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Original Article

Toxicity Test of Nanosilver Particles on Zebrafish (*Danio rerio*) Embryonic Development

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Abstract

Contamination of the environment with Nanosilver particles (AgNPs) has caused considerable concern. At present there is little information of AgNPs toxicity, especially in aquatic environments. The purpose of this study was to evaluate toxic effects of AgNPs on Zebrafish (*Danio rerio*) embryonic development. Toxicity test of AgNPs on the embryos was conducted in concentration of 0.05, 0.1, 0.5, 1 and 5 µg/ml. Standard water was used as control. Observation for mortality, morphological and functional abnormalities at 4, 24, 48 and 72 hours post fertilization (hpf) were performed. The results showed that AgNPs at 1 and 5 µg/ml had significant lethal effect on the embryos. Calculated LC50 at 48 hpf of AgNPs to the embryos was 1.78 µg/ml. In concentration of 1 µg/ml, decreased movement rate and hatchability of the embryos were significantly different when compared to the controls. No remarkable toxic effect was found in the embryos that were exposed to 0.05 0.1 and 0.5 µg/ml AgNPs when compared to the controls. In summary, colloidal AgNPs could be a potential cause of the adverse effects involving mortality and altered physiological functions to aquatic embryos. Silver ion might play an important role in toxic mechanism.

Keywords: *Danio rerio*, nanosilver particles, toxicity, zebrafish embryos

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บทคัดย่อ

การทดสอบความเป็นพิษของอนุภาคนาโนซิลเวอร์ต่อพัฒนาการของตัวอ่อนปลาม้าลาย

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ปัญหาการปนเปื้อนสู่สิ่งแวดล้อมของอนุภาคนาโนซิลเวอร์ได้รับความสนใจและกังวลเป็นอย่างมาก ในปัจจุบันมีข้อมูลเพียงเล็กน้อยในการศึกษาความเป็นพิษของอนุภาคนาโนซิลเวอร์ โดยเฉพาะอย่างยิ่งความเป็นพิษต่อสิ่งมีชีวิตและสิ่งแวดล้อมในน้ำ การศึกษาในครั้งนี้เป็นการศึกษาผลของความเป็นพิษของอนุภาคนาโนซิลเวอร์ต่อการพัฒนาของตัวอ่อนปลาม้าลาย (***Danio rerio***) ที่ความเข้มข้น 0.05 0.1 0.5 1 และ 5 ไมโครกรัมต่อมล. และใช้สารละลายมาตรฐานเป็นกลุ่มควบคุม โดยจะทำการสังเกตอัตราการตาย ความผิดปกติทางโครงสร้างและการทำหน้าที่ของอวัยวะต่างๆในช่วงการพัฒนาของตัวอ่อนปลาม้าลาย ที่เวลา 4 24 48 และ 72 ชั่วโมงหลังการผสม จากผลการทดลองพบว่าตัวอ่อนปลาม้าลายที่ได้รับ อนุภาคนาโนซิลเวอร์ที่ความเข้มข้น 1 และ 5 ไมโครกรัมต่อมล. มีอัตราการตายมากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ จากการคำนวณค่าความเป็นพิษเฉียบพลันของอนุภาคนาโนซิลเวอร์พบว่ามีค่าเท่ากับ 1.78 ไมโครกรัมต่อมล. ในกลุ่มทดลองที่ความเข้มข้น 1 ไมโครกรัมต่อมล.ของอนุภาคนาโนซิลเวอร์พบตัวอ่อนมีอัตราการเคลื่อนไหวและการฟักเป็นตัวลดลงอย่างมีนัยสำคัญ ส่วนในกลุ่มทดลองที่ความเข้มข้นที่ 0.05 0.1 และ 0.5 ไมโครกรัมต่อมิลิลิตรของอนุภาคนาโนซิลเวอร์ ไม่พบความเป็นพิษใดๆ เมื่อเทียบกับกลุ่มควบคุม จากผลการทดลองข้างต้นอาจสรุปได้ว่า อนุภาคนาโนซิลเวอร์สามารถที่จะทำให้เกิดความเป็นพิษต่อตัวอ่อนของสัตว์น้ำได้ โดยความเป็นพิษดังกล่าวอาจจะเกิดจากซิลเวอร์อิออนที่เกิดจากอนุภาคนาโนซิลเวอร์

