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# Developmental Toxicity of Zinc Oxide Nanoparticles on the Early Life Stage of Java Medaka (*Oryzias javanicus* Bleeker, 1856)

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## ABSTRACT

With a high likelihood of being discharged into aquatic habitats, zinc oxide nanoparticles have been widely employed in a variety of industrial and commercial goods. Concerns over their effects on the environment and human health have grown. This study evaluated the developmental toxicity of zinc oxide nanoparticles **(**ZnO NPs) on the embryo Java medaka (*Oryzias javanicus*). With three replicates for each treatment group, the Java medaka embryos were subject to various concentrations of ZnO NPs (10, 25, 50, 100, and 150 µg/L). The heartbeat of treated embryos was increased compared to the control group at 5-, 8-, and 11-days post-exposure (dpe). However, the hatching and mortality of embryos decreased when the concentrations of ZnO NPs increased. Meanwhile, deformities such as low pigmentation, edema (yolk sac and pericardial edema), and spinal deformities were observed in the embryo and larva during the exposure time. Compared to previous studies, ZnO NPs show severe toxicity to selected endpoints at lower concentrations in the embryos of Java medaka.

*Keywords*: Early life stage, Medaka, nanoparticles, ZnO NPs

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## INTRODUCTION

Developing new techniques for manufacturing goods and using these novel nanoparticles (NPs) has advanced in the USA, Europe, and Japan following the identification of carbon-60 fullerenes ($C_{60}$), carbon nanotubes, and quantum dots. The United States National Nanotechnology Initiatives defined NPs as substances with at least one dimension falling within the range of 1 and 100 nm (Kashiwada, 2006), and these particles can be nano-films, nano-wires and nano-tubes or NPs, while the application of these materials is known as nanotechnology (Handy et al., 2008; Patibandla et al., 2018). Different types of NPs could be categorized based on their structural constituents. Carbon-based NPs, like carbon nanotubes and carbon-black, fall into one category. Inorganic NPs, including aluminum (Al), bismuth (Bi), cobalt (Co), zinc oxide (ZnO), and copper oxide (CuO), form another category. Organic-based NPs, for example, dendrimers, micelles, liposomes, and polymer NPs, constitute a separate category. Additionally, composite-based NPs are another type that can be categorized based on their structural constituents (Amin et al., 2021). The hazards and advantages of these new materials have been extensively debated. The advantages of NPs are huge, and their benefits are still being studied.

At room temperature, these materials possess a wide band-gap semiconductor characterized by a band-gap energy of 3.3 eV (Sabir et al., 2014), excellent stability, resistance to corrosion, and the ability to catalyze photosynthesis (Hao et al., 2013). These materials are also non-migratory, fluorescent, piezoelectric, capable of absorbing light and scattering ultraviolet light (Li et al., 2018), diverse nanostructures (Bai et al., 2010). Despite having numerous applications, ZnO NPs are the third most widely produced nanoparticles worldwide, with annual production falling behind only silicon dioxide NPs ($SiO_2$ NPs) and titanium dioxide NPs ($TiO_2$ NPs) (García-Gómez et al., 2020; Rajput et al., 2018). These nanoparticles are frequently combined to produce sunscreens to ensure enhanced protection against ultraviolet (UV) radiation. However, ZnO NP usage may overtake $TiO_2$ NPs shortly by being able to absorb both UV-A and UV-B radiation; it provides enhanced protection and increased opacity (Wong et al., 2010). The use of ZnO NPs in cosmetics, medication delivery systems, therapies, and biosensors has been incorporated into human's daily lives. Several studies have been reported on the possible toxicity of ZnO NPs to aquatic environments; the key emphasis has been on acute toxicity studies of aquatic species. At the same time, the chronic toxicity studies of ZnO NPs were surprisingly scarce. Meanwhile, most of those studies focus on the early life stage of zebrafish because of their well-known biology and short life span. For instance, Bai et al. (2010) reported that the embryos of zebrafish dead after exposing to ZnO NPs at 50 and 100 mg/L, retarded the hatching at 1-25 mg/L, and caused abnormality after the 96-hour post-fertilization (hpf) exposure.

