Effects of Paracetamol on the Development of Zebrafish (Danio rerio)

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ABSTRACT

The misuse of paracetamol is becoming more prevalent worldwide. Due to non-compliance with recommended dosage and regulations, paracetamol consumption can result in serious health issues such as liver necrosis, kidney damage, heart damage, and hematological changes. This study sought to investigate the impact of paracetamol on the development of zebrafish embryos, which are often used as a model for assessing the effect of drug exposure on animals. The results indicated that paracetamol negatively affects the hatching and survival rates of zebrafish. Additionally, paracetamol exposure caused spinal abnormalities, pericardial edema, hypopigmentation, reduced heart rate, and spontaneous movement in zebrafish larvae. The developmental abnormalities in zebrafish were more significant with higher concentrations and longer exposure times. These findings may provide valuable insights into the detrimental impact of paracetamol on aquatic animals.

Keywords: Danio rerio, embryo development, paracetamol, pigmentation, zebrafish

INTRODUCTION

Paracetamol usage has been on the rise globally, likely due to the widespread availability of this medication (Alchin et al., 2022; Franzelliti et al., 2015). Its popularity is due to the perception among the public that it is a safe and effective pain reliever (Ishizuka et al., 2020; Jóźwiak-Bebenista & Nowak, 2014). This trend is further...
exacerbated during the ongoing COVID-19 pandemic, in which people tend to self-treat by purchasing and consuming paracetamol (Leal et al., 2021).

In Indonesia, paracetamol is widely available and belongs to over-the-counter (OTC) drugs that can be obtained without a prescription from a medical professional (Kuswinarti et al., 2020). It falls under the category of non-opioid drugs, along with several non-steroidal anti-inflammatory drugs (NSAIDs), and is legally regulated by the World Health Organization (WHO) (Freo et al., 2021). The misuse of paracetamol by the public has become a global concern, with numerous studies investigating attitudes toward self-medication with paracetamol and other analgesics. For instance, Chakraborty et al. (2015) conducted a study on the prevalence of paracetamol abuse among Indian students, while Faqihi and Sayed (2021) investigated the practice of self-medication with analgesics among students at Jazan University in Saudi Arabia. In addition, Hidayati and Kustriyani (2020), as well as Kuswinarti et al. (2020), have examined the issue of paracetamol overuse within different communities in Indonesia. Several studies have demonstrated that dependence and overdosing on paracetamol, which occurs when it is consumed improperly and not according to the recommended dosage, can lead to health problems such as liver necrosis, kidney damage, heart damage, and hematological changes (Chiew et al., 2020; Hodgman & Garrad, 2012; Mostafa et al., 2022). Incorrect doses of paracetamol in pregnant and lactating women can also endanger both the mother and fetus. Experimental and epidemiological research has found that prenatal exposure to paracetamol can disrupt fetal development and pose risks for developing neurological, reproductive, and urogenital disorders. Prenatal paracetamol exposure has been linked to an increased risk of neurological and behavioral disorders, such as attention deficit hyperactivity disorder (ADHD), autism spectrum disorders, delayed language skills, and reduced intellectual abilities (Leppert et al., 2019; Liew et al., 2016; Skovlund et al., 2016). These findings suggest that paracetamol can easily cross the placenta and blood-brain barrier (Koehn et al., 2020).

The extensive use of paracetamol is closely associated with the increased environmental waste of this medication, particularly in aquatic environments. Many studies have investigated the presence of paracetamol contamination in these environments, including studies by Patel et al. (2022). Recent research has revealed the presence of paracetamol contamination in Jakarta Bay, with concentrations of 610 ng/L and 420 ng/L (Koagouw et al., 2021). These levels are exceptionally high compared to those found in other areas, such as the study by Shigei et al. (2021), which found paracetamol with a 40–70 ng/L concentration in the Zarqa River in Jordan. Groundwater, a drinking water source in Atlanta, Georgia, in America, was also contaminated with paracetamol (Al-Kaf et al., 2017).
The contamination of paracetamol in the aquatic environment indicates its accumulation in aquatic organisms. The pharmacologically active compounds in paracetamol can be harmful to aquatic organisms and have the potential to accumulate in the food chain. According to a study by Rivera-Utrilla et al. (2013), medical contaminants with a molecular mass of <500 Da have varying complex molecular contents and may have an affinity with other pollutants in the aquatic environment, such as heavy metals and microplastics. These factors are further influenced by human anthropogenic activities, rising water temperatures, and increasing rate of water acidification (Daniel et al., 2022).

