Effects of Nanometer Sized Silver Materials on Biological Toxicity During Zebrafish Embryogenesis

Min-Kyeong Yeo† and Misook Kang*

Department of Chemistry, College of Science, Yeungnam University, Gyeongsan, Gyeongbuk 712-749, Korea
**E-mail: mskang@ynu.ac.kr*
†Department of Environmental Science and Engineering, KyungHee University, Yongin, Gyeonggi 449-701, Korea
Received January 18, 2008

Commercial nanometer sized silver is widely used for its antibacterial effect; however, nanoparticles may also have ecotoxicological effects after being discharged into water. Nanometer sized silver can flow into aquatic environments, where it can exert a variety of physiologically effects in living organisms, including fish. The present study aimed to investigate the effect of nanometer sized silver on the development of zebrafish embryos, analyze the properties of commercial nanometer sized silver and define the toxicity relationship between embryogenesis and hatched flies. The commercial nanometer sized silver was analyzed in the Ag^+ ion form. The hatch rate decreased in the nano-silver exposed groups (10 and 20 ppt); furthermore, the hatched flies had an abnormal notochord, weak heart beat, damaged eyes and curved tail. The expression of the Sel N1 gene decreased in the nano-silver exposed groups, and the catalase activities of the exposed groups increased relative to those in the control group. Therefore, the Ag^+ ions in commercial nanometer sized silver could accumulate in aquatic environments and seriously damage the development of zebrafish embryos.

Key Words : Nanometer sized silver, Biological toxicity, Zebrafish embryos

Introduction

Nanometer sized silver materials are now used in various areas, including cloths, cosmetic materials, tooth pastes and washing machines.[1,2] The major property of nano-silver materials is their antibacterial effect.[3,4] However, there is no information regarding this antibacterial effect of silver nano-material when used by ordinary people. Furthermore, scientists worry about the toxicity of nanometer sized material, but little is known about its pathway into the cell. Lundborg *et al*.[5,6] found that rat and human alveolar macrophages had impaired function due to aggregates of ultrafine carbon particles, which may be linked to increased infection risk and decreased protection of sensitive lung cells, which were different from the common image of nano-material. Silver is known as a safe metal; however, the antibacterial effect does not result from silver metal, but rather the silver ion. . It is widely known that the silver ions in nano-silver washing machines made by the company SEC (http://www.sec.co.kr) are produced by applying an electric current to a silver metal plate in the machine, where they have an antibacterial effect on the washed clothes.[7] The silver nanoparticles used in washing machines, cleaner products and coated textures will largely be discharged into sewer systems. These silver products will probably be persistent and continue to have bactericidal effects. Of course, some of the contaminant will form AgCl, which could ultimately form a deposit. AgCl has a lower antibacterial effect than Ag ions, but may also attach to underwater organisms, since AgCl particles are ultrafine and similar to TiO_2 powder (−100 nm).[8,9] For these reasons AgCl could pose a potential hazard to ecosystems, including the bacteria used in sewage treatment.[10,11] Abnormal notochord development during embryogenesis was observed in zebrafish exposed to nano-silver. Selenoprotein N1 (Sel N1) is related to vertebra development, as well as heart disease in zebrafish.[12] The relationship of the Sel N1 gene with nano-silver was also investigated. Moreover, the anti-oxide effects were investigated with regard to the catalase (EC: 1.11.16) activity. Catalase decreases the presence of free radicals, but some damage still occurs because the role of catalase is to protect enzymes of living organisms when free radical concentrations are increased within the cell.[13-15]

To investigate these hypotheses, the constituent materials of nano-silver particles were initially investigated using TEM and X-ray diffraction methods. Secondly, the biological effects of nano-silver in aquatic environments were assessed. Therefore, several exposure groups were investigated: 1) Control, 2) Nano-silver (Ag 10 ppt) and 3) Nano-silver (Ag 20 ppt). The animals used in this study were zebrafish (*Danio rerio*, wild type). The biological effects of nano-silver during embryogenesis and after hatching were investigated. Also, the catalase activity and expression of the Sel N1 gene were investigated.