คำสำคัญ: *Danio rerio* อนุภาคนาโนซิลเวอร์ ความเป็นพิษ ตัวอ่อนปลาม้าลาย

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Introduction

Nanosilver particles (AgNPs) have been used extensively in variety of research and industrial fields. Nowadays AgNPs can be found in a lot of daily consumer products. Some products containing AgNPs such as detergents and wound dressings are increasingly and broadly used and possibly end up in the environment during waste disposal. Because of the wide and rapid use, the contamination of the environment with AgNPs has caused considerable concern. Several studies reported that nanoparticles were exposed to the environment (Hund-Rinke et al., 2006; Moore, 2006; Wiench et al., 2009; Van Hoecke et al., 2011) and had significant effects on aquatic life organisms. Luoma and colleagues (2008) reported that AgNPs either in nanoparticle or ion form could enter and accumulate in the living aquatic organisms. Environmental effects of nanoparticles are still questionable and remain unsolved.

Zebrafish (*Danio rerio*) is one of the most widely used laboratory species because they enable embryological manipulations and large-scale genetics studies. Small housing space, reduced costs of husbandry and experimental materials, and minimized volumes of waste for disposal are the advantages of the use of zebrafish in various disciplines. Zebrafish embryos have been used as screening tests for chemical toxicity, small molecule and drug discovery (Teraoka et al., 2002; MacRae and Peterson, 2003; Scholz, 2008) because of their small size, which allow reasonable sample sizes and series of experimental replicates in single plate environment. In addition, the transparency of the embryos can be clearly and easily observed for the development and malformation of embryonic stages (Spitsbergen and Kent, 2003).

At present, there is little information of AgNPs toxicity especially in aquatic environment. Therefore, the purpose of this study was to examine the ecotoxic effects of AgNPs on Zebrafish embryonic development.

Materials and Methods

Preparation and characterization of AgNPs: High concentration of colloidal AgNPs solution was

synthesized via chemical reduction process according to the method previously described (Maneewattanapinyo et al., 2011). Briefly, a 0.094 M aqueous solution of silver nitrate ($AgNO_3$; Merck) was prepared with soluble starch (Merck) as a stabilizer. An aqueous solution of 0.07M sodium borohydride ($NaBH_4$; Merck) reducing agent with the soluble starch solution as a solvent was sequentially prepared. By mixing both solutions, the $AgNO_3$ solution was added dropwise to the $NaBH_4$ solution under a vigorous stirring. A dark cloud appeared and turned to yellowish brown within a few seconds. When all reactants were completely added, the solution turned dark brown. The purification of the AgNPs was precipitated by using the centrifugation. Then, the precipitates of AgNPs were washed three times with DI water and adjusted the same volume before dilution. Synthetic AgNPs was very pure (99.96%) and Ag ions were very low in concentration (less than 0.04%). The AgNPs solutions were diluted with distilled water to obtain various concentrations of AgNPs prior to use. The particle morphology of AgNPs was observed using JEOL JEM-2010 analytical transmission electron microscope. The AgNPs had a spherical configuration which had a primary particle diameter of 10-20 nm. The plasmon extinction of AgNPs measured by Ocean Optics Portable UV-Visible spectrometer (USB 4000-UV-VIS detector) showed that the size of distribution of AgNPs was narrow (Maneewattanapinyo et al., 2011).

***Experimental animals*:** Wild type Zebrafish, 6 months old, were purchased from Veterinary Medical Aquatic Research Center (VMARC), Chulalongkorn University and acclimated for more than one week prior to the experiment. The adult zebrafish were maintained in 62.5-liter glass aquaria filled with 20-liter tap water. Air pump with aquarium foam sponge filter was used for the aeration system. Water temperature, pH, and dissolved oxygen (DO) were measured daily during the experiment. Average water temperature was maintained at 27-29^{0}C with 14/10 hour of light/dark cycle. All fish were fed twice daily commercially dry flake food and blood worms throughout the experimental period. All procedures were approved by the ethics committee of Chulalongkorn University Animal Care and Use Committee (CU-ACUC).

