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Japanese medaka exposed to gold nanoparticles: Only embryonic exposure generates irreversible hatching failure, developmental failure, and mortality of sac-fry

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ABSTRACT

This study evaluated irreversible toxicity effects of gold nanoparticles (AuNPs) during the short-term (only embryonic stage) and long-term (both embryo and sac-fry stages) exposures of Japanese ricefish, *Oryzias latipes* (medaka) embryos and sac-fry. Embryos and sac-fry exposed to AuNPs at 8 and 15 days post-fertilization exhibited mortality, developmental failure, and abnormal appearance, and sac-fry additionally exhibited hatching failure and abnormal behavior. Embryos damaged by AuNPs during the embryonic stages failed to hatch and died, despite being raised under AuNP-free conditions after embryonic exposure. This study demonstrates that AuNPs have irreversible effects on *O. latipes* embryos and sac-fry, including the embryonic stages, regardless of the length of exposure. This result may be critical for predicting the potential continuous effects of AuNPs when the exposure duration of fish is short but includes the embryonic stages. To the best of our knowledge, this is the first study to test the toxicity of AuNP exposure on the embryos and sac-fry of *O. latipes*.

1. Introduction

AuNPs have been used in colorants (CEC, 2008), biomedical diagnostics, cancer research (Perreault et al., 2012), and many other applications. The Working Party on Manufactured Nanomaterials of the Organisation for Economic Cooperation and Development (OECD) has recommended a list of representative manufactured nanomaterials and included gold nanoparticles (AuNPs) as a new item in 2010 (OECD, 2010).

To date, eight published studies have investigated the effect of fish embryo exposure to AuNPs (Table 1; Harper et al., 2008; Bar-Ilan et al., 2009; Browning et al., 2009; Asharani et al., 2011; George et al., 2011; Harper et al., 2011; Truong et al., 2012a,b). All of these studies used *Danio rerio* as the test species, with test durations of 120 h post-fertilization (hpf), except for Asharani et al. (2011), who used 72 hpf. Four of these studies demonstrated that the surface charge and size of AuNPs had significant effects on the test species (Harper et al., 2008, 2011; Truong et al., 2012a,2012b). Harper et al. (2008) assessed the effect of different sizes (0.8 and 1.5 nm) and surface groups (neutral charge = 2-(2-mercaptoethoxy)ethanol, positive charge = *N*,*N*,*N*-trimethylammoniumethanethiol (TMAT), and negative charge = 2-mercaptoethanesulfonate) of AuNPs. As a result, the authors demonstrated that the small size and positive surface charge of AuNPs induced higher toxicity-related mortality and deformities in fish than AuNPs of large size with neutral or negative surface charges. Furthermore, Harper et al. (2011) tested three sizes (0.8, 1.5, and 15 nm) and four surface groups (neutral charge = 2,2-mercaptoethoxyethoxyethanol, neutral charge = 2,2-mercaptoethoxyethanol, cationic charge = *N*,*N*,*N*-TMAT, and anionic charge = 2-mercaptoethanesulfonate) of AuNPs and obtained the same results as Harper et al. (2008). Truong et al. (2012b) found that 1.2 nm 3-mercaptopropionic acid-functionalized AuNPs induced morbidity and mortality in fish raised in low ionic media. Additionally, Truong et al. (2012a) showed that 1.5 nm AuNPs functionalized with 2-mercaptoethanesulfonic acid (MES) or TMAT caused hypo-locomotor activity in fish. However, the other four studies recorded no significant effects of AuNPs on the fish (Bar-Ilan et al., 2009; Browning et al., 2009; Asharani et al., 2011; George et al., 2011). Browning et al. (2009) found that increased AuNP accumulation in embryos was related to AuNP concentration; however, developmental defects did not increase proportionally. In addition, Bar-Ilan et al. (2009) found that the sublethal toxic effects of triphenylphosphine monosulfonate functionalized AuNPs had no significant effects on fish mortality. The remaining studies also found that AuNPs did not induce any morphological or physiological defects (George et al., 2011) or any indication of toxicity (Asharani et al., 2011) in the test fish. However, these studies primarily focused on understanding how long-term exposure affects mortality or abnormality in fish (specifically, embryo and sac-fry stages). There is limited information on the

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Table 1
Toxicity data for zebrafish (*Danio rerio*) embryo and sac-fry stages exposed to AuNPs in previous studies.