Experimental Section

The characterizations of nano-sized silver material. The Nano-silver material was purchased from N corporation (Korea) and diluted with water to a concentration of 500 ppm. This type of silver is widely used within 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, a small drop of the supernatant then placed on copper grids and dried at room temperature. Nano-silver specimens were prepared by placing several drops of nano-silver solution onto glasses and dried at 35 ± 5 °C, and then subjected to X-ray diffraction (XRD, model PW 1830, Philips), with nickel-filtered *CuKα* 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. Also, nano-silver solution was added to HCl (1:1) to generate a precipitate, which was treated as outlines above. The prepared precipitates, including nanometer-sized silver, were analyzed by powder X-RD. 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.

Experimental animals. Zebrafish (*Danio rerio*, wild-type), approximately 7-8 months old and bred in our laboratory, were used. The zebrafish breeding conditions, development stage, morphology and hatching rate were examined according to previous research.[16] A 60 L glass water tank contained the aquatic environment, which was filtered by a carbon filter. The water temperature was maintained at 28 ± 1 °C; with a light/dark cycle 14/10 h. Adult fish were fed blood worms, dry flake food and brine shrimp. Eggs were laid and fertilized within 1 h of the beginning of the light cycle, which provided large populations of synchronously developing embryos. The embryos were collected, pooled and rinsed with several times. Embryonic staging was carried out according to the standardized staging series set forth by Kimmel *et al*.[17] The embryos were immersed in exposure or vehicle control solutions at the 64- to 256-cell stages, and 2.5 hour post-fertilization (hpf). Dead embryos were removed to avoid contaminating the test solutions. Embryos were observed with a microscope (Olympus, SZ61, Japan) to determine the morphological effects.

Chemicals exposure during development stage. The nano-silver stock solution was diluted with city water, which was allowed to stand for 24 hours to evaporate the chlorine. The final nano-silver exposure concentrations were 10 and 20 ppt. Each group of embryos were placed in 1 L glass beakers and maintained in a carbon-filtrated water system at 28 ± 1 °C. Each group contained 300 variable embryos.

Embryos were randomly divided into the following groups: Group 1 was the general control group; Groups 2 and 3 were exposed to two nano-silver concentrations (10 and 20 ppt, respectively). Embryos were observed at 2, 5, 8, 22, 27, 32, 48, 52 and 72 hpf, which were time points based on known developmental stages (Kimmel *et al.*, 1995). Dead embryos were removed during development. The hatching rates were calculated at 72 hpf for the experimental and control groups.