The aquatic invertebrate species *Daphnia magna* and *Thamnocephalus platyurus* have both been involved in ZnO NP toxicity studies, and comparable effective concentration ($EC_{50}$) and lethal concentration ($LC_{50}$) values were reported in the literature for them (Blinova et al., 2010; Heinlaan et al., 2008; Zhu et al., 2009). For instance, 3.20 and 0.18 mg/L were reported as 48-hr $LC_{50}$ values for *D. magna* and *T. platyurus* (Blinova et al., 2010; Heinlaan et al., 2008). Meanwhile, for the exact NPs, the 48-hr $EC_{50}$ was 2.60 mg/L for *D. magna,* and the 24-hr $LC_{50}$ was 0.14 for *T. platyurus* (Blinova et al., 2010). Although the toxicity of ZnO NPs was reported in different studies on some living organisms in their habitat, a comprehensive deficit assessment in evaluating the toxicity of ZnO NPs and their long-term effect at environmentally relevant concentrations still exists.

According to widespread consensus, the growing fish embryo or larvae are the optimal phase in the lifespan of a bony fish because they are specifically susceptible to low levels of environmental pollution (Bai et al., 2010; Wu et al., 2010). Meanwhile, following unique characteristics associated with Java medaka (*O. javanicus*), such as their excellent osmotic adaptation to a wide range of salinity from freshwater and brackish water to salt water, their small size, transparent body, and their short life cycle, these fish were chosen as a test organism instead of other model organisms. Moreover, Java medaka is essential to estuarine ecosystems, providing a feeder fish for commercially, recreationally, and scientifically valuable species like the mudskipper. As a top dweller, the Java medaka is also exposed to pollutants introduced to the aquatic ecosystem by atmospheric deposition. Furthermore, the partial-life test of medaka fish has been frequently used to determine the developmental impact of various substances or to evaluate compounds derived from complicated environmental mixtures (Wu et al., 2010). Hence, this study was carried out to ascertain the developmental toxicity of ZnO NPs in the embryonic stage of the Java medaka.

## MATERIALS AND METHODS

### Java Medaka Culture and Embryo Selection

Java medaka breeding groups were collected from the estuary region of the Sepang River in Selangor, Malaysia. Then, they were maintained in overflow containers and fed fresh *Artemia nauplii* (brine shrimp) larvae three times daily. They were maintained under 14 hr light and 10 dark periods at 26°C. Prior to the experiment, newly fertilized cluster eggs were collected directly from the female body and washed several times to eliminate any remaining substances on the surface of the egg. Healthy embryos at less than 8 hpf were then selected for subsequent experiments.

### Zinc Oxide NPs Stock Preparation and Characterization

Sigma-Aldrich (USA) supplied the uncoated ZnO NPs with a stated particle size of less than 50 nm and more than 97% purity.

Stirring was used to produce a stock solution of ZnO NPs (10 mg/L), which was then diluted to exposure concentrations. Embryos were induced to five distinct exposure levels of ZnO NPs (10, 25, 50, 100, and 150 µg/L). In our earlier work, the characterization of ZnO NPs for size in powder form using an X-ray diffraction spectrometer (XRD), shape or morphology using transmission electron microscopy (TEM), and size distribution and surface charge using dynamic light scattering (DLS) was already reported (Amin et al., 2021).

**Embryo Bioassays**

The exposure protocol was modified from the guidelines of the Organization for Economic Co-operation and Development (OECD) (2013) for testing chemicals through the fish: early life stage toxicity test. A single embryo at the blastula stage was placed in each of the test wells of a 24-well multi-plate. Each of the twenty wells contained 2 ml of ZnO NPs test solution, while the remaining four contained 2 ml of water per well. A total of 150 embryos had been utilized in the exposure groups, and 30 embryos were used in the control group for the triplicate experiment.

Based on the distinctive characteristics of the early life stage of *Oryzias latipes* (Japanese medaka), the embryonic process of Java medaka was observed (Iwamatsu & Kobayashi, 2002). At 5-, 8-, and 11-days post fertilization (dpf), embryos' heartbeats were counted over a 15-s (in three replicates) to estimate heart rate. A stereo microscope (Olympus Corporation, Japan) equipped with a digital camera was used to inspect the abnormalities in embryos and newly hatched larvae beginning at 11 dpf (also known as 1-day post-hatch [dph]) and continuing until the final day of the experiment, hatching numbers were measured daily. Unhatched embryos were no longer monitored after 20 days because they were regarded as hatching failures.