Zebrafish embryos were collected and rinsed several time with tap water. Healthy fertilized embryos were selected and pooled in the standard water. Standard water was prepared by the combination of 0.1176 g $CaCl_2 2H_2O$, 0.0493 g $MgSO_4 7H_2O$, 0.0259 g $NaHCO_3$ 0.0023 g and 1 liter deionized water (OECD, 2006). Embryonic staging was performed following standard protocols (Kimmel et al., 1995). For embryonic toxicity test, cleavage (two or four-cell) stage embryos were divided and immersed in 5 different concentrations of AgNPs (0.05, 0.1, 0.5, 1 and 5 µg/ ml) and standard water solution as control. Twelve healthy embryos were transferred to the wells of a 96 well plate along with 1 ml of AgNPs and control solutions to determine mortality, development, morphology and hatching rate using light stereomicroscopy. Total number of dead embryos was recorded at 4, 24, 48 and 72 hours for mortality rate. Morphological and physiological abnormalities including organ deformation, growth retardation, scoliosis, deformity of yolk sac, movement rate, heart rate, circulatory disturbance and reduced pigmentation were determined at different stages (24, 48 and 72 hpf). Hatching rate and time of hatching were recorded at 72 hpf (Nagel, 2002; Umwelt Bundes Amt, 2006; OECD, 2006).

Statistical analysis: All data in graphs were presented as mean ± standard error (SE). Data were analyzed using analysis of variance (ANOVA). Values of $p< 0.05$ were considered as level statistical significance. All statistical analyses were carried out using the SPSS statistical software for Windows, version 12.

Results

The cumulative mortality, morphological and physiological abnormalities of zebrafish embryos were evaluated 4, 24, 48 and 72 hpf. At 4, 24 and 48 hpf, normal development of somite, eye and tail were observed in control embryos. The number of coagulated eggs was determined after 4, 24 and 48 hours. Coagulated or death embryos were white opaque appearance and showed dark appearance under the stereomicroscopy (Fig 1). The highest concentrations

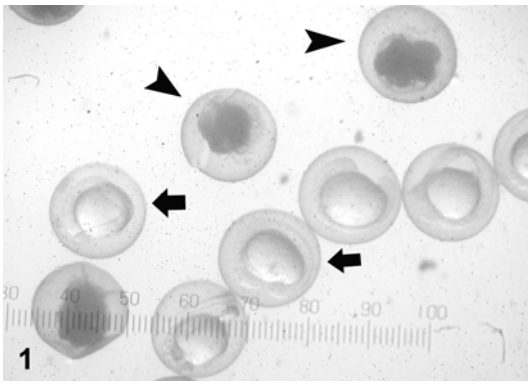

Figure 1 Embryos were exposed to 1 µg/ml AgNPs at 48 hpf. Controlled embryos showed normal development (Bold arrows). Coagulated embryos revealed brownish flakes (arrowheads).

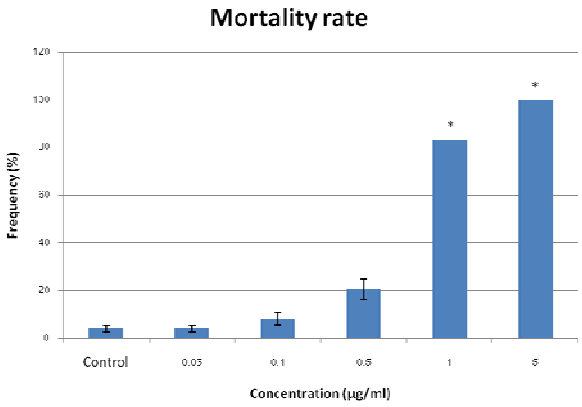

Figure 2 Effects of AgNPs in terms of mortality at 72 hpf. A significant increase in mortality was observed in 1 and 5 µg/ml of AgNPs treated embryos. Results are expressed as mean±SE. All experiments were repeated

three times.