Test AuNPs			Test duration	Results	References
Size (nm)	Conc. (mg/L)	Note			
0.8 and 1.5	0–250	Surface groups (MEE(−), TMAT(+), MES(neutral))	120 hpf	• Positive surface charge, small size → higher toxicity • Toxicity of TMAT(+)-AuNPs was revealed from 80 ppb to 250 ppm.	Harper et al. (2008)
3, 10, 50 and 100	0–49.24	Unfunctionalized and TPPMS-functionalized	120 hpf	• No significant effects	Bar-Ilan et al. (2009)
11.6	0–12.6	–	120 hpf	• No significant effects	Browning et al. (2009)
10	0–0.025	–	120 hpf	• No significant effects	George et al. (2011)
15–35	0–0.1	–	72 hpf	• No significant effects	Asharani et al. (2011)
0.8, 1.5 and 15	0–250	Surface groups (MEE(neutral), MEEE(neutral), TMAT(+), MES(−))	120 hpf	• Positive surface charge, small size → higher toxicity • Toxicity of TMAT(+)-AuNPs was revealed from 80 ppb to 250 ppm for 0.8 and 1.5 nm and from 10 ppm to 250 ppm for 15 nm. • Low ionic media → increase of toxicity	Harper et al. (2011)
1.2	0–50	3-MPA functionalized	120 hpf	• Toxicity of 3-MPA-AuNPs was revealed from 0.08 mg/L to 50 mg/L. • Inhibition of hypolocomotor activity by MES and TMAT	Truong et al. (2012b)
1.5	0–50 for MES and 10 for TMAT	Surface groups (TMAT(+) and MES(−))	120 hpf	• Toxicity of TMAT(+)-AuNPs was revealed at 10 mg/L and toxicity of MES(−)-AuNPs was revealed at 50 mg/L.	Truong et al. (2012a)

relationship between the length of exposure (e.g., short- versus long-term in developmental stages) and different stages in the life cycle of the test species on the final toxicological effects. Truong et al. (2012a) found that exposure of *D. rerio* to AuNPs at 5 dpf caused mortality and behavioral abnormalities that persisted into adulthood (until 117 dpf). Information is required about the toxicological effects of AuNPs on sac-fry exposed at different developmental stages.

This study was conducted on embryo and sac-fry stages because *D. rerio* typically hatch between 2 and 3 dpf. The irreversible toxicity effects of AuNPs were assessed during the short-term (only embryonic stage) and long-term (both embryo and sac-fry stages) exposures of *Oryzias latipes*. To the best of our knowledge, this is the first study to test the toxicity of AuNP exposure on the embryos and sac-fry of *O. latipes*.

2. Materials and methods

2.1. Nanoparticle characterization and analytical procedures

AuNPs (particle size, 10 nm; concentration, 5×10^{12} particles/L; <0.01% tannic acid; <0.04% sodium citrate; Sigma-Aldrich) in a red colloidal suspension were maintained in a dark refrigerator. A morphological image of the AuNPs was obtained using a field emission transmission electron microscope (FE-TEM; JEM 2100F; Jeol, Japan). Samples were handled by drop casting an aliquot of the AuNP dispersion onto a 300-mesh carbon-coated copper grid, which was dried under ambient conditions. Before exposure, AuNPs were dispersed in the fish embryo-rearing solution (1 g/L NaCl, 0.03 g/L KCl, 0.04 g/L $CaCl_2$, 0.163 g/L $MgSO_4$, and 0.001 g/L methylene blue) and suspended with a pipette. The particle size distributions of the hydrodynamic diameter of the AuNPs dispersed in the embryo-rearing solution were measured using an electrophoretic light scattering spectrophotometer (ELS; ELS-Z2; Otuska Electronics).

2.2. Fish

O. latipes (Japanese ricefish (medaka), orange-red type) specimens were provided by the National Academy of Agricultural Science (NAAS, Suwon, Korea). The fish were maintained in a square glass tank (300 × 450 × 300 mm) with aeration. The fish were cultured in dechlorinated tap water (pH, 7.0 ± 0.2; hardness, 65 ± 4.5 mg/L $CaCO_3$), at a constant temperature of 25 ± 1 °C and under a photoperiod of 16:8 h (light:dark). The fish were fed twice per day with Tetra Min (Tetra Werke, Germany) and brine shrimp (OSI PRO80TM, USA). Fertilized *O. latipes* eggs were collected from the broodstock on the day of the experiment, and then separated into 9–10 developmental stages (between late morula stage and early blastula stage) under a stereomicroscope.

2.3. Short-term exposure test (only fish at the embryonic stage were exposed, followed by change in media)

Fig. 1 presents the experimental design of this study. The short-term fish exposure test was conducted to assess the recovery of newly hatched larvae exposed to AuNPs as embryos, according to modified OECD guidelines (No. 212) for chemical testing (OECD, 1998). The test was performed until 15 dpf, without providing any food or oxygen supply, and the exposure solution was replaced with NP-free embryo-rearing solution at 8 dpf, which was when the control embryos hatched. Three hundred microliters of the exposure solution was placed in a 96-well microplate (ID, 7 mm; height, 10 mm; and volume in each well, 0.33 mL). Each well contained one embryo. The test temperature and photoperiod were the same as those during the initial culture phase. Exposure concentrations were 0, 1, 2, 3, 4, and 5×10^{10} particles/mL AuNPs. The fish were observed daily under a dissection microscope. The evaluated endpoints for embryos and sac-fry were mortality,