**Statistical Analysis**

One-way analysis of variance (ANOVA) was used with a 95% confidence interval to compare the heart rate at 5, 8, and 11 dpe, mortality, and hatching rates between the control and treatment groups by using GraphPad Prism (version 8.0.2) for Windows, which is developed by GraphPad Software (USA). The significance of the test results was determined at a $P$-value $< 0.05$. Additionally, the Shapiro-Wilk test was performed to assess the normal distribution of the data. The data are expressed as the mean ± standard deviation (SD).

## RESULTS

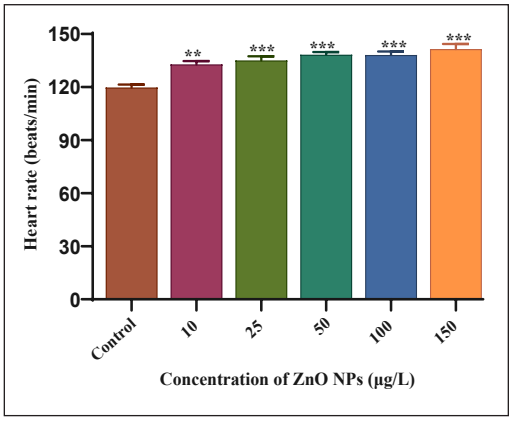
During the embryonic stage of Java medaka, the toxic effects produced by ZnO NPs on developmental endpoints were found to be dependent on the concentration.

The heart rate (y-axis) of embryos exposed to ZnO NP concentration (x-axis) at 5, 8, and 11 dpe is shown in Figure 1. Compared to the control, there was a significant concentration-related increase in the heart rate of exposed embryos to ZnO NPs. For instance, in contrast to control, at 5 dpe to 10 µg/L (132.8 ± 3.27 heart beats

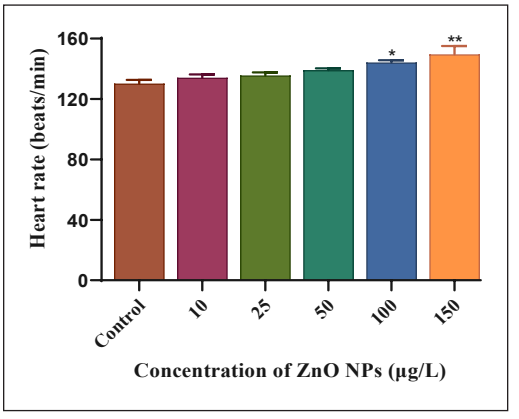
per minute (HBpM), $P < 0.03$), 25 µg/L (135.06 ± 4.00 HBpM, $P < 0.001$), 50 µg/L (138.30 ± 2.52 HBpM, $P < 0.001$), 100 µg/L (138.13 ± 3.40 HBpM, $P < 0.001$), and 150 µg/L (141.46 ± 5.00 HBpM, $P < 0.001$) showed significantly higher HBpM (Figure 1a). However, at 8 dpe to 100 µg/L (144.24 ± 2.61, $P < 0.01$) and 150 µg/L (149.63 ± 9.52 HBpM, $P < 0.03$), and 11 dpe to 10 µg/L (160.09 ± 1.85 HBpM, $P < 0.01$), 50 µg/L (161.18 ± 1.11 HBpM, $P < 0.01$), and 100 µg/L (160.9 ± 2.28 HBpM, $P < 0.01$) showed significantly higher HBpM compared to control group (Figures 1b and 1c).