Using animal models is an alternative to studying the effects of paracetamol contamination in aquatic environments. Folarin et al. (2019) examined the effect of paracetamol and diclofenac on African freshwater fish *Clarias gariepinus* and found disturbances in liver function and anti-oxidative enzyme stress. Blue mussels (*Mytilus edulis*) exposed to paracetamol for 24 days showed disrupted reproductive processes, including modulation of several important genes such as estrogen receptor 2 and vitellogenin (Koagouw et al., 2021). Studies on zebrafish (*Danio rerio*) have also examined the effects of paracetamol (Cedron et al., 2020; Xia et al., 2017; Xu et al., 2010). These studies have shown that paracetamol affects zebrafish larvae’s hatching rate and survival rate (Xia et al., 2017; Xu et al., 2010) and can cause edema and pigmentation disorders (Cedron et al., 2020). The abundance of research papers on the effects of paracetamol on zebrafish highlights the importance of using zebrafish as an animal model, especially considering their habitat in rivers, which are closely related to water pollution caused by human activities (Xia et al., 2017).

This study aimed to examine the effects of paracetamol on zebrafish development up to 72 hr post-fertilization (hpf). The development of zebrafish larvae, including measuring the intensity of eye pigmentation and spontaneous movement abnormalities, was observed. The findings from this study can be utilized as a reference for understanding the effects of paracetamol on humans and aquatic animals.

**MATERIALS AND METHODS**

**Preparation of Brine, Egg Media, and Paracetamol Solutions**

Three types of solutions were utilized in this study: brine solution, egg media, and paracetamol solution (PT. Mersifarma, Indonesia). All the solutions were prepared and dissolved using reverse osmosis (RO) water to maintain a stable pH. The brine solution was prepared by adding 35 g of salt (PT. Duta Kencana Swaguna, Indonesia) to one liter of water and stirring it with a spatula until it dissolved. For the egg medium solution, 1.5 ml of brine solution, one drop of methylene blue (Merck, India), and one liter of water were mixed and stirred until the mixture became homogeneous. The paracetamol solution was prepared using 500 mg of paracetamol tablets (PT. Mersifarma,
Indonesia). Based on previous studies, the paracetamol stock solution was then dissolved using the egg media solution to obtain a dosage of 3 and 5 mM (Cedron et al., 2020; Nogueira et al., 2019; Xia et al., 2017).

**Paracetamol Exposure**

The study started with the process of spawning zebrafish to obtain their eggs. A 30 × 20 × 15 cm³ aquarium was filled with water that had been aerated for 24 hr, and a spawning vessel was added. Broodstock zebrafish, approximately 3 months old and exhibiting good morphology and movement, were chosen for the experiment. Two male zebrafish were placed outside the mating chamber area, while one female was placed inside. The male and female fish were put together in spawning containers and left overnight in a dark environment. The following day, the fish were exposed to natural lighting and lamps for one hour to stimulate spawning. Once a successful spawning process was identified by the number of eggs in the aquarium, the parental fishes were returned to the original aquarium, and the fish eggs were harvested and placed in a petri dish filled with egg water media. The harvested eggs were then washed with egg water media to remove any dirt or debris and selected using a microscope for the study. A total of 20 healthy eggs with 2 replicates (40 eggs in total) were each placed in three Petri dishes containing 3- or 5-mM paracetamol solution, and egg water media was used as a control. These dishes were kept at room temperature for 72 hpf or three days. This methodology follows Arias-Alpizar et al. (2021) and Halder et al. (2010).

**Observation of Developmental Abnormalities**

Observation of zebrafish development was carried out at 24, 48, and 72 hpf, utilizing a Leica DM 500 microscope (Germany) with a magnification of 4 × 10 – 10 × 10 (Cedron et al., 2020; Kimmel et al., 1995; Nogueira et al., 2019). The study focused on survival rate, hatching rate, and observable morphological changes. Morphological parameters such as spinal abnormalities, pericardial edema and blood clots, eye pigmentation, heart rate, and spontaneous movements were observed before and after hatching.

