Determination of lethal concentration (LC50) of silver nanoparticles (AgNPs) produced by chemical methods in Zebrafish (*Danio rerio*)

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Abstract

The silver nanoparticles (AgNPs) pose significant risks to aquatic ecosystems due to their persistence and toxicity, as well as challenges related to their toxicological classification and regulation. Upon release into water, AgNPs undergo oxidation, forming toxic ionic silver (Ag^+), which exacerbates their harmful impact. To determine the lethal dose of AgNPs in this experiment, Zebrafish as a biological model animal were exposed to different concentrations of a type of colloidal AgNPs for 96 hours. Based on the results, The LC50 value for AgNPs in adult female zebrafish was determined to be 4.47 mg/L. The results showed that with increasing concentration of silver nanoparticles and also exposure time, the percentage of zebrafish mortality increased. The highest mortality rate was observed at the highest concentration of silver nanoparticles. In some treatments, fish showed signs of respiratory stress, during the hours they were exposed to AgNPs. Lower concentrations of silver nanoparticles elicited no observable behavioral alterations or mortality in the studied fish specimens.

Keywords: Biological model animal, Model organism, Zebra danio, Toxicity, Biosynthesis

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Introduction

The rapid expansion of nanoparticle research, particularly concerning noble metals like silver, has raised concerns about the potential toxicity of nanomaterials (NMs) on human and environmental health, as well as their appropriate toxicological classification and regulation. Silver nanoparticles (AgNPs), typically <100 nm, possess unique properties compared to bulk silver due to their size and shape, influencing their optical, thermal, and catalytic behavior. Consequently, AgNPs are widely used in microelectronics and as antimicrobial agents in consumer and medical products. However, incomplete removal of AgNPs during wastewater treatment leads to their accumulation in aquatic environments, where they can oxidize into toxic ionic silver (Ag^+) (Ihtisham *et al*., 2021), potentially causing adverse health effects (Gao, 2016) and ecotoxicological impacts (Yeo and Kang 2008; Qiang et al., 2020; Pilaquinga *et al*., 2021; He *et al*., 2022). In 2015, over 410 products contained AgNPs, with a global production exceeding 550 tons (Saleeb *et al*., 2019). While most forecasts focus on market value, available scientific literature estimates the global annual production of silver nanoparticles at 400 to 800 tons as of recent years (Pulit-Prociak and Banach 2016; Calderón-Jiménez *et al*., 2017).

The Zebrafish (*Danio rerio*), a small, omnivorous freshwater fish in the Danionidae family, is a widely used and biologically superior vertebrate model organism in developmental biology and human disease research compared to other model fish such as Clownfish (*Amphiprion* sp.), Medaka (*Oryzias latipes*), Swordtail (*Xiphophorus hellerii*), and Platy (X. *maculatus*) (Khiabani *et al*., 2014; Khiabani, Anvarifar, and Mousavi-Sabet 2016; Walter, Lu, and Savage 2019; Wang and Cao 2021; Khiabani 2024). However, each offers unique advantages, and their suitability depends on the specific scientific context. Zebrafish value stems from several advantages: genetic similarity to humans, rapid development, small size, high fecundity, ease of husbandry and genetic manipulation, and low maintenance costs. Approximately 84% of genes known to be associated with human diseases are present in zebrafish, making them highly relevant for studying human genetic disorders and diseases. Zebrafish have about 26,000 genes, with 71.4% orthologous to human genes (Kolb, Hildebrandt, and Lawrence 2018; Khiabani and Esteghlalian 2021). Consequently, zebrafish are employed in basic biological, environmental, ornamental aquacultural, and biomedical research (Fowler *et al*., 2019). Findings from zebrafish studies are highly relevant to both human and fish biology (Aleström, Holter, and Nourizadeh-Lillabadi 2006; Howe *et al*., 2013; Khiabani 2019). Zebrafish also exhibit regenerative capabilities (Akimenko *et al*., 1995; Goldshmit *et al*., 2012), and numerous transgenic strains have been generated through genetic modification (White *et al*., 2008), allowing for the study of induced disease pathologies

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(Siccardi III *et al*., 2009). However, many researchers use both zebrafish and medaka or other model organisms as complementary models to validate findings and explore mechanisms not evident in a single species. LC50 (Lethal Concentration 50%) is a common measure of environmental toxicity, representing the concentration of a substance in air or water that causes death in 50% of a test population during a specific exposure period (e.g., 4-hour inhalation or water immersion). Used for inhalation or aquatic exposure, it is measured in ppm, ppb, mg/l or mg/m³. The acute toxicity data provide information that is useful to identify the mode of action of a substance and also help with comparison of dose response among various chemical substances. The 96-h LC50 tests are conducted to assess the vulnerability and survival potential of organisms to particular toxic chemical substances. Chemical agents with lower LC50 values are more toxic because their lower concentrations result in 50% mortality in organisms (Tarkhani *et al*., 2012). According to the Organization for Economic Cooperation and Development (OECD) guidelines for the testing of chemicals, a traditional experiment involves groups of animals exposed to a concentration (or series of concentrations) for a set period of time (usually 4 hours). The animals are clinically observed for up to 14 days (OECD, 2019).

