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Toxicity of gold nanoparticles on the survival and hatching rates of African catfish (*Clarias gariepinus*) embryo and larvae

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Abstract

Aim : African catfish (*Clarias gariepinus*) was selected model organism in this study to investigate the toxic effects of gold nanoparticles (AuNPs) on its embryonic and larval development.

Methodology : Mortality, hatching rate and malformation of *C. gariepinus* embryo and larvae exposed to AuNPs were observed and recorded.

Results : The study found that as AuNPs concentration increased, the embryo's hatching rate and larvae's survival rate decreased. A higher concentration of AuNPs ($>10 \text{ mg l}^{-1}$) induced toxicity in fish embryo. In contrast, exposure to AuNPs induced embryonic malformations such as pericardial edema, yolk sac edema, neck, and head defects.

Interpretation : The results obtained in the study provides additional details with the present understanding of possible ecotoxicological effects of AuNPs and reinforce to closely monitor the release of AuNPs into the aquatic environment.

Key words: African catfish, *Clarias gariepinus*, Embryo toxicity, Gold nanoparticles

Characterization of gold nanoparticles (AuNPs)

Induced spawning of catfish to obtain fertilized eggs and hatchling for toxicity studies

African catfish embryo toxicity assay conducted for different concentration of AuNPs (0.1, 0.5, 1, 5, 10, 50 and 100 mg l^{-1})

- Incubation period
- Hatching rate
- Embryo abnormality

African catfish larval toxicity assay conducted for different concentration of AuNPs (0.1, 0.5, 1, 5, 10, 50 and 100 mg l^{-1})

- Survival rate of larvae at 24 hrs
- Survival rate of larvae at 48 hrs

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Introduction

The rapid advancement in the field of nanotechnology has extended its applications into various fields, including electronics, optics, textiles, biomedical, and environmental remediation (Oberdorster *et al.*, 2005; Shen *et al.*, 2014). Due to increase in the surface-to-volume ratio, nanoparticles show unique physical-chemical and high specific surface properties. Bulk gold is considered inert, but at the scale of nanoparticles, gold has completely different properties due to its surface resonance excitation characteristics (Aillon *et al.*, 2009; Jenkins *et al.*, 2013; Marsich *et al.*, 2011). The widespread use of gold nanoparticles (AuNPs) are due to simple synthesis protocol, external surface modifications (covalent) (Khlebtsov and Dykman, 2011; Lewinski *et al.*, 2008; Longmire *et al.*, 2008), easily modified surface charges (Longmire *et al.*, 2008), extreme resistance to oxidation (Longmire *et al.*, 2008) and fast detection (Fadeel and Garcia-Bennett, 2010). Numerous methods can be used to detect AuNPs, such as fluorescence microscopy, differential interference contrast microscopy, transmission electron microscopy (TEM), optical microscopy, optical coherence tomography, photoacoustic imaging, X-ray scattering, multiphoton surface plasmon resonance microscopy, two-photon luminescence, polarized resonance scattering and gamma radiation using neutron activation (Boisselier and Astruc, 2009; Khlebtsov and Dykman, 2011). In addition, AuNPs have many desirable optical properties, including photobleaching resistance (Fadeel and Garcia-Bennett, 2010), easily adjustable surface plasmon absorption and dispersion (vis-NIR) (Khlebtsov and Dykman, 2011; Longmire *et al.*, 2008) and extreme nano-sized light dispersion (Fadeel and Garcia-Bennett, 2010).

At present, AuNPs are used to strengthen solar cells and act as flash memory devices in liquid crystal form (Prime *et al.*, 2009; Tsoukalas, 2009). It has several applications in the biomedical field, including drug delivery, gene therapy, photothermal and radiotherapy, biosensing as well as cancer contrast agents, diagnostic tracers, enzyme immobilization and cell imaging. (Bracamonte *et al.*, 2011; Chandra *et al.*, 2010; Li *et al.*, 2011; Nimesh, Gupta and Chandra, 2011; Pissuwan *et al.*, 2011; Zhang *et al.*, 2010). Other applications include purification of water and hydrogen, pollution control and carbon monoxide oxidation catalysts (Hashmi and Hutchings, 2006; McPherson and Thompson, 2009; Sardar *et al.*, 2009). Notwithstanding their enormous potential benefits in ecological, biomedical and industrial applications, data on the impact of AuNPs on aquatic organisms and environment is meagre. Previous studies have reported that AuNPs circulate in the body for an extended period of time without being rejected by the immune system of the body. Health and safety information on the use of AuNPs is currently limited, while gold as a component is generally considered inert and biocompatible (Longmire *et al.*, 2008). The toxicological effects of AuNPs are poorly understood. However, few researchers have raised concern about toxicity of nanoparticles,