**Statistical Analysis**

Quantitative data on survival rate, hatchability, spinal abnormalities, spontaneous movements, pericardial edema, and heart rate were analyzed using Microsoft Excel 2013. Pigmentation analysis was conducted utilizing Image J. Statistical analysis was carried out using two-way analysis of variance (ANOVA), with a significance level of 0.05. The Tukey’s honestly significant difference (HSD) test was then employed to determine the location of the significant differences.

**RESULTS AND DISCUSSION**

**Effect of Paracetamol on Hatching and Survival Rate**

Paracetamol is a teratogen that negatively affects the hatching rate of zebrafish.
Statistical analysis indicated that each treatment group’s hatching rate differed significantly ($p<0.05$). Tukey’s HSD analysis showed that the paracetamol concentrations of 3- and 5-mM considerably impacted the hatching rate compared to the control group. However, there was no significant difference between the hatching rates of the 3- and 5-mM concentrations, as illustrated in Figure 1.

![Figure 1](image.png)

*Figure 1. Effects of paracetamol exposure on the hatching rate of zebrafish*

In this study, the evaluation of the hatching rate began at 48 to 72 hpf. The control group exhibited a higher hatching rate than those exposed to 3- and 5-mM paracetamol concentrations. These findings suggest that paracetamol delays the hatchability of embryos into larvae. The percentage of hatching rate at 72 hpf for embryos exposed to 3- and 5-mM concentrations was 52.5 and 42.5%, respectively, of the total embryos. Moreover, this study found that most embryos exposed to paracetamol experienced hatch failure and died. These results are consistent with Jyotsna’s (2016) research on a concentration of 10 mM paracetamol and Glasco et al. (2022) study on a concentration of 3.9 mM paracetamol.

The hatching period of zebrafish usually occurs within 72 hpf, as observed in the control group. However, this study showed a lower hatching rate, particularly in embryos exposed to 5 mM paracetamol. This finding is similar to the study conducted by Xia et al. (2017), which reported that paracetamol reduced egg hatchability and affected cell survival (Cedron et al., 2020). The exposure to paracetamol was carried out until 72 hpf, and there was no transfer to a drug-free medium, which is consistent with the findings of Kantae et al. (2016), who reported that paracetamol accumulation was present in the larvae after more than 2 hr of exposure. Paracetamol induces reactive oxygen species (ROS) and disrupts apoptosis, leading to spinal abnormalities in the embryo. Consequently, the movement of the embryo’s spine, crucial in the hatching process, is disrupted (Glasco et al., 2022; Xia et al., 2017).

Similar to the hatching rate, the larval survival rate decreases over time. The statistical analysis demonstrated significant differences in the survival rate among each treatment group ($p<0.05$). According to Tukey’s HSD analysis, 3- and 5-mM paracetamol had a significant effect on the survival rate compared to the control, with 5 mM treatment demonstrating a more significant effect, as depicted in Figure 2, which shows that the survival rate of zebrafish exposed to 3- and 5-mM
Paracetamol decreased sharply at 72 hpf, to 70 and 22.5% of the total embryos, respectively. These results are consistent with studies conducted by Cedron et al. (2020) and Rosas-Ramírez et al. (2022), where paracetamol reduced the hatchability and survival rate of zebrafish embryos and larvae by up to 75%.

