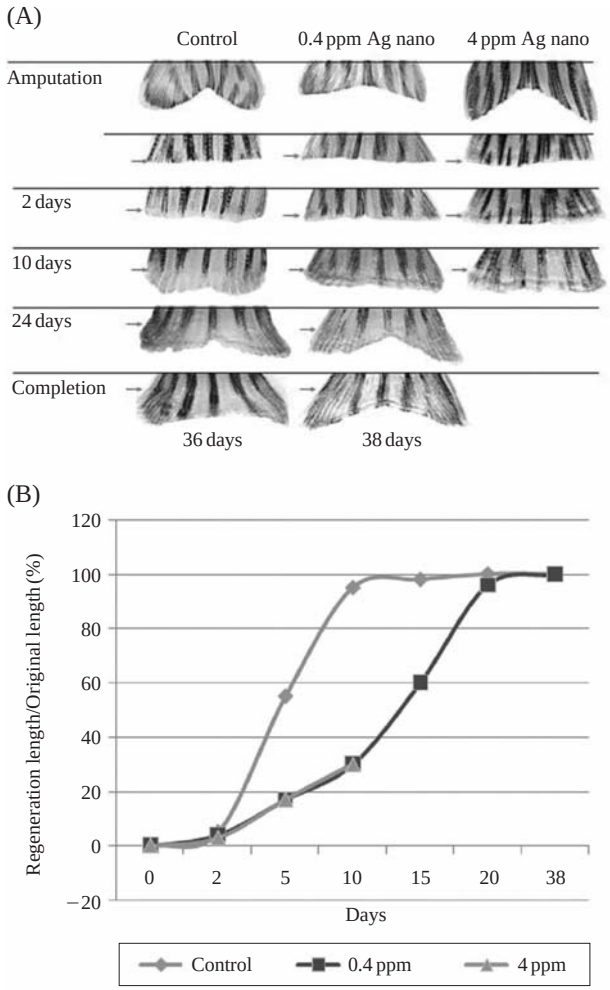

Figure 2. Whole-mounts of caudal fins exposed to nano-sized silver at 0.4 and 4 ppm and regenerated at 2, 10, 24 and 36 days postamputation are shown in (A). The groups exposed to nano-silver at 0.4 and 4 ppm showed a clear defect in fin regeneration by 10 days postamputation. Fin regeneration was slower in the group exposed to the lower concentration (0.4 ppm). Fin regeneration in the control group was complete by 10 days (B). Arrows indicate the amputation plane. The magnification is 15×.

antibacterial applications. Our TEM and SEM analyses showed that the nanometer-sized silver remained on the Ti support material (Figure 1). If the nanometer-sized materials rejoin to form a complex, however, their properties are likely to change. Silver ions that bond to other materials may result in a secondary contaminant that has an impact on the ecosystem. In addition, the properties of the nanometer-sized materials are likely to be lost upon binding to another material. Nanometer-sized silver that is not bound to another molecule can enter cells. The relationship between size and migration into cells observed in this study corresponds to reports from several other researchers[12-14].

Ag ions are a type of metal ion, and metal ions, such as Mg^{2+}, act as cofactors for enzymes that cleave or synthesize DNA[15,16], using nucleotides as substrates[16,17]. In addition, Ag^{+} is known to undergo strong covalent binding with DNA. Furthermore, the pBR322 plasmid has been reported to suffer DNA damage when exposed to Ag^{+}. The increased DNA damage was believed to be caused by free radicals produced from the oxidation of ascorbate by molecular oxygen, where the Ag^{+} ion played a catalytic role[17].

This study found that caudal fin regeneration in zebrafish was significantly inhibited by nano-sized silver at 4 ppm (Figure 2A), which may be due to nanometer-sized silver passing through the cell membrane. Ag^{+} ions might bind DNA, resulting in DNA damage. Furthermore, delay of caudal fin regeneration was shown in zebrafish exposed to 0.4 ppm nano-sized silver, compared to unexposed fish (Figure 2B), although caudal fin regeneration was eventually completed.