including AuNPs (Almeida *et al.*, 2011; Dreaden *et al.*, 2011). Because of these issues, AuNPs toxicity in non-target species and environment needs to be studied in depth.

As AuNPs are readily taken up by the cells, they are used as new tools in medical diagnostic research and drug delivery systems (Azzazy and Mansour, 2009; Chithrani *et al.*, 2006; Yih and Al-Fandi, 2006). Several studies have reported the cytotoxic effect of different sizes and coating properties of AuNPs. Pan *et al.* (2007) reported that smaller AuNPs are more toxic. Connor *et al.* (2005), on the other hand, found no toxic effects on AuNPs-exposed human leukaemia cell lines. Adverse effects of cytoskeletal components and decreased cell growth in human dermal fibroblasts along with increased levels of reactive oxygen in exposed bivalves have been reported (Pernodet *et al.*, 2006; Tedesco *et al.*, 2008). The cytotoxicity of AuNPs in human cells has been elucidated and the findings have shown that they are non-toxic up to 250 mM, while ionic gold demonstrated detectable cytotoxicity at 25 mM (Connor *et al.*, 2005). The earlier studies did not investigate the toxicity of AuNPs (4-18 nm in diameter) with respect to cell viability, pro-apoptotic effects, oxidative stress, and inflammatory response (Connor *et al.*, 2005; Khan *et al.*, 2007; Shukla, 2005). Brandenberger *et al.* (2010) confirmed that non-functionalized 13-20 nm-diameter gold particles do not cause immediate adverse effects. Very few toxicological reports and data are available on the toxicity of AuNPs in animal models, especially aquatic organisms. Hence, this study was carried out to investigate the possible toxic effects of various concentrations of AuNP on the African catfish embryo and larvae. Embryo and larvae stages are considered as the most vulnerable stage in the life cycle of fish contaminants (Westernhagen, 1988). This is valuable for examining the toxicity of any chemicals that could impact the environment and human health (Frayssie *et al.*, 2006; Zhu *et al.*, 2008). The findings of this investigation will contribute to the recent understanding of nanoparticles' toxicological consequences, supporting the safe use of nanomaterials and protecting the aquatic environment.

Materials and Methods

AuNPs synthesis and characterization : Gold nanoparticles (AuNPs) were prepared following the sodium citrate reduction method (Turkevich *et al.*, 1951 and Frens, 1973). Two hundred ml of 0.01% hydrogen tetrachloroaurate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, Sigma) in water was vigorously stirred and heated to boil. In the reaction mixture, sodium citrate (1% in aqueous solution) was added and boiled. The light-yellow colour solution completely changed its color to deep blue and eventually red wine after one minute. The reaction was stopped after boiling the solution for 5 min. The total volume of the reaction mixture was then adjusted to 200 ml and stored at 4°C for further use. The AuNPs synthesized were characterized using a 350 to 800 nm wavelength UV-VIS spectrophotometer (DU8000 Beckman Coulter, USA). The size of AuNPs was measured by transmission electron microscope

(JEM-2100, JEOL Ltd., Japan).

Maintenance of brood fish: This study was carried out at the Aquaculture Research Facility, AIMST University, Kedah Darul Aman, Malaysia. Circular cement tanks were filled with tap water and stored for 48 hr before prior to experiments to remove the chlorine present in the water. Sexually mature male and female African catfish, weighing 1 - 2 kg, 40-50 cm were purchased from the local fish market in Sungai Petani, Kedah Darul Aman, Malaysia. Brood fish were acclimatized at 28°C under natural light conditions in 500 l circular cement tanks.