This study aimed to: (1) examine the correlation between zebrafish survival rates and the toxicity of water-soluble silver nanoparticles, and (2) identify the lethal concentration (LC50) of chemically synthesized silver nanoparticles in zebrafish to support future scientific research.

Materials and methods

To determine the lethal concentration (LC50) of silver nanoparticles colloidal solution, first a pilot study was performed on zebrafish casualty rate and then, based on the pilot study, the required concentrations in the main study were determined. The colloidal solution of silver nanoparticles (AgNPs) used in this study was a product of Company Zist Shimi Azma Roshd Iran (Zist Shimi Azma Roshd Co. Cat. No. NZ-AGNP-10-CS), and its technical data sheet is included in Table 1.

The lethal range was defined by the lowest concentration causing observable 96-hour mortality and the concentration resulting in 100% mortality. At this stage, in accordance with Organization for Economic Cooperation and Development (OECD, 2019) Test Guideline No. 203, before inducing silver nanoparticles to the target fish, a test to determine the lethal concentration (LC50) of silver nanoparticles was performed on similar fish (in terms of lifespan and approximate weight).

All steps of fish adaptation before the experiment, temperature and feeding were performed by the method previously described for group c (control) (Khiabani *et al*., 2020a; Khiabani *et al*., 2020b).

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Table 1: Technical data sheet for silver nanoparticles colloidal solution.

Properties	Chemicals/Test Methods	Value	Unit
Formula	Silver	Ag	-
Formula weight	-	107.86	g/mol
Appearance	-	Solution	-
Color	-	Black	-
Particle size	TEM	10	nm
Size distribution	DLS	6-35	nm
λmax	Spectrophotometer	395-410	nm
Optical density	Spectrophotometer	1.4	-
pH	pH meter	7	-
Purity	Atomic absorption	99.9	%
Morphology	TEM	Spherical	-
Dispersant	Water	-	-
Stabilizer	Trisodium citrate	1500	ppm

Before creating oxidative stress conditions resulting from exposure of zebrafish to silver nanoparticles, the temperature of the tanks was stabilized at 25°C and other experimental conditions (tank aeration and water quality) were the same as in the rearing stage. The tanks were exposed to a 14-hour light/10-hour dark cycle. Following Bilberg *et al*. (2012), LC50 determination test involved continuous mortality monitoring; fish were considered dead upon cessation of gill cap movement and lack of response to mechanical stimuli (Bilberg *et al*., 2012). Dead fish were immediately removed to avoid possible water deterioration. Fish mortality during AgNPs exposure was recorded at 24, 48, 72, and 96 hours. In this study, 70 adult zebrafish (female: Identification through visual assessment of the conspicuous egg sac) samples with an average weight of 0.32 ± 0.00 grams and a total length of 3.07±0.15 cm were used. The specimens received no food, and the test media was not renewed during the assay. To this end, Test A (range-finding test) and Test B (determining the lethal range) were conducted as follows. For this purpose, 7 fish were transferred to each 5-liter tank containing different concentrations of commercial AgNPs including 0 (control), 1.75, 2.32, 4.68, and 9.37 mg/liter (total of 5 tanks). Following on the results of test A, fresh fish were subjected to test B in 5-liter tanks (7 fish per tank) with silver nanoparticle concentrations of 0 (control), 3.5, 4, 4.5 and 5 mg/L (Table 2). LC50 values with 95% confidence intervals were determined at 24-, 48-, 72-, and 96-hours using probit analysis (SPSS version 23) based on cumulative mortality recorded at the same time points. Acute toxicity tests followed Hotos and Vlahos (1998) (Hotos and Vlahos 1998).

Results

The results concerning the toxicity of varying silver nanoparticle (AgNPs) concentrations in zebrafish (*Danio rerio*) are given in Table 2. No losses were observed in the control group, which was devoid of silver

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nanoparticles. Analysis of Table 2 reveals a positive correlation between silver nanoparticle concentration and mortality rates in fish. Specifically, the greatest number of mortalities occurred following a 96-hour exposure period. In Test A (range-finding test), group 5, which had an AgNPs concentration of 9.37 mg/L, exhibited 100% mortality within 24 hours. Conversely, groups 1 through 4 of Test A showed no mortality throughout the 96-hour observation period. So, the results suggest a high degree of AgNPs toxicity to the zebrafish model employed in this study. In Test B (determining the lethal range), group 9, which had an AgNPs concentration of 4.5 mg/L, exhibited 100% mortality within 96hours. Conversely, groups 6 through 8 of Test B showed no mortality throughout the 96-hour observation period. Table 3 details the lethal concentration (LC10-90) values of AgNPs for zebrafish at specified time intervals following exposure. The LC50 value for AgNPs in adult female zebrafish was determined to be 4.47 mg/L (Table 3).