From 11 dpe, the hatching of embryos was recorded. Hatching of embryos was started in control at 13 dpe with 30% of hatchability. With increasing concentrations of ZnO nanoparticles in the treatment groups, there was a reduction in the hatching rate of embryos. Figure 2 shows that the percentage of hatching success of Java medaka's embryos treated to ZnO NPs was inversely related. None of the individual embryos hatched when exposed to 150 µg/L at the end of the experiment (21 dpe). The lowest hatching success was observed at 100 µg/L with 63.33%, significantly ($P < 0.01$) lower than control at 100%. The percentage of hatching success of exposed embryos in treatment groups was decreased from 96.66, 90, 83.33, 63.33, and 0%. Meanwhile, some embryos failed to hatch after exposure to ZnO NPs.

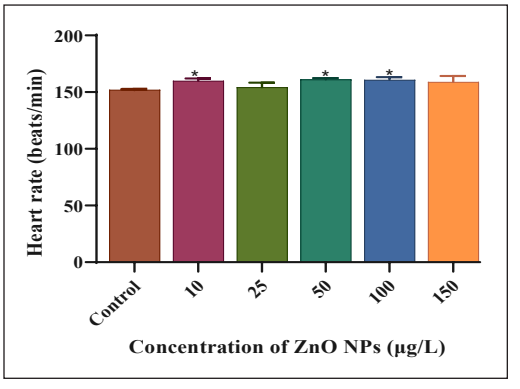
Throughout the experiment, no death embryos were recorded within the control group. However, concentration-dependent mortality was observed in treatment groups.




(a)




(b)




(c)

*Figure 1.* Heart rate of Java medaka's embryos at (a) 5-, (b) 8-, and (c) 11-days post-exposure to different concentrations of zinc oxide nanoparticles (ZnO NPs) *Note*. Data expressed as means ± SD with n = 3. *, **, *** shows significant difference at $P < 0.05$, $P < 0.01$, and $P < 0.001$ between control and treatment groups

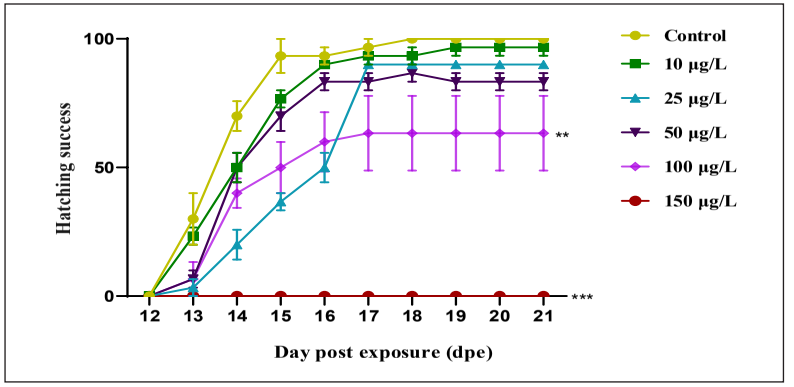



*Figure 2.* The hatching percentage of Java medaka's embryos exposed to different concentrations of zinc oxide nanoparticles, with n = 3

*Note.* **, *** shows a significant difference at $P < 0.01$ and $P < 0.001$, respectively, between control and treatment groups

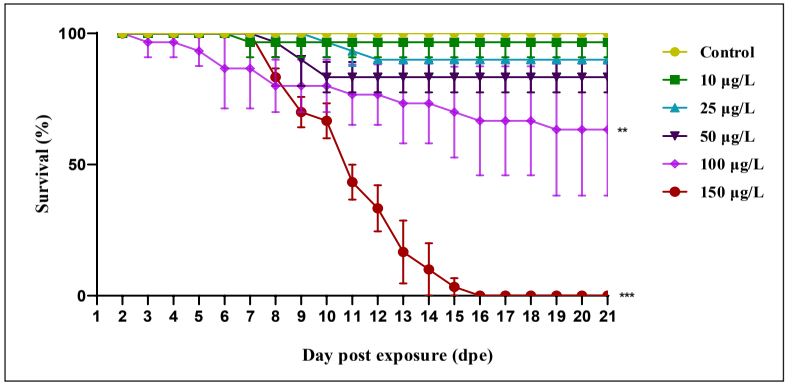



*Figure 3.* The survival rate of Java medaka's embryo exposed to different concentrations of zinc oxide nanoparticles, with n = 3

*Note.* *, **, *** shows a significant difference at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, between control and treatment groups