Biological effects of nanometer sized silver. Samples were taken from each group and treated as described below. Five zebrafish were homogenized in 1 mL phosphate buffer (0.1 M, pH 7.3). The homogenate was centrifuged at 9000 × g for 5 min at 4 °C. The supernatant, containing the enzyme, was reacted with H_2O_2 for 1 min at 37 °C. The reaction was stopped with 32.4 mmol/L ammonium molybdate, and the enzyme activity measured at 405 nm with a UV-Vis spectrophotometer (UV-1601PC, Shimadzu, Japan). The expression of the sepn1 gene in wild-type zebrafish was assessed by RT-PCR performed on total RNA samples isolated from 72 hpf flies (n = 30), using a modification to the method described by Deniziak *et al.*[12] Total RNA was obtained using the easy-BLUE™ Total RNA Extraction Kit (iNtRON Biotechnology, Inc.) according to the manufacturer's instruction. Reverse transcription was performed using 8 u of a One-Step RT-PCR premix kit (iNtRON Biotechnology, Inc.), ~200-500 pg of each total RNA matrix and 10 pmol of AL 299 reverse primer hybridizing in the 3 region of the SelN coding sequence (5'-GAGAGCTCTTGACAGAGG-3'). The reaction was conducted at 45°C for 60 min, in a 20 μL reaction mixture, containing 50 mM KCl, 4 mM $MgCl_2$, 50 mM Tris–HCl pH 8.3, 10 mM DTT, 1 mM dNTPs mix and 20 u RNasin (Promega). For each RNA sample, a "RT control" (reaction without reverse transcriptase) was performed. Reverse transcribed cDNAs (5 μL) were then amplified in a 50 μL PCR reaction, containing 10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/mL BSA, 200 μM each dNTP, 20 pmol of each primer and 1 u Taq Polymerase (Qbiogene). PCR was conducted under the following conditions: one cycle of denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 45 s, 65 °C for 1 min and 72 °C for 2 min; amplification was terminated by one cycle of elongation at 72 °C for 10 min. The following oligonucleotides were used in the PCR reaction: sepn-ex1, hybridizing to the 3 region of exon 1 (5'-GGGACTCCATCCAGCAG-ACG-3') and sepn-ex3, a reverse primer complementary to exon 3 (5'-TGCAGGGTCAGCGTCTCTCC-3'). Amplification products were analyzed by electrophoresis on a 1.2% agarose gel. The band intensities of the amplification products were obtained using gel documentation and analysis systems (UVITEC, Cambridge, UK). All graphs and statistical analyses were carried out using Excel 2003 (Microsoft, USA). All data were collected for groups consisting of 300 embryos. Each experiment was conducted in triplicate. Data were analyzed using a paired one-tailed Student's *t*-test to determine the lowest statistically significant concentration relative to an unexposed baseline control.

Results

Characteristics of commercial nanometer sized silver material. TEM images of the nanometer sized silver material are shown Figures 1A and B. The silver particles, supported by Ti (Figure 1A) 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 supporter material was proven to be titanium oxide. When HCl was introduced into the commercial nanometer sized silver solution, a precipitate formed (Figure 2A). The precipitate according to X-RD analysis was AgCl (Figure 2C),

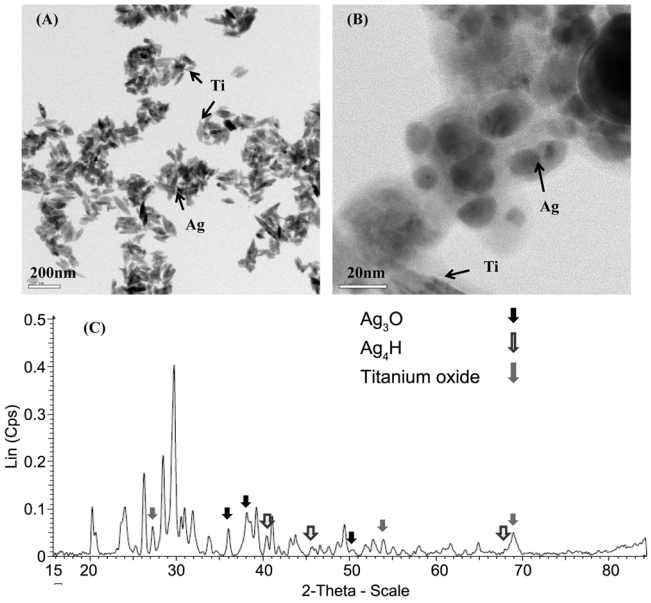

Figure 1. HRTEM images of nanometer sized silver are shown in (A) and (B). The nano-silver sol (A), and extension images (B). The X-RD pattern is shown in (C). There are several compounds (Ag_3O, Ag_3H and titanium oxide) in the nanometer sized silver material.

with a size of above 800 nm (Figure 2B). From these results, the presence of silver ions in the commercial nanometer sized silver solution was confirmed.