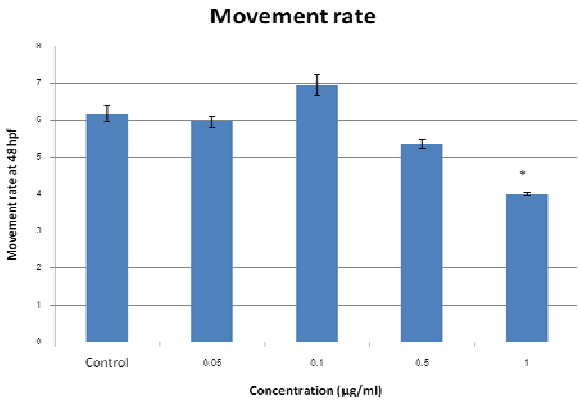

Figure 3 Effects of AgNPs on movement rate. A significant decrease in movement rate was observed in 1 µg/ml of AgNPs treated embryos. Results are expressed as mean±SE. All experiments were repeated three times.

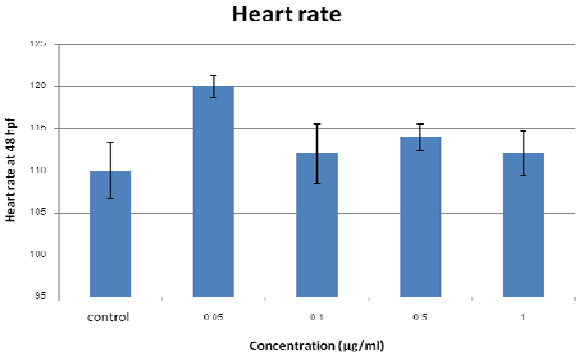

Figure 4 Effects of AgNPs on the heart rate. No significant difference between treated groups and controls. Results are expressed as mean±SE. All experiments were repeated three times.

of AgNPs (5 µg/ml) showed the mortality rate of 75 and 100% at 4 and 24 hpf, respectively (Data not shown). At 48 hpf, a significant increase in mortality was observed in 1 and 5 µg/ml of AgNPs treated embryos (Fig 2). Calculated median lethal concentration (LC50) at 48 hpf of AgNPs to the zebrafish embryo was 1.78 µg/ml. Abnormality of morphology and function were also found in embryos treated with AgNPs. At 1 µg/ml concentration of AgNPs, a significant decrease in movement and hatching rate was observed (Fig 3 & 5). Moreover,

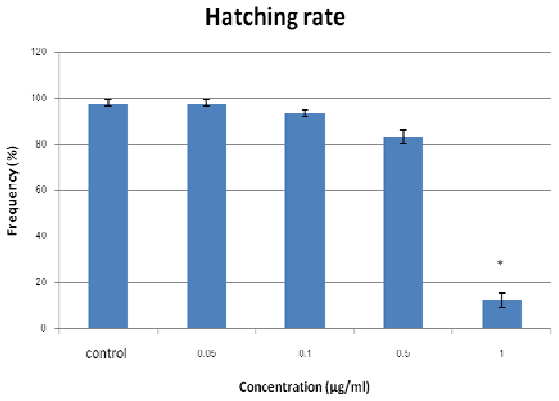

Figure 5 Effects of AgNPs on hatching rate from the total number of surviving embryos in each concentration. The hatching rate significantly decreased in groups exposed to 1 µg/ml of AgNPs when compared to the control. Values are expressed as mean±SD of three experiments.

other defects including tail deformation and weak heartbeat were found in some treated embryos (Table 1). No significant difference in heart rate between the control and treated groups (Fig 4) was found. There were no significant abnormalities in embryos treated with other lower concentrations of 0.05, 0.1, 0.5 µg/ml AgNPs compared to the controls (Table 1). The mortality rate and other abnormalities of embryosexposed to 0.05 µg/ml AgNPs were comparable with control samples, suggesting an absence of toxic effects. Therefore, no observed effect concentration (NOEC) of AgNPs in the present study was 0.05 µg/ml.