Table 2: Results concerning the toxicity of varying silver nanoparticle (AgNPs) concentrations in Zebrafish (*Danio rerio*).

Test	Groups number	Number of fish	AgNPs concentration (mg/L)	Mortality (No.)			
				24h	48h	72h	96h
A	1	7	0	0	0	0	0
	2	7	1.75	0	0	0	0
	3	7	2.32	0	0	0	0
	4	7	4.68	0	0	7	7
	5	7	9.37	7	7	7	7
B	6	7	0	0	0	0	0
	7	7	3.5	0	0	0	0
	8	7	4	0	0	0	0
	9	7	4.5	0	0	0	7
	10	7	5	0	0	0	7

Table 3: Lethal concentrations (LC10-90) of AgNPs in Zebrafish at various time points post-exposure.

Point	Concentration of AgNPs (mg/L)			
	24h	48h	72h	96h
LC10	6.04	6.04	3.94	3.83
LC20	6.31	6.31	4.19	4.01
LC30	6.51	6.51	4.37	4.15
LC40	6.68	6.68	4.52	4.30
LC50	6.84	6.84	4.66	4.47
LC60	7.00	7.00	4.80	4.67
LC70	7.17	7.17	4.96	4.92
LC80	7.37	7.37	5.14	5.24
LC90	7.65	7.65	5.38	5.27

In some treatments, fish showed signs of respiratory stress (increased operculum movement), during the hours they were exposed to AgNPs (Table 4). The number of fish showing these symptoms was checked and counted at the end of every 24 hours of the entire treatment period (4 days).

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Table 4: Status of respiratory stress (increased operculum movement) in Zebrafish following exposure to AgNPs.

Test	Groups number	Number of fish	AgNPs concentration (mg/L)	Increased operculum movement (No.)			
				24h	48h	72h	96h
A	1	7	0	0	0	0	0
	2	7	1.75	0	0	0	0
	3	7	2.32	0	0	1	1
	4	7	4.68	0	3	5	7
	5	7	9.37	4	0	0	0
B	6	7	0	0	0	0	0
	7	7	3.5	0	0	1	2
	8	7	4	0	0	0	1
	9	7	4.5	0	0	3	5
	10	7	5	0	0	4	5

Discussion

The results indicated a positive correlation between the concentration of silver nanoparticles (AgNPs), the duration of exposure, and the mortality rate. This suggests that both toxin concentration and exposure duration are significant factors influencing aquatic toxicity. In the range-finding test (test A), it was determined: The administration of 9.37 mg/L of AgNPs resulted in rapid and complete mortality, with 100% of the study population succumbing within the initial 24-hour observation period. Administration of 4.68 mg/L of AgNPs induced 100% mortality in the fish population within 72 hours. Consequently, determining the lethal range (test B) was designed to determine the lethal concentration (LC50). The lethality concentration test showed that the concentration of 4.5 mg/L of AgNPs used in this study produced the highest lethality in adult female zebrafish (*Danio rerio*) over 96 hours of exposure. The LC50 of AgNPs for zebrafish was found to be 4.47 mg/L (Table 3). Toxicological investigations of AgNPs in zebrafish remain in the exploratory phase, primarily utilizing acute and chronic exposure experimental designs (Bai and Tang 2020). Griffitt *et al*., assessed the toxicity of metallic NPs and their corresponding soluble metals in zebrafish via 48-hour static bioassays. Their findings revealed that the 48-hour LC50 values for AgNPs (26.6 nm) were determined to be 7.07 mg/L and 7.20 mg/L in adult and juvenile zebrafish, respectively (Griffitt *et al*., 2008). In the study of Bita *et al*. (2016), the median lethal concentration of silver nanoparticles synthesized from sargassum algae on common carp (*Cyprinus carpio*) was determined to be 11.34 mg/L (Bita, Balouch, and Mohammadian 2021). Ostaszewska *et al*. (2016) Reported a median lethal concentration of LC50 of 96 hours during exposure of the Siberian sturgeon (*Acipenser baerii*) to silver nanoparticles at 15.03 mg/ L (Ostaszewska *et al*., 2016). In another study, the LC50 values for common carp exposed to two types of silver nanoparticles, Nanosil (less than 100 nm) and Nanocid (18 nm), were