The effects of nanometer sized silver on the development stages of zebrafish. The effect of nanometer sized silver on the embryogenesis zebrafish is shown Figure 3. The embryos were evaluated 48 hpf (hours post fertilization) during the Long-pec stage. In the control group, black colored eyes, vacuolated differentiating cells in the notochord, and a yolk sack approximately equal to the volume of the head were observed. However, the groups exposed to nanometer sized silver very seriously injured flies were detected, and many had abnormal notochord development, which were very short and curved in both (10 and 20 ppt) groups. Furthermore, the eyes were not developed in some flies (Figures 3B-2 and C-1). At this stage, the size of the yolk sack was approximately equal to the head, but the size of the head was smaller than the yolk sack in fish with damaged eyes. Figure 4 denotes the accumulation of specific abnormal morphologies/total numbers of surviving individuals (%). Almost all the individuals in the nano-silver (10 ppt and 20 ppt) exposed groups had abnormal properties, including weak heartbeats, edema and abnormal notochords. Especially, there were increased edema and weak heartbeats in the 20 ppt nanometer-sized silver exposed group compared with those in the 10 ppt and control groups. The hatching rates were significantly decreased in both the nano-silver (10 ppt and 20 ppt) exposed groups compared to the control (Figure 5A). The catalase activities in the hatched flies of both the treated groups were increased compared to the control, while that in the 20 ppt nano-silver group was significantly greater than in the control group (Figure 5B). The RT-PCR results of the Sel N-1 gene expression in the hatched zebrafish are noted in Figure 6. Sel N1 and N2 are located on exons 1and 3, respectively. Line 0 in Figure 6 denotes the expression marker. β-actin is shown at 536 bp, Sel N1 is found at 317 bp and Sel N2 is at 196 bp. The data

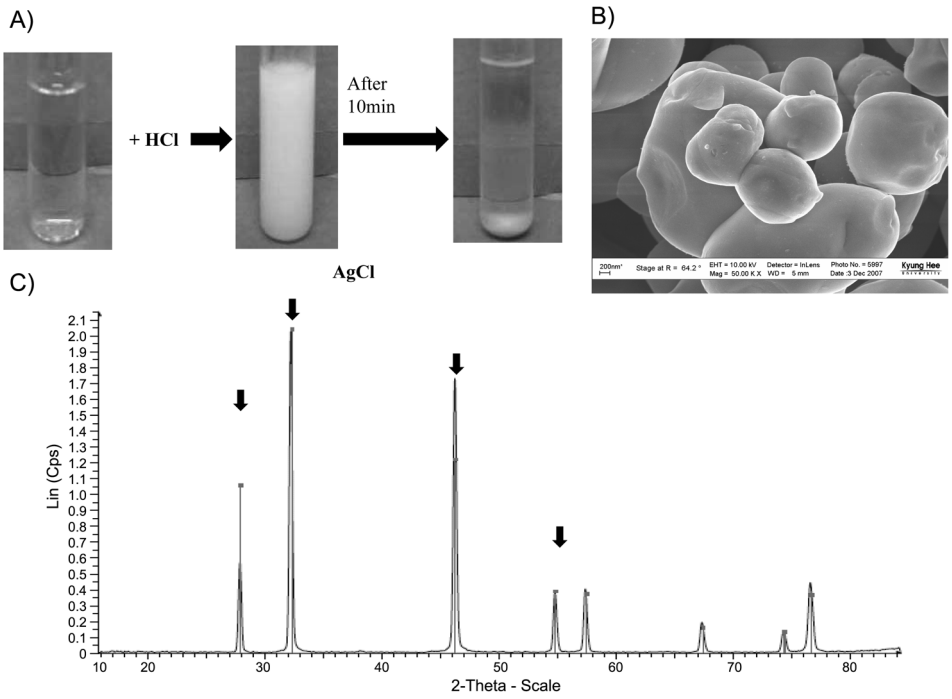

Figure 2. The property of nanometer sized silver materials. The process for the formation of the nano silver precipitate is shown in (A). The FE-SEM analysis of the precipitate (B); the X-RD pattern of the precipitate (C). The sediment analysis yielded AgCl by X-RD, which suggests the presence of Ag ions in the nanometer the silver solutions.