Permeation and accumulation of nano-sized silver particles was seen in organelles, for example mitochondria, nuclei and blood vessels, when cells were exposed to nano-silver at 0.4 or 4 ppm (Figure 3 and 4). Most of the mitochondria had been destroyed or become swollen, and nano-sized silver materials accumulated in blood vessels. Moreover, the damage was observed in the mitochondria-rich cell types of gill and muscle (Figure 4). It has been demonstrated that silver nanoparticles attack mitochondria, which are redox active organelles. Nanoparticles may alter ROS production and thereby interfere with antioxidant defenses[18,19]. Moreover, ROS are an important factor in the apoptotic process. The generation of excess ROS induces mitochondrial membrane permeability and damages the respiratory chain, triggering apoptosis[17,20]. Increased DNA damage in zebrafish embryogenesis after exposure to Ag^{+} was believed to be caused by production of free radicals[21] and increased catalase activity[5]. After exposure to nano-sized silver, the residual silver in muscle and testis could not be measured after 100 hpe. This result is similar to observed silver levels in teleost tissue showing the order of silver concentration, from highest to lowest, as liver > gills ≥ intestines > white muscle[22].

Exposure to heavy metals also modulates lysozyme levels but the nature of the modulation can be complex. In plaice, exposure to sub-lethal mercury concentrations reduced plasma lysozyme activity[23], whereas activity levels were unchanged in the blue gourami[24]. Nonetheless, exposure of blue gourami to

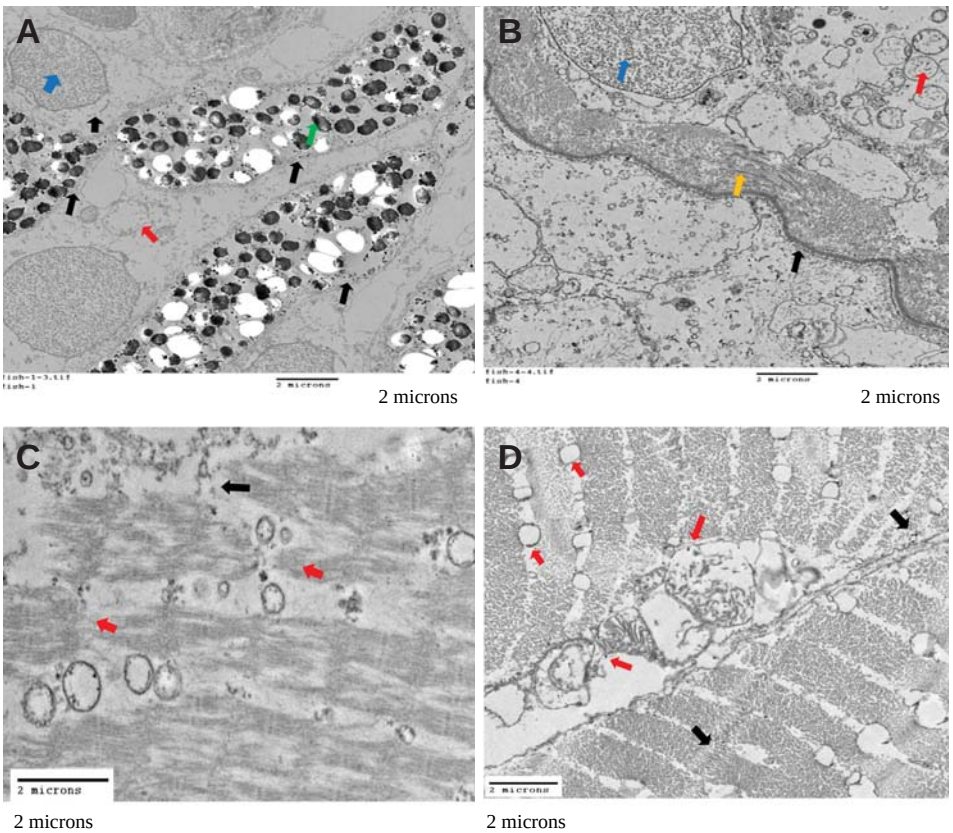

Figure 3. TEM images of caudal fins (A and B), gill (C) and muscle (D) from zebrafish exposed to nano-sized silver. Fins were regenerated for 10 to approximately 36 days at 28˚C. The yellow arrow indicates fibroblasts during regeneration. Note the large mitochondria with empty matrices (red arrow), nano-sized silver in the nucleus (blue arrow), accumulated nano-sized silver in blood vessels (green arrow) and nano-sized silver particles (black arrow).

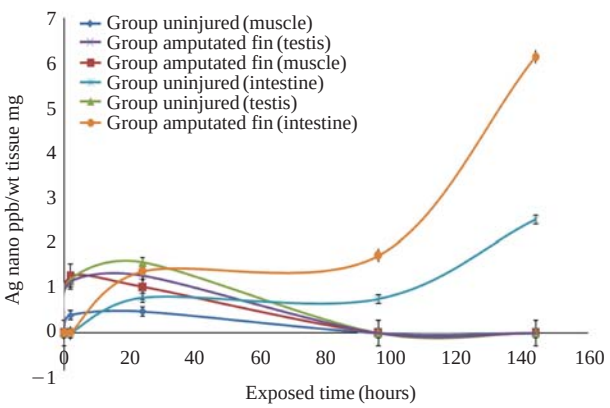

Figure 4. Residual concentration of silver in muscle, testis and intestine. Values are mean ± S.D., n=10 zebrafish per observed groups. Observations were made after 2, 24, 96 and 144 exposure.

mercury did increase lysozyme activity in the kidney[24].