Production of fertilized fish eggs: Based on the external morphological characteristics, two matured males and female fish were selected. Both female and male fish were intramuscularly administered with 0.4 ml of Ovaprim kg⁻¹ body. The eggs were stripped from the female fish after 12 hr of latency period and the testes were surgically removed from the male fish. The stripped eggs were mixed with sperm suspension prepared from testis. The eggs were then stirred gently for 2 min and the eggs were washed twice with fresh water and transferred immediately for embryo toxicity assay to experimental containers. To collect hatchlings for larval toxicity tests, a part of the fertilized eggs were placed in a rectangular glass tank.

Embryo and larval toxicity studies: Embryo toxicity study was designed according to the standard guidelines of OECD (1998). Water quality parameters were determined by the methods described in APHA (2012), and their values are as follows: temperature 26.0 ± 2.0 °C, pH 7.2 ± 0.4, dissolved oxygen 7.2 ± 0.1 mg l⁻¹, total alkalinity (as CaCO₃) 27.0 ± 2.0 mg l⁻¹, hardness 32.0 ± 5.0 mg l⁻¹, nitrate 2.4 ± 0.1 mg l⁻¹, nitrite 0.01 mg l⁻¹, ammonia 0.01 ± 0.001 mg l⁻¹ and chloride 6.2 ± 1.1 mg l⁻¹. Stock solution of AuNPs were diluted to concentrations of 0.1, 0.5, 1, 5, 10, 50 and 100 mg l⁻¹. Twenty-five fertilized eggs were placed in each Petri dish containing different concentrations of AuNPs. Fertilized eggs exposed to water were treated as control. Each control and treatment groups were performed in 3 replicates. The Petri dishes were covered with lids and placed in the ambient temperature and light conditions. Hatching time and number of dead embryos for both control groups and exposed AuNPs were recorded. Hatchlings in the control and AuNPs exposed groups were randomly selected immediately after hatching and again at 24 hr after hatching. Embryos were tested for possible morphological deformities commonly found in toxicological studies under a microscope (Boudreau *et al.*, 2004; Peters *et al.*, 2007).

For larval toxicity study, hatchlings were placed in Petri dishes containing 0.1, 0.5, 1, 5, 10, 50 and 100 mg l⁻¹ of AuNPs. Hatchlings exposed to water served as. Each control and AuNPs exposed groups were performed in three replicates. Petri dishes were examined after 24-48 hr and the number of dead larvae was counted and recorded. Larvae were considered to be dead when

they became enlarged, white, opaque and did not move even when agitated with a plastic rod.

Statistical analysis: The data presented in this study on embryo hatching and fish larvae survival rate is the average of three replicates ± standard deviation (SD). The results were analyzed by One-way variance analysis (ANOVA) followed by Duncan multiple range test at 5 % significant using version 20 of SPSS

Results and Discussion

Characterization of AuNPs : Characterization of AuNPs using transmission electron microscopic (TEM) images and size distribution is shown in Fig. 1. AuNPs were spherical in shape with diameter of 36.47 ± 7.42 nm. On adding AuNPs to water used for incubation and larval bioassay induced the formation of aggregates. The study revealed that the AuNPs aggregates settled in the water column rapidly. A coating of AuNPs aggregates was found at the bottom of all the exposed Petri dishes after an incubation period of 24 hr. The incubation media was clear and all the AuNPs aggregates were settled. UV-Vis spectra of citrate-stabilized AuNPs showed two different peaks that matched different particle shapes. Spherical-shaped AuNPs showed a single maximum absorption peak at 521 nm, while urchin-shaped AuNPs showed maximum absorption at 695 nm.

Mortality and hatching rate of embryos exposed to AuNPs:

Table 1 demonstrates the toxicity of AuNPs to the viability of African catfish embryos. In the embryo toxicity assay, embryo mortality was increased by increasing the concentrations of AuNPs from 5 to 100 mg l⁻¹, but these differences ($P > 0.05$) did not vary significantly between control and treatment groups. The incubation periods of fertilized eggs were determined as 25 hr for both control and treated groups up to 10 mg l⁻¹. The incubation period decreased slightly in treatment groups of 50 and 100 mg l⁻¹.