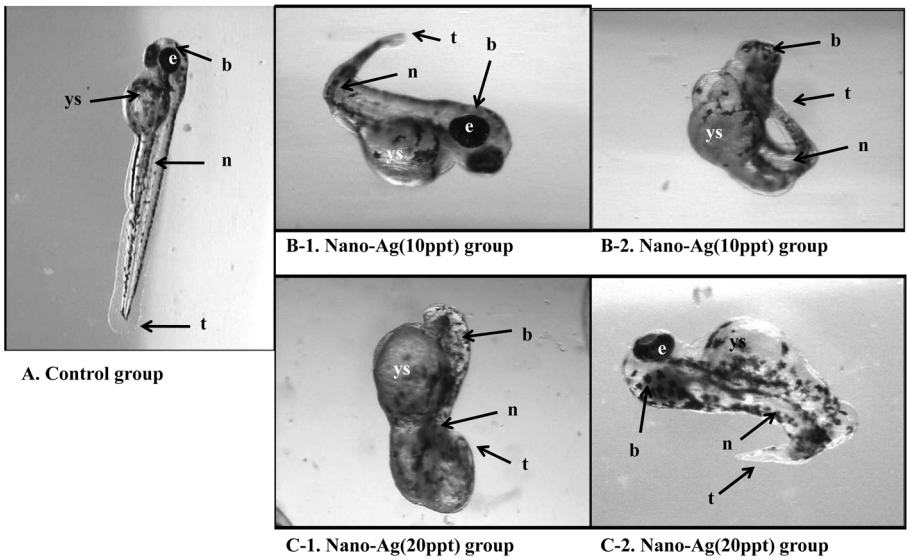

Figure 3. The effects of nanometer sized Ag on the development of zebrafish. Embryos were exposed to Ag 10 ppt (B-1, B-2) and Ag 20 ppt (C-1, C-2). These images show exposed zebrafish at 48 hpf. *Abbreviations*: b, brain; e, eye; n, notochord; t, tail; ys, yolk sac.

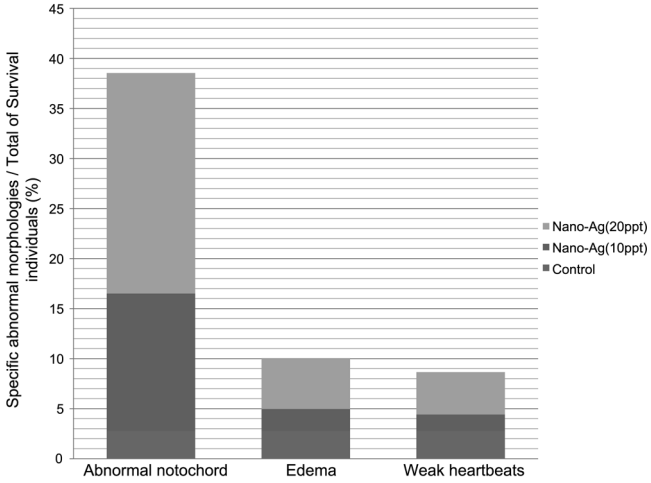

Figure 4. Rates of specific abnormal morphologies among surviving embryos are shown. The properties of abnormal morphologies are weak heartbeats, edema and abnormal vertebra.

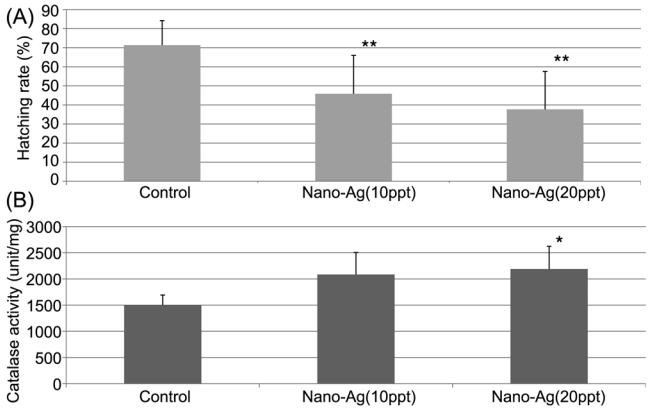

Figure 5. The effects of nanometer sized silver on the hatching rate (A) and catalase activity (B). The hatching rate decreased in groups exposed to nanometer sized silver (10 ppt, 20 ppt) compared to the control group. The catalase activities in the nano-silver exposed groups are also shown to be increased. In particular, the catalase activity in the 20 ppt nano-silver exposed group is significantly greater than that in the control group.