Discussion

The effects of AgNPs on lethal response and embryogenesis on the zebrafish were evaluated in different time points. The groups exposed to high doses (1 and 5 µg/ml) of AgNPs showed acute severe injury and death. The increased mortality rate of the treated embryos was observed with increase in concentration. Hatching rate decreased significantly in 1 µg/ml exposed group compared with the control. Morphological and physiological abnormalities

Table 1 Compiled data table of morphological and physiological abnormalities among surviving embryos after 72 hpf in each concentration of AgNPs and control

Morphological and physiological abnormalities	Concentration of nanosilver particles (µg/ml)					
	Control	0.05	0.1	0.5	1	5
Head deformation	0	0	0	0	0	-
Tail deformation	0	0	0	0	1	-
Heart deformation	0	0	0	0	0	-
Somite deformation	0	0	0	0	0	-
Otolith deformation	0	0	0	0	0	-
Scoliosis	0	0	0	0	0	-
Yolk sac deformation	0	0	0	0	0	-
Growth retardation	0	0	0	0	0	-
Reduced pigmentation	0	0	0	0	0	-
Weak heartbeats	0	0	0	0	1	-
Circulatory disturbance	0	0	0	0	0	-

including decreased movement rate, tail deformation and weak heartbeat were also detected in some treated embryos of 1 µg/ml treated group.

Several studies investigated the toxicity effects of silver nanoparticles in various aquatic test organisms. Asharani et al. (2008) reported that 5-20 nm diameter of silver nanoparticles with capping agents (Ag-bovine serum albumin and Ag-starch) in zebrafish embryos had the LC50 of 25-50 µg/ml and could cause mortality, hatching delay, abnormal body axes, twisted notochord, slow blood flow, pericardial edema, cardiac arrhythmia, decreased heart rate and growth retardation. Kennedy et al. (2010) studied the toxicity of nanoparticles of AgNO3 with varying primary particle sizes of 10-80 nm in *Daphnia magna*, *Pimephales promelas* and *Pseudokirchneriella subcapitata*. At 48 hpf, the LC50 values were in range of 0.002 - 0.126 µg/ml. The results from this study concluded that more ionic Ag+ or smaller size were detected, more toxic abnormality was observed in the embryos. In the present study, nanosilver particles had a spherical colloidal configuration with the primary particle diameter of 10-20 nm. Size distribution of particles was narrow. Releasing status of Ag+ ions from colloidal AgNPs was absent or in very low level. The LC50 AgNPs suspension in our study was 1.78 µg/ml.

Differences in the mortality, LC50 values and developmental abnormalities in each study mentioned above might be a result of various factors involving the releasing status of Ag+ ion during the preparation or experiment. The releasing of Ag+ from AgNPs is one of the important factors that can bind to DNA and possibly cause DNA damage due to oxidative stress formation. Ag+ ions can also induce the impairment of ion regulation and increase mortality (Guadagnolo et al., 2000). The value of 48-hour mean lethal concentration (LC50-48hpf) of AgNPs in Asharani et al., (2008) was higher than those reported in the present experiment. The higher values might be related to low level of Ag+ due to the using of bovine serum albumin and starch as AgNPs capping agents. The binding of AgNPs and capping agents resulted in decline in the Ag+ releasing level from the nanoparticles. When compare the value of LC50-48 hr in the present work with the studies of Kennedy et al. (2010), which used AgNPs with high level of Ag+ ions, the greater LC50 was observed in our study. In the present study, the level Ag ions were very low in concentration, showing less than 0.04% in 99.96% purity of AgNPs.

Functional abnormalities induced by AgNPs were also investigated in the present study. The abnormality of embryonic movement was observed in the groups exposed to AgNPs. Nanoparticles are known to have direct effects on nervous system. Earlier reports had suggested that the invasion of nanoparticles during early embryonic stages could distribute and deposit in the brain leading to interference of nervous system function and signal transduction processes (Oberdorster et al., 2004). The decreased hatching was also detected in AgNPs treated embryos. DNA damage and genetic abnormality due to the direct effects of AgNPs or Ag+ ion might be the cause of the interruption of embryonic development, resulting in abnormal hatching activity (Yeo et al., 2008).

In conclusion, exposure to colloidal AgNPs could cause mortality and some altered physiological functions, namely decreased movement and hatching rate in treated zebrafish embryos. Ag+ ion might play an important role in these serious damages. The pathogenesis after exposure to AgNPs in aquatic organisms remains unclear. Further studies including the pathological evaluation of affected embryos and other molecular techniques to elucidate the toxic mechanisms should be performed. The AgNPs used in this study was in colloidal form that could easily disperse and flow into aquatic environments, leading to possible adverse effects on aquatic organisms. Therefore, the control of the release of untreated AgNPs waste into the environment should be given special attention to for clean environment and good quality of life.

Acknowledgements

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