There was a slight increase in hatching at 1, 5 and 50 mg l⁻¹ treatment and a decline in hatching at 100 mg l⁻¹, but the difference was not significant ($P > 0.05$). The survival rate of embryos exposed to various AuNPs concentrations for 48 hr showed a gradual decline as AuNPs concentration increased. The exposed embryo's decreased survival rate ($P < 0.05$) was significant compared to the control group. The results showed that the AuNPs-exposed embryos developed embryonic toxicity and dose-dependent mortality increased.

Survival rate of larvae exposed to AuNPs: The survival rate of catfish larvae was observed at 24 and 48 hrs post-exposure to AuNPs. After 24 hr exposure, the survival rate decreased in all tested concentrations of AuNPs. Nevertheless, no significant difference was observed in the survival rate between the AuNPs concentration of 0.1-50 mg l⁻¹. Significantly low survival of larvae (16.7 %) was noticed with 100 mg l⁻¹ AuNPs exposed group. After 48 hr of exposure, 32% of larvae survived at 0.1 mg l⁻¹ AuNPs and

Fig. 1 : Characterization of gold nanoparticles (AuNPs): (a) Visual appearance of 40 nm diameter of colloidal AuNPs; and (b) UV-VIS spectra of 40 nm diameter of colloidal AuNPs. Spherical (A) and urchin-shaped (B); (c) Transmission electron microscopy image shows that AuNPs exhibit homogeneous distribution in size and (d) Size distribution chart.

Table 1 : Hatching and survival rate of African catfish, *C. gariepinus* embryo and larvae exposed to different concentration of gold nanoparticle

Concentration mg l ⁻¹	Incubation period (hr)	Hatching rate (%)	Survival of embryo at 48 hr of exposure	Larvae survival at 24 hr of exposure	Larvae survival at 48 hr of exposure
0	25	84.4 ± 1.6 ^a	72.2 ± 3.6 ^a	100 ± 0.0 ^a	95.3 ± 5.7 ^a
0.1	25	83.2 ± 4.5 ^a	66.3 ± 5.4 ^a	94.4 ± 2.5 ^a	31.9 ± 2.9 ^b
0.5	25	84.4 ± 2.3 ^a	62.1 ± 3.2 ^a	93.6 ± 10.9 ^a	23.8 ± 0.4 ^c
1	25	89.5 ± 4.4 ^a	62.3 ± 2.4 ^a	88.6 ± 9.6 ^a	25.2 ± 4.5 ^c
5	25	88.5 ± 3.4 ^a	62.4 ± 7.5 ^a	85.2 ± 7.6 ^a	24.3 ± 1.1 ^c
10	25	83.9 ± 8.6 ^a	57.7 ± 16.5 ^a	83.2 ± 8.3 ^a	5.4 ± 2.5 ^d
50	24	88.3 ± 4.8 ^a	53.9 ± 10.9 ^a	72.2 ± 7.2 ^b	7.9 ± 2.0 ^d
100	22	77.1 ± 4.9 ^b	52.4 ± 11.4 ^a	16.7 ± 5.9 ^c	0

Each value is mean of three replicates ± SD and the values in the column bearing different superscripts are statistically significant (P < 0.05).

100% larval mortality at the highest concentration (100 mg l^{-1}) was observed. Overall, the findings indicated time- and dose-dependent survival rate of the embryo and larvae exposed to AuNPs.

Malformations in the embryos after exposure to AuNPs :

A number of malformations were noticed in AuNPs exposed groups and the abnormalities observed are shown in Fig. 2. Typical malformations included irregular head shape, lordosis, yolk sac edema, body arquation, skin ulceration, pericardial edema and haemorrhage pericardial. The observations revealed pericardial edema and lordosis as the most significant malformations among the AuNPs exposed embryos.

The use of nanomaterials is widespread, especially in consumer products that are used for daily life. Silver and gold nanoparticles are widely used among the nanoparticles available. Their use in products such as washing machines, personal care products, and clothing leads to accumulation in sewage treatment plants and ultimately the aquatic environment. Nanomaterial contaminants have detrimental effects on human health and the environment despite their useful applications. The toxicity of nanoparticles has been documented in aquatic organisms, especially in fish (Kashiwada, 2006). Chi *et al.* (2009) revealed the toxic effect of nanoparticles at a gene and protein molecular level. The toxicity of AuNPs towards a wide range of aquatic organisms from plants to vertebrates has been reported. These include aquatic plants, freshwater algae, blue mussel, *Mytilus edulis* and zebrafish (Dedeh *et al.*, 2015; Lapresta Fernandez *et al.*, 2012).