for the control group are shown in 1-1 (β-actin), 1-2 (Sel N1) and 1-3 (Sel N2). The data lines for the 10 ppt nanometer-sized silver exposed group are noted at 2-1 (β-actin), 2-2 (Sel N1) and 2-3 (Sel N2). Data for zebrafish treated with 20 ppt of nanometer sized silver are shown in 3-1 to 3-3 (Figure 6A). The band intensity of the amplification products was obtained using gel documentation and analysis systems (Figure 6B). Gene expressions were made relative to β-actin by calculating the relative band intensities. Values are represented as Sel N1 or Sel N2/β-actin expression ratios (n = 30). In the exposed groups (10 and 20 ppt), the Sel N2 gene expression decreased compared to the control group. In particular, when exposed to a high concentration (20 ppt), the Sel N gene expression was greatly reduced. This phenomenon was shown for both Sel N1 and Sel N2. Furthermore, the Sel N2 expression in the 20 ppt exposed group was about 38% that of the control group.

Discussion

The material analysis of the commercial nanometer sized silver demonstrated that silver is unstable in ion form (Figures 1 and 2). The largest application of commercial nanometer sized silver is in antibacterial products. It is widely known that the antibacterial effect of silver ions is better than that of stable metal silver. Silver ions generated electrically have good antibacterial effects.[18] Textiles have the antibacterial property that is used for underwear, sacks and baby wear in Korea. At present, the texture of the coating or attachment methods of nanometer sized silver is still under development.[19] In the present study, the nanometer sized silver ions in commercial products are discussed (Figures 1 and 2). Silver ions could increase the antibacterial effect; moreover, the nanometer size results in an increased

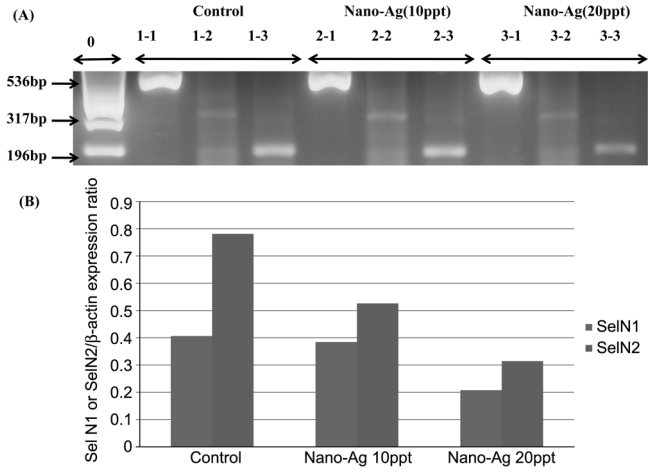

Figure 6. RT-PCR results for the Sel N-1 and Sel N-2 genes in hatched zebrafish under nanometer sized silver exposed conditions (10 ppt and 20 ppt). (A) The line 0 denotes the marker. β-actin is at 536 bp, Sel N1 is found at 317 bp and Sel N2 is at 196 bp. The data for the control group are shown in 1-1 (β-actin), 1-2 (Sel N1) and 1-3 (Sel N2). The data for the 10 ppt nanometer sized silver exposed group are 2-1 (β-actin), 2-2 (Sel N1) and 2-3 (Sel N2). The data lines for the 20 ppt nanometer sized silver exposed group are denoted by 3-1 to 3-3. (B) Band intensities of the amplification products were obtained using gel documentation and analysis systems. The gene expression was evaluated relative to β-. Values are represented as Sel N1 or Sel N2/β-actin expression ratios in zebrafish (n = 30).

surface area. Furthermore, from the analyses, the nanometer sized silver was found to remain on the support material (Ti) (Figure 1). If the rejoined nanometer sized materials form a complex, the properties of such materials will be likely to change.