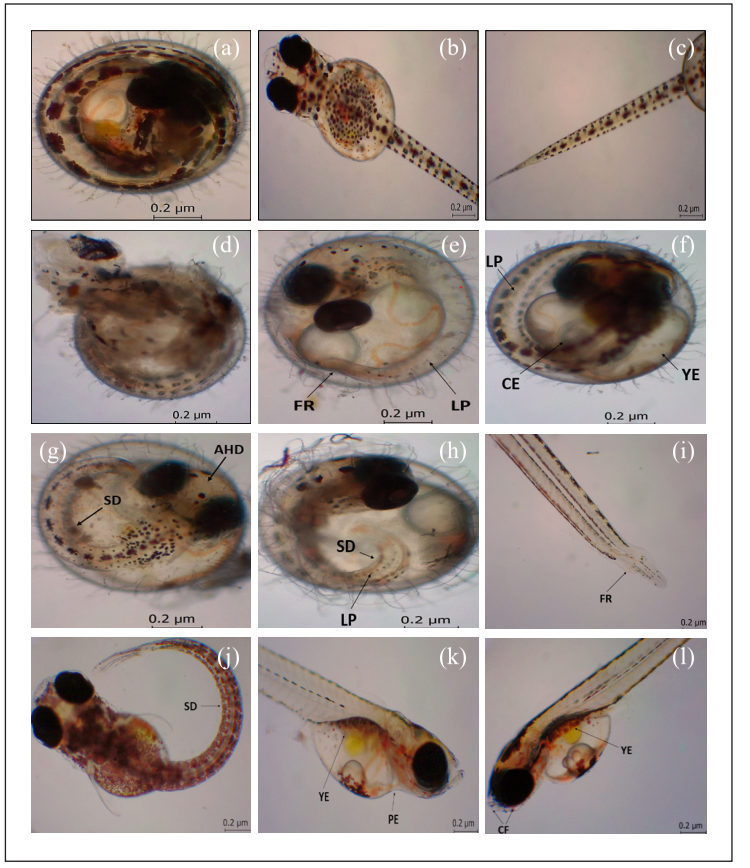



*Figure 4.* Abnormalities of Java medaka's embryos exposed to different concentrations of zinc oxide nanoparticles (ZnO NPs). (a, b, and c) control group; (d) failed hatching at 100 µg/L ZnO NPs; (e, j, and k) 25 µg/L ZnO NPs; (f and l) 50 µg/L ZnO NPs; (h) 100 µg/L ZnO NPs; (i) 10 µg/L ZnO NPs

*Note*. LP = Low pigmentation; FR = Fin rot; SD = Spinal deformities; CE = Cranial oedema; YE = Yolk sac oedema; CF = Craniofacial deformities

In contrast to control, significantly ($P < 0.001$) higher mortality was observed at 150 µg/L (100%) and 100 µg/L (36.66%) at the end of the experiment. However, a low mortality rate was observed at 10 µg/L (3.33%) and 25 µg/L (10.00%) (Figure 3).

The control embryos were observed to be normal (Figures 4a, 4b, and 4c). ZnO NPs-treated Java medaka embryos underwent numerous morphogenesis malformations during the embryonic exposure experiment, and these deformities depended on the exposure concentration. Observed abnormalities included low pigmentation, fin rot, spinal deformities, cranial edema, yolk sac edema, abnormal head deformities, abnormal pigmentation, lateral body curvature, hatching failure, and death of Java medaka embryos. However, after hatching in treatment groups, tail malformation, spinal deformities, edema, and short lifespan of juveniles were observed in post-hatched juveniles (Figure 4). Furthermore, edema (yolk sac and heart edema) and low pigmentation were frequently observed in treated embryos.