In our study, nano-sized silver induced variation in the p53 signal pathway. Expression of the genes for Bax, PICs, Scotin, and Siah, which encode mitochondria-associated products, doubled. Expression of the genes that cause apoptosis, such as CASP9, CASP3 and ICF were altered, with expression of the ICF gene decreasing two-fold (Figure 5 and Table 1). This results are consistent with those showing that nano-

Table 1. Genes related to apoptosis showing expression changes. Genes with statistically significant expression changes were identified ($P < 0.05$).

Gene description	Gene symbol	Regulation profile (fold change)
transformed 3T3 cell double minute 2 homolog (mouse)	mdm2	2.05
Tumor protein p53	p53	2.15
bcl2-associated X protein	bax	3.08
phosphatidylinositol glycan, class C	pigc	2.69
phosphatidylinositol glycan, class P	pigp	2.53
seven in absentia homolog 1 (Drosophila)	siah1	2.15
insulin-like growth factor 1	igf1	0.38
insulin-like growth factor binding protein 3	igfbp3	2.22
caspase 3, apoptosis-related cysteine protease	casp3	0.77
caspase 9, apoptosis-related cysteine protease	Casp9 (zgc 101776)	1.11

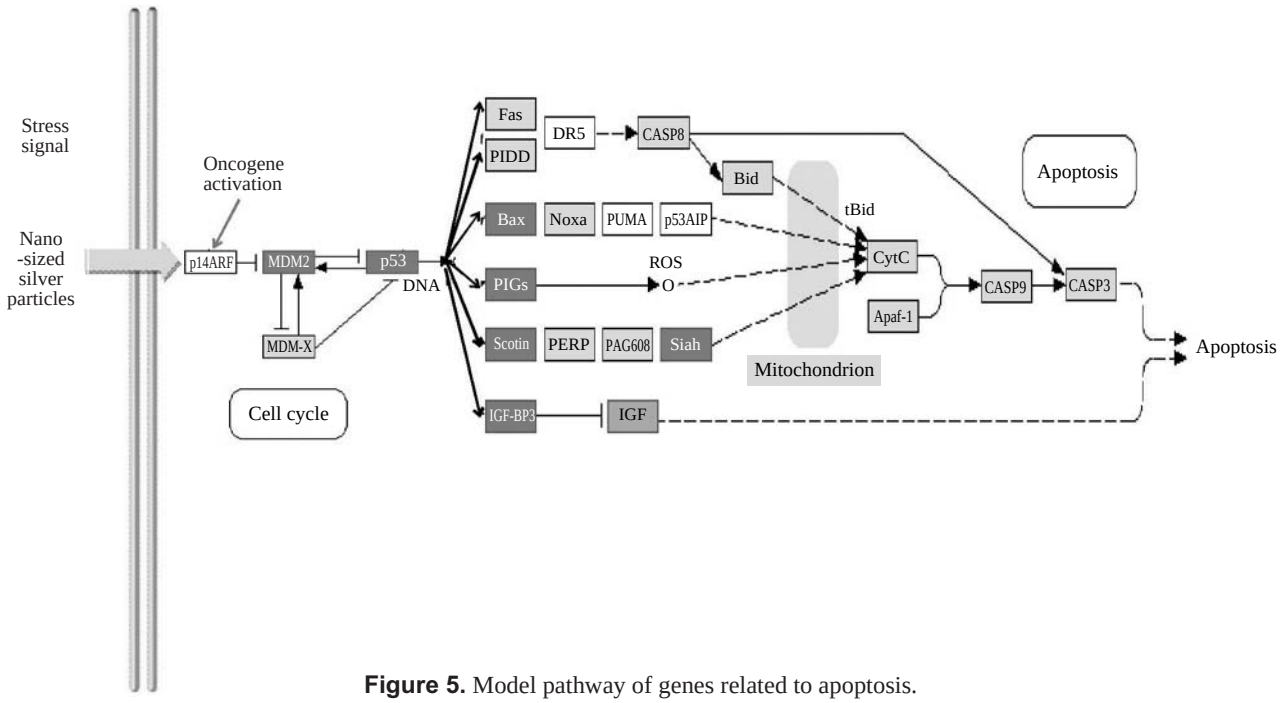

Figure 5. Model pathway of genes related to apoptosis.

sized silver activated CASP3 and CASP9 to induce mitochondria-dependent apoptosis in SGC-7910 human gastric cancer cells[25]. Thus the upregulation of these three genes can lead to increased apoptosis. The increased apopotosis in the groups exposed to nano-sized silver are hypothesized to be the result of injury to the cell. Moreover, if mitochondria are the target of nano-sized silver, mitochondria-rich cells would be especially susceptible to damage.