From this result, it is suggested that nanometer sized silver materials should be in the ionic form for use in antibacterial applications. However, silver ions could join with other materials, resulting in a secondary contaminant that may flow into another ecosystem. Upon binding with another material, the properties of the nanometer sized materials are likely to be lost. However, nanometer sized silver not bound to another material could enter a cell. The size relationship observed in this study has been reported by several other researchers.[8,20,21]

The Ag ion is a type of metal ion; metal ions, such as Mg^{2+}, act as cofactors to enzymes that utilize nucleotides as substrates and synthesize or cleave DNA.[22,23] Also, Ag^+ is known to undergo strong covalent binding with DNA. Furthermore, the PBR 322 plasmid when exposed to Ag^+ has been reported to suffer DNA damage. The increased DNA damage was believed to be due to the free radicals produced from the oxidation of ascorbate by molecular oxygen, where the Ag^+ ion played a catalytic role.[24]

In this study, exposure to nanometer sized silver caused zebrafish embryos to develop abnormally (Figure 3), which may be due to nanometer sized silver entering the embryo membrane. Ag^+ ions could bind DNA, resulting in possible DNA damage. Abnormal notochords and very weak heart beat were observed; furthermore, some of the flies had no or damaged eyes. Specifically, when the concentration of nano-silver was high (20 ppt), the noted abnormalities increased. The extent of exposure is suggested to be related to the observed abnormalities. In particular, the presence of abnormal notochords was increased in both the nanometer silver exposed groups (10 and 20 ppt). The Sel N gene has a relationship with abnormal notochord development and heart disease during the early development stages in zebrafish[12] and humans.[7] In this study, the Sel N1 gene expression was decreased in the nanometer silver exposed groups compared to the control group. Moreover, the Sel N2 gene expression was decreased in the nanometer silver exposed groups relative to control group. Therefore, the nanometer sized silver material is suggested could enter the nucleus and; thereby, affect the gene expression. Furthermore, this result is similar with the reported DNA damage associated with Ag^+ metal ion,[24] but the authors did not report the type of gene damage. The zebrafish is a species of vertebrate that live in an aquatic environment and have homologous genes with humans.[25] In this study, the aquatic environment consisted of civil water used in a washing machine with an electrically produced Ag^+ product. Of course, other ions or material are present in civil water, but all of the Ag^+ ions will not bind with other ions. Since civil water formed via several filtering steps, a lot of material and ions have to be removed to produce water suitable for drinking.

Ag^+ ions in commercial nanometer sized silver could flow into an aquatic environment, where they could possibly cause DNA damage during the early development stages in zebrafish. This may be the cause of the decreased hatching rate observed in the nanometer silver exposed groups in this study. Although anticancer activity is associated with its covalent bonding to the N7 centers of guanine and adenine in DNA,[26] metal ions, including Ag^+, could covalently bind with DNA,[24] and the resultant anticancer activity could be affected by metal ion binding. In this study, the antioxidation enzyme catalase activities were increased in the nanometer sized silver exposed groups (10 and 20 ppt). This result, like that of the increased DNA damage, was believed to be due to the free radicals produced,[24] while it is the role of catalase to remove free radicals. The increased catalase activities in the Ag^+ exposed groups are suggested to be the result of DNA damage due to free radicals.

Conclusion

In conclusion, exposure to commercial nanometer sized silver significantly decreased the hatching rate of zebrafish embryos; furthermore, the hatched embryos exposed to nanometer sized silver had abnormal notochord, weak heart beat and curved tail. In the nanometer sized silver exposed group, the Sel N1 and Sel N2 gene expressions were also decreased compared to those in the control groups. The catalase activities of the exposed groups increased relative to those in the control group. The commercial nanometer sized silver was in the ion form, and as a result, could flow into

aquatic environments, resulting in serious damage to the development of zebrafish embryo.