## DISCUSSION

Acute toxicity studies of aquatic species have received the most attention in the research that has been published regarding the possible harmful impacts of ZnO NPs on aquatic ecosystems. Meanwhile, the long-term toxicity studies of ZnO NPs were surprisingly scarce. Previous research has found that ZnO NPs pose serious risks and have greater toxicity than other NPs in the aqueous ecosystem. Several developmental markers were evaluated in this study to investigate the potential developmental toxicity of ZnO NPs in the embryonic stage of Java medaka. The findings showed that ZnO NPs were hazardous to the endpoints throughout the embryo's development and induced abnormalities in the embryo and larvae. It is the first instance in which the developmental toxicity of ZnO NPs in Java medaka's embryos has been documented. Furthermore, it is noteworthy that in this study, we exposed Java medaka embryos for 21 days, significantly longer than the average freshwater fish embryo growth period of 5 days for zebrafish. Prolonged exposure may have consequences beyond the acute ones and may be useful in understanding the mechanism of ZnO NP toxicity.

The early-life stage of medaka bioassays is a sensitive laboratory model for assessing the impacts of contaminants (Wu & Zhou, 2012). Significant increases in toxicity occurred when medaka embryos were subjected to 10–150 µg/L of ZnO NPs. Our result was in accordance with previous studies—for example, P.-J. Chen et al. (2013) demonstrated that the toxicity of ZnO NPs increased by increasing exposure concentration on *O. latipes* (Japanese medaka) embryos. Meanwhile, the ZnO NPs killed zebrafish embryos at 50 and 100 mg/L, retarded hatching at 1-25 mg/L, and caused malformation after 96 hpf exposure. However, at exposure levels of 0.10 and 1 mg/L, ZnO NPs did not hinder the growth of *Dunaliella tertiolecta*. The lowest concentration at which an effect

was observed was 0.50 and 3 mg/L, and the $EC_{50}$ after 96 hr was determined to be 2.42 and 4.45 mg/L for ZnO NPs with sizes of 100 and 200 nm, respectively (Hou et al., 2018). Studies have also demonstrated that the toxicity of ZnO NPs on bacteria (Sirelkhatim et al., 2015), algae (Suman et al., 2015), invertebrates (Ates et al., 2013; Khoshnood et al., 2016; Xiao et al., 2015), and vertebrates (Fernández et al., 2013; Murthy et al., 2022) are also influenced by their concentration. In our study, higher toxicity was observed in lower concentrations of ZnO NPs in the early life stage of Java medaka compared to other studies that involved fish as model organisms (Table 1).

Our studies show that the heart rate of Java medaka at the early life stage typically rises as they grow up. Concentration-dependent increases in heart rate were observed in exposed embryos of Java medaka. Increases in heart rate after exposure to ZnO NPs were also mentioned in the literature. Cong et al. (2017) demonstrated that the heart rate of *Oryzias melastigma* (marine medaka) exposed embryos increased by increasing the concentration of ZnO NPs. In contrast, Wu et al. (2010) conducted a study where the embryos of

Table 1
*Comparison of zinc oxide nanoparticles (ZnO NPs) concentrations in toxicological studies on different species of fish*

| Species | Life stage | Concentration of ZnO NPs | Duration of exposure (Days) | References |
|---|---|---|---|---|
| Java medaka (*Oryzias javanicus*) | Embryo | 10, 25, 50, 100, and 150 µg/L | 21 | Current study |
| Java medaka (*Oryzias javanicus*) | Embryo | 0.10, 0.25, 0.50, 1, 5, and 10 mg/L | 4 | Amin et al. (2021) |
| Marine medaka (*Oryzias melastigma*) | Embryo | 0.01, 0.1, 1, and 10 mg/L | 20 | Cong et al. (2017) |
| Zebrafish (*Danio rerio*) | Embryo | 1, 5, 10, 25, 50, and 100 mg/L | 4 | Bai et al. (2010) |
| Zebrafish (*Danio rerio*) | Embryo | 1, 5, 10, 20, 50, and 100 mg/L | 4 | Zhao et al. (2013) |
| Zebrafish (*Danio rerio*) | Embryo and larva | 0.1, 0.5, 1, 5, 10, 50, and 100 mg/L | 4 | Zhu et al. (2009) |
| Carp (*Cyprinus carpio*) | Juvenile | 50 mg/L | 30 | Hao et al. (2013) |
| Rainbow trout (*Oncorhynchus mykiss*) | Adult | 1, 10, and 100 mg/L | 4 | Taherian et al. (2019) |
| *Lobeo rohita* | Adult | 2, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, and 55 mg/L | 4 | Aziz et al. (2020) |
| Common carp (*Cyprinus carpio*) | Adult | 0.382, 0.573, and 1.146 mg/L | 4 | Rajkumar et al. (2022) |
| Tilapia (*Oreochromis niloticus*) | Adult | 1 and 10 mg/L | 14 | Kaya et al. (2016) |

Japanese medaka were exposed to silver nanoparticles. They discovered a notable increase in heart rate among the exposed embryos, with the intensity of the increase being directly related to the concentration of exposure (ranging from 100 to 400 µg/L).