Materials & Methods

Characterization of Nano-sized Silver Material

Nano-silver material was purchased from the N corporation (Korea) and diluted with water to a concentration of 500 ppm. This type of silver is widely used in nano-silver products in Korea, including baby bottles, socks and underwear. A HRTEM (High Resolution Transmission Electron Microscope, JEOL, Japan), with an accelerating voltage of 300 kV was used to study the structure and morphology of the nano-silver. For TEM imaging, a nano-silver solution was centrifuged, and a small drop of the supernatant was placed on copper grids and dried at room temperature. Nano-silver specimens were prepared by placing several drops of nano-silver solution onto glass and drying at 35±5˚C. Samples were subjected to X-ray diffraction (XRD, model PW 1830, Philips), with nickel-filtered *CuKa* radiation (30 kV, 60 mA), at angles ranging from 5 to 70˚, at a scan speed of 10˚ /min and time constant of 1 s. A diffraction angle of 25.0˚ was selected to evaluate the crystalline structure of the sample. The size, shape and composition of the precipitates were observed by scanning electron microscopy (SEM, model JEOL-JSM35CF). The power and working distance were set to 15 kV and 39 cm, respectively.

Fish Care, Regeneration, Time-Lapse, and Temperature-Shift Experiments

Fish handling and breeding were performed according to standard procedures[26]. Regeneration experiments were performed on caudal fins that were amputated at an approximately 50% proximal-distal level. For time-lapse experiments, the amputated fish were kept in individual 500-mL beakers (1 fish/beaker) with 400 mL of distilled water supplemented with 0.3 g/L Instant Ocean Sea Salt (Marine Biotech, Beverly, MA). The water was filtered through 0.45 μm mesh, denitrified by bacterial filtration, and finally disinfected by ultraviolet light exposure. Throughout the experiments, fish were deprived of food. Fish were fed once daily, with live artemia. At least five fish from each treatment group were used in each experiment. Phenotypic comparisons between the control and exposed groups were performed on fish challenged to regenerate at 28˚C. The same fin ray of each fish was photographed at different time intervals

during the course of the time-lapse experiments.

Chemical Exposure During Development Stage

The nano-silver stock solution was diluted with distilled water. The final nano-silver exposure concentrations were 0.4 and 4 ppm.

Measurement of Fin Regeneration

Fins were observed with a microscope (Olympus, SZ61, Japan) to determine the morphological effects. Measurements were performed on fins while the fish's eyes were covered with wet tissue. The regenerate length was measured as the distance between the amputation plane and the tip of the regenerate, including the distal muscle.

Histological Preparation and Ttransmission Electron Microscopy (TEM) of Tissue

Tissue were fixed at 4˚C in 2% glutaraldehyde in sodium phosphate buffer, post fixed in 1% osmium tetroxide, dehydrated through graded ethanol solutions and then embedded in Embed812-Araldite502 resin (EMS). For transmission electron microscopy, ultra-thin sections (from 60 to 70 nm of depth) were mounted on copper grids, and then stained in lead citrate and uranyl acetate solutions for examination. Samples were observed using a Field Emission transmission electron microscope (FE TEM, H-7600, operated at 80 kV, Hitachi, Japan).

Measurement of Total Silver Concentration in Tissue

Total silver (Ag) was measured in tissues after microwave digestion (Q 15, Questron, USA) with 10 mL nitric acid (HNO_3) and 2 mL 10% hydrogen peroxide (H_2O_2)[27]. Silver concentrations were determined using an ICP spectrophotometer (LEEMAN ABS. INC., U.S.A).

Microarray Analysis

RNA was isolated from young zebrafish (52 hours after fertilization) exposed to nano-silver particles using an Agilent Low RNA Input Linear Amplification kit PLUS (Agilent Technology, USA). Reference RNA was isolated from non-treated zebrafish of the same age. Expression profiles were analyzed by GenomicTree, Korea, using an Agilent Zebrafish Oligo Microarray Kit V2 (44 K) chip.

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