References

1. Lee, H. J.; Yeo, S. Y.; Jeong, S. H. *J. Mater. Sci.* **2003**, *38*, 2199.
2. Harper, *T. Nano Korea* (<http://www.nanotechweb.org/>), 2003.
3. Hamouda, T.; Hayes, M. M.; Cao, Z.; Tonda, R.; Johnson, K.; Wright, D. C. *J. Infect. D* **1999**, *180*, 1939.
4. Sondi, I.; Salopek-Sondi, B. *J. Colloid Interf. Sci.* **2004**, *275*, 177.
5. Lundborg, M.; Johansson, A.; Lastbom, L.; Camner, P. *Environ. Res.* **1999**, *81*, 309.
6. Lundborg, M.; Johard, U.; Lastbom, L.; Gerde, P.; Camner, P. *Environ. Res.* **2001**, *86*, 244.
7. Rederstorff, M.; Krol, A.; Lescure, A. *Cell Mol. Life Sci.* **2006**, *63*, 52.
8. Möller, W.; Hofer, T.; Ziesenis, A.; Karg, E.; Heyder, J. *Toxicol. Appl. Pharm.* **2002**, *182*, 197.
9. Yeo, M. K.; Jo, Y. H. *J. Environ. Sci.* **2007**, *22*, 189.
10. Reijnders, L. *J. Clean Prod.* **2006**, *14*, 124.
11. Yeo, M. K.; Lee, J. Y. *J. Environ. Sci.* **2006**, *15*, 471.
12. Deniziak, M.; Thisse, C.; Rederstorff, M.; Hindelang, C.; Lescure, A.; Thisse, B. *Exp. Cell Res.* **2007**, *313*, 156.
13. Chitra, K. C.; Latchoumycandane, C.; Mathur, P. P. *Toxicology* **2003**, *185*, 119.
14. Kabuto, H.; Hasuike, S.; Minagawa, N.; Shishibori, T. *Environ. Res.* **2003**, *93*, 31.
15. Kabuto, H.; Amakawa, M.; Shishibori, T. *Life Sci.* **2004**, *74*, 2931.
16. Yeo, M. K. *Kor. J. Env. Hlth.* **2003**, *29*, 1.
17. Kimmel, W.; Ballard, S.; Ullman, B. K.; Schilling, T. *Dev. Dynam.* **1995**, *203*, 253.
18. Berger, T. J.; Spadaro, J. A.; Chapin, S. E.; Becker, R. O. *Antimicrob. Agents Ch.* **1976**, *7*, 357.
19. Chung, H.; Kim, B.; Yang, H. *J. Kor. Soc. Cloth. Text.* **2005**, *29*, 805.
20. Lambert, A. L.; Mangum, J. B.; DELorme, M. P.; Everitt, J. I. *Toxicol. Sci.* **2003**, *72*, 339.
21. Renwick, L. C.; Donaldson, K.; Clouter, A. *Toxicol. Appl. Pharm.* **2001**, *172*, 119.
22. Brooker, R. J.; Slayman, C. W. *J. Biol. Chem.* **1983**, *258*, 8833.
23. Black, C. B.; Huang, H. W.; Cowan, J. A. *Coordin. Chem. Rev.* **1994**, 135, 165.
24. Hossain, Z.; Huq, F. *J. Inorg. Biochem.* **2002**, *91*, 398.
25. Miriam, A.; Moriarty, E. D.; Martin, L. B.; Maura, G. *Biochem. Biophys. Res. Commun.* **2008**, *367*, 124.
26. Bloemink, M. J.; Reedijk, J. In *Metal Ions in Biological Systems*; Sigel, A.; Sigel, H., Eds.; Marcel Dekker: New York, 1996; Vol. 32, p 641.