However, the zebrafish embryos showed a concentration-dependent decrease in heart rate after exposure to silver NPs in the study of Asharani et al. (2008), leading them to conclude that the cardiac cycle was disrupted by increasing concentration of silver NPs. Similarly, Cong et al. (2017) and Wu et al. (2010) suggested that the effects of ZnO and silver NPs on the embryos could also cause an increased heart rate. Moreover, NPs are also suspected of interacting with the endothelium of the arteries, having an immediate impact on the atherosclerotic plaque, or serving as a location for the development of thrombi and myocardial infarctions (A. Peters et al., 2001; Gatti et al., 2004; Khandoga et al., 2004).

Through fish embryogenesis, hatching is considered to be an important stage. In the current study, rising ZnO NP concentrations impacted Java medaka hatching. Concentration-dependent hatching rate inhibition for ZnO NPs was also reported on marine medaka (Cong et al., 2017), zebrafish (Bai et al., 2010; T.-H. Chen et al., 2014; Zhao et al., 2013; Zhu et al., 2009), and yellow stripe goby (Li et al., 2018). The blocking of the chorion pore by the adsorption of ZnO NPs limits oxygen and nutrient supply, which could affect the activities of the proteolytic enzyme. The enzyme known as proteolytic is released by the growing embryo's glandular cells during the normal hatching of teleost embryos, which then consumes the chorion (Bai et al., 2010; Cong et al., 2017). Then, P.-J. Chen et al. (2013) trapped the iron NPs inside the chorion and reported that the transport membrane of oxygen ($O_2$) was affected, which finally delayed the hatching of Japanese medaka. He further mentioned that due to hypoxia-induced by stabilized nanoscale zerovalent iron (CMS-nZVI), the hatching of Japanese medaka was retarded.

An increase in exposed embryo mortality was observed compared to the control. The increase in mortality after exposure to ZnO NPs on the embryo of yellow stripe goby (*Mugilogobius chaulae*) (Li et al., 2018), zebra fish embryos-larva (Bai et al., 2010; Zhao et al., 2013), Java medaka (Amin et al., 2021), marine medaka (Cong et al., 2017) were also reported in the literature. In the current study, numerous deficits have been detected at the embryonic and larval stages after exposure to ZnO NPs, along with embryo death. The malformations include low pigmentation, fin rot, spinal deformities, cranial edema, yolk sac edema, abnormal head deformities, pigmentation, lateral body curvature, and tail malformations. Among all the observed morphological abnormalities, the presence of edema (specifically pericardial and yolk sac) and low pigmentation were frequently observed abnormalities across all the treatment groups. Similarly, Cong et al. (2017) showed that ZnO NP exposure causes fin rot, spinal abnormalities, craniofacial malformations, and edema (yolk sac) in the early life stage of marine medaka. In a study

by Wu et al. (2010), abnormalities such as tail, spinal cord flexure, and truncation were observed after exposing Japanese medaka to silver NPs.

Additionally, tail abnormalities, spinal deformities, and edema were observed after exposing different fish species' embryos to ZnO NPs (Bai et al., 2010; Li et al., 2018; Zhu et al., 2009). According to a recent investigation, tiny particles might be taken up by the medaka chorion and then transferred into the yolk sac and body, raising the possibility that they could remain persistent during the embryo's growth (Wu et al., 2010). Once iron NPs are ingested by the chorion, they may begin redox cycling iron due to pH differences between the chorion's exterior and interior or between different organs, which accelerates the production of internal reactive oxygen species (ROS) and results in oxidative damage and developmental toxicity (P.-J. Chen et al. (2013). Additionally, earlier research has shown that several *Vibrio polysaccharide* gene (*VPS*) mutations, such as the *vps18* mutant in zebrafish and the *vps11* mutation in medaka fish, may decrease pigmentation and result in the most severe abnormalities (Golling et al., 2002; Yu et al., 2006). Because of their size and physicochemical characteristics, nanoparticles may interact differently with physiologically important macromolecules like DNA, as suggested by Balbus et al. (2007) and the abnormalities observed in the current study.