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reported as 73.8 mg/L and 0.43 mg/L, respectively (Tarkhani *et al*., 2012). The authors suggested that the difference in LC50 values may be attributed to the variation in nanoparticle size. Furthermore, research indicates that biologically synthesized silver nanoparticles exhibit lower toxicity compared to those produced via chemical methods (Bilberg *et al*., 2012). Currently, the acute toxicity of environmental chemicals (including NPs) to fish is most commonly estimated through a short-term test on zebrafish embryos or adults (Bai and Tang 2020). Because the acute toxicity results of embryos and adults are very consistent with each other, the fish embryo toxicity tests have been acknowledged as one of the promising alternative approaches to classical acute fish toxicity testing for their advantages such as transparency during embryo stages (Embry *et al*., 2010). Olasagasti *et al*. (2014) determined the 48-hour LC50 for 18 nm silver nanoparticles (AgNPs) in deionized water to be 0.94 mg/L using 3-day-old zebrafish embryos. Another study investigated the acute toxicity of polyvinylpyrrolidone (PVP)-coated AgNPs (81 nm) to zebrafish, reporting a 48-hour LC50 value of 84 μg/L (Olasagasti *et al*., 2014).

The lethal dose of silver nanoparticles in aquatic animals appears to be determined by both the method of synthesis (chemical or biological) and the resulting particle size. Basically, Young fish are more susceptible, and different species respond unlike to concentrations of chemicals agents (Boateng *et al*., 2006). Exposure of zebrafish embryos to silver nanoparticles (AgNPs, 5–20 nm) has been associated with adverse developmental outcomes, including spinal deformities, cardiac arrhythmia, concentration-dependent mortality, and transcriptional (specifically HIF4 and Pxmp2) perturbations in developing embryos (Asharani *et al*., 2008; Bai and Tang 2020; Qiang *et al*., 2020). As an example, Ašmonaitė *et al*. (2016) demonstrated that Ag+ exhibits greater toxicity than AgNPs with respect to zebrafish embryo survival. The LC50 values were determined to be 0.235 mg/L for Ag+ and 0.306 mg/L for AgNPs (Ašmonaitė *et al*., 2016).

On the other hand, adult zebrafish (D. *rerio*) exposed to 20–30 nm AgNPs showed greater toxicity compared with dissolved Ag+ ions released from silver nitrate due to higher silver association in fish gills, and higher thickening of the gill filaments (Griffitt *et al*., 2009). Dose-dependent toxic effects of AgNPs, Zinc oxide nanoparticles (ZnONPs), and Copper nanoparticles (CuNPs) have been demonstrated in zebrafish (Bai *et al*., 2010). Specifically, Griffitt *et al*. (2013) reported dose-dependent accumulation of AgNPs in zebrafish gill and eviscerated carcass tissues (Griffitt *et al*., 2013).

Kovrižnych *et al*. (2013) investigated the acute toxicity of 31 distinct nanomaterials, encompassing eight pure metals and 10 metal oxides, in adult zebrafish. Their findings indicated that exposure to metal and metal oxide nanoparticles resulted in cumulative

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mortality. Specifically, CuNPs and AgNPs exhibited toxicity to zebrafish, with median lethal concentration (LC50) values approximated at 3 mg/L (Kovrižnych *et al*., 2013). Studies have indicated the induction of the inflammatory response, metal-detoxification processes, and oxidative stress (Bai and Tang 2020). The toxicity of AgNPs has been attributed to several mechanisms, including interactions with cell membranes, induction of cellular reactive oxygen species (ROS), release of Ag+ ions, disruption of cellular signaling pathways, and interactions between AgNPs and cellular proteins and/or enzymes (Bouallegui *et al*., 2018; Mao *et al*., 2018). A comparative analysis of our research findings with those of other researchers indicates that nanoparticle toxicity exhibits considerable variability. This variability is influenced by factors such as the experimental species, the nanoparticle synthesis methodology, the developmental stage of the specimens, their reproductive maturity and sex, the route of administration, and the characteristics of the rearing environment. However, the dose of acute and sub-lethal toxicity of AgNPs and the molecular mechanism of toxicity are not currently fully understood (Shabrangharehdasht, Mirvaghefi, and Farahmand 2020). Up to now, studies conclude that a lower concentration of silver nanoparticles does not induce any toxicity and behavioral change in aqua biota (Banu *et al*., 2021). The respiratory toxicity of zebrafish was observed after Nanosilver exposure with symptoms such as an increased rate of operculum movement and surface respiration (Bilberg *et al*., 2012). This confirms the state of respiratory stress (increased operculum movement) in zebrafish after exposure to AgNPs reported in this study.

Ethical considerations

This study adhered to OECD standards and ethical guidelines for zebrafish (*Danio rerio*) research. The number of samples used in the experiment was kept to a minimum (without replications) and, as mentioned in the introduction, this experiment was designed to generalize the results in planning for further fundamental research by this team.

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