Nanoparticles' toxicity varies depending on the size, charge, and bioavailability of nanoparticles. Therefore it is recommended that nanomaterials be described in-depth prior to the toxicity study in order to achieve a better understanding of the toxicity experiments (Balbus *et al.*, 2007; Li *et al.*, 2011). In this study, two differently shaped AuNPs characterized were spherical and urchin shaped. However, only spherical shaped AuNPs were used for TEM and toxicity assays. TEM was utilized to determine the morphology and size of the particles. Moreover, most of the AuNPs that were dissolved in water became aggregated and settled down within 24 hr, thus the incubation medium was clear and transparent. A similar observation was reported by Lee and Ranville (2012), where AuNPs aggregated and settled in nanoparticle mixed water within 24 hr. They also reported that only negatively charged AuNPs aggregated and settled down. Thus, it is clear that the spherical shaped AuNPs with an optimum size of 36.47 nm used in this study for the toxicity assays were negatively charged and relatively more aggregated.

Most studies indicate that AuNPs are not cytotoxic and some that they minimize production of ROS (Connor *et al.*, 2005; Khan *et al.*, 2007; Shukla *et al.*, 2005). Nonetheless, few studies have suggested that nano-metals can cause a wide range of sub-

Fig. 2 : Malformations (irregular head shape, lordosis, yolk sac edema, body arquation, tissue ulceration, pericardial edema and pericardial hemorrhage) induced by AuNPs. (A) 8 cell stage embryo from control treatment; (B) Embryo from control treatment; (C) Hatchling with tail curvature and pericardial edema exposed to 0.5 mg l^{-1} ; (D) Hatchling with irregular head shape and lordosis exposed to 0.5 mg l^{-1} ; (E) Notochordal abnormality (body curvature) lordosis exposed to 1 mg l^{-1} ; (F) Yolk sac edema and body arquation exposed to 5 mg l^{-1} ; (G) Hatchlings with scoliosis exposed to 10 mg l^{-1} ; (H) Yolk sac edema and body arquation with short tail exposed to 50 mg l^{-1} ; (I) Hatchling with bag shaped yolk sac with skeletal flexure and vertebral truncation exposed to 100 mg l^{-1} and (J) Hatchling with pericardial edema exposed to 100 mg l^{-1} .

lethal effects in fish, including breathing toxicity, tissue trace element disruptions Na^{+} K^{+} -ATPase, inhibition and oxidative stress. In a variety of organs including gill, liver, intestine, and

brain, nano metals have also induced organ pathologies. Toxicity of nanoparticles is more pronounced, especially at embryo and larval stages. The intake of nanoparticles by embryo is more than that of larval stage. Toxicity assays performed in cell lines are reliable; however, toxicity differs between artificial cell lines and live animals. Developmental toxicity of AuNPs cannot be determined in a human embryo; hence a better animal model is necessary to elucidate the toxic effects of AuNPs.

The results of the study showed that African catfish embryos and larvae, exposed to AuNPs showed significant decrease in hatching and survival rate in a dose- and time-dependent manner. Lordosis and body arcuation malformations are referred as excessive inward curvature of lumbar and cervical regions of the spine accompanied by abnormal calcification of afflicted vertebrae (Dickson, 2004). These malformations were observed in most of the treated embryos, especially at high concentrations of AuNPs. Similar effects were observed in zebrafish embryos exposed to silica nanoparticles, where embryo and larvae malformations and mortality had increased, leading to embryonic developmental toxicity that alters larval locomotive activity (Duan *et al.*, 2013). The present findings clearly implicate that AuNPs are toxic to catfish embryos and larvae; however, the mode of AuNPs toxicity in embryo and larvae of catfish is not yet elucidated. In conclusion, 24 hr exposure to AuNPs triggered embryonic developmental toxicity, leading to severe effects on larval survival. Consequently, the results indicate that exposure to AuNPs can pose a potential risk to aquatic organisms. Therefore, further studies are needed for early embryonic and larval studies and safety assessment of AuNPs on the relationship between exposure, adverse effects and biological mechanisms.

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