Therefore, more research is required to ascertain if ZnO NPs can suppress or influence the expression of vps genes. Furthermore, silver NPs (Ag NPs) caused spinal cord deformities associated with detectable hemostasis (coagulated blood) in Japanese medaka (Wu et al., 2010). The proper development of the embryonic stage is hampered by hemostasis because it limits the number of nutrients transported by the blood from the yolk to the embryonic organs or tissues. Therefore, yolk sac edema abnormalities were more observed in the current study. Meanwhile, different studies also observed edema during the embryonic stage of fathead minnow (Laban et al., 2010), zebrafish (Lee et al., 2007; Zhao et al., 2013), and Japanese medaka (Wu & Zhou, 2012) when they were exposed to different NPs. Disturbance in osmoregulation caused by toxicants is the common reason for edema (Kiener et al., 2008). According to L. E. Peters et al. (2007), osmoregulation and the disturbed circulatory system were thought to be responsible for several malformations observed in medaka. The disturbance of the circulatory system and osmoregulation by ZnO NPs can be correlated with these abnormalities (Bai et al., 2010; Li et al., 2018; Zhu et al., 2009).

## Java Medaka (*O. javanicus*) as a Nano-ecotoxicology Model

With findings acquired in a matter of days, this experiment indicates that Java medaka embryos may be employed as high productivity, high efficiency, economical, and highly responsive to the wide range of toxicants for examining the toxicity of NPs. Several organisms that study the effects

of NPs have a limited understanding of the genotoxic or metabolic effects due to the range of tests that may be performed or the model used. The zebrafish test organism has already been used in several nano-ecotoxicological studies (Bai et al., 2010; Xiong et al., 2011; Zhu et al., 2008). However, the model organism utilized in this work has several benefits, particularly for examining how NPs affect the environment. Java medaka shares many advantages with the well-known model organism Japanese medaka (*O. latipes*), such as their distribution, availability all year round, small size (3–4 cm), brief life span, and life cycle, rapid growth rate, readily identifiable, and easily cultured under laboratory conditions. Another notable feature of Java medaka is their sizable, see-through eggs and embryos, enabling convenient observation of their developmental stages (Amal et al., 2018, 2019; Salleh et al., 2017; Wittbrodt et al., 2002; Woo & Yum et, 2001).

Eggs fertilization and development occur externally. Daily, Java medaka produces a range of 30 to 50 eggs, with a potential total of 3,000 eggs during the mating season. The spawning event lasts less than one minute and closely regulates light cycles. Identifying and observing sexually active females of Java medaka is easy due to the presence of attachment filaments that secure the eggs to their bodies. Furthermore, the Java medaka is an ideal test organism as it reaches sexual maturity within a mere 100 days from hatching. It is much easier to collect eggs for subsequent research at the exact time of spawning, especially for those investigations involving extremely early stages of embryo formation up until later stages (Imai et al., 2007; Ismail & Yusof et al., 2011; Wittbrodt et al., 2001).

## CONCLUSION

To the best of our knowledge, this study reported the developmental toxicity of ZnO NPs in the initial growth phase of Java medaka for the first time. Heart rate, hatching, mortality, and deformities of embryos were criteria to assist the developmental toxicity of ZnO NPs on the embryonic development of Java medaka. ZnO NPs show concentration-dependent toxicity to all developmental endpoints. The results demonstrate that Java medaka embryos show more sensitivity compared to the well-known model organism zebrafish to low concentrations of ZnO NPs, showing the potential of Java medaka embryos as an in vivo study to demonstrate the bioavailability and toxicity of NPs. The present study highlights tasks focused on the environmental-related concentration of NPs on fish embryos to manage their ecological risk.

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