**Spinal Abnormalities**

A scoring method was utilized to assess the severity of spinal abnormalities, with a score of 0 for normal straight spine, 1 for mild curvature (<10°), 2 for moderate curvature (10°–30°), 3 for severe curvature (>30°), and 4 for a vertebra that failed to form. At 72 hpf, exposure to paracetamol at a concentration of 3 mM resulted in moderate spinal curvature (score 2), whereas exposure to 5 mM paracetamol led to severe spinal curvature (score 3). Although spinal abnormalities were observed from 24 hpf at 3 mM, no formation of failed (score 4) spines was observed in this study. The results indicate that exposure to paracetamol affected the development of zebrafish. At 24 hpf, spinal abnormalities were observed, which may cause delays in hatching. Statistical analysis showed significant differences in each exposure treatment \( p < 0.05 \). Tukey’s HSD analysis revealed that paracetamol concentrations of 3 and 5 mM significantly affected spinal abnormalities compared to the control group (Figure 3). At 24 hpf, spinal curvature occurrence (Figure 3) was found in embryos exposed to 3- and 5-mM paracetamol, accounting for 7.5% and 10% of the total embryos, respectively. The percentage of these abnormalities increased with age. At 48 hpf, larvae exposed to 5 mM paracetamol showed a spine abnormality increase of up to 67.5%. By 72 hpf, larvae exposed to paracetamol 3- and 5-mM showed spinal abnormalities, including 80 and 92.5% of the total embryos, respectively (Figure 4). These findings are consistent with the study reported by Raharjeng et al.
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(2021), which suggests that developmental abnormalities in zebrafish embryos may or may not be related to bone. The results of this study show that higher concentrations and duration of paracetamol exposure led to increased spinal abnormalities, in line with previous studies by Cedron et al. (2020), Nogueira et al. (2019), and Xia et al. (2017). These spinal abnormalities suggest that paracetamol affects the development of the spinal cord, which has also been shown in the research by Cedron et al. (2020).

Zebrafish embryos have neural crest cells (NC Cells), pluripotent cells formed during the early developmental period of vertebrates, specifically at the border of the neural tube (Cedron et al., 2020). Upon closure of the neural tube, NC cells differentiate into various types of cells, such as sensory nerves, autonomic nerves, pigment cells, bone, and cartilage. Rosas-Ramírez et al. (2022) found that NC cells activate the soxE gene, which determines the development of NC tissue.

When paracetamol enters the human body, it undergoes biotransformation mediated by cytochrome P450 and converts into \(N\)-acetyl-\(p\)-benzoquinonemine (NAPQI) (Cedron et al., 2020; Rosas-Ramírez et al., 2022). NAPQI is a metabolite compound that is produced during the process of xenobiotic metabolism of paracetamol/analgesia. NAPQI is typically eliminated by a conjugation
reaction with glutathione (GSH) in the liver, but high doses of paracetamol can lead to the accumulation of NAPQI, which can cause mutations in the soxE gene in NC cells, affecting the process of cell specification and differentiation (Bastiaan Vliegenthart et al., 2015; Glasco et al., 2022; Gum & Cho, 2013). Additionally, NAPQI induces apoptosis in differentiated cells, such as those in the spinal column, leading to neurological abnormalities characterized by the bending of the body or tail of a fish (Cedron et al., 2020; Rosas-Ramírez et al., 2022).

**Pericardial Edema**

A scoring system was implemented to evaluate the severity of pericardial edema. The score starts at 0 for normal/no edema and increases as follows: 1 for mild edema (enlargement < 10%), 2 for moderate edema (enlargement 10%–45%), 3 for severe edema (enlargement 46%–70%), and 4 for very severe edema (>70% enlargement). None of the groups showed pericardial edema at 24 hpf, but at 48 hpf, the 3 mM group had mild pericardial edema (score 1), while the 5 mM group showed severe edema (score 3). At 72 hpf, exposure to 3 mM paracetamol resulted in moderate edema (score 2), whereas, at 5 mM exposure, severe edema (score 4) was observed. Statistical analysis showed significant differences in each treatment \( p<0.05 \). Tukey’s HSD analysis showed that concentrations of 3- and 5-mM paracetamol significantly affected pericardial edema compared to controls. However, there was no significant difference in pericardial edema between concentrations of 3- and 5-mM (Figure 5). The control group showed no pericardial edema. At 48 hpf, exposure to 3- and 5-mM paracetamol caused pericardial edema in 62.5 and 82.5% of the total embryos, respectively. 3- and 5-mM paracetamol exposure caused pericardial edema in 87.5 and 92.5% of total embryos at 72 hpf. Figure 6 shows the increased pericardial edema at 72 hpf on exposure to paracetamol 3 and 5 mM. The results are consistent with those of Cedron et al. (2020) and Kang et al. (2020).

Pericardial edema in this study (Figure 6) is believed to be caused by impaired transport and membrane permeability from exposure to paracetamol. Paracetamol is known to reduce water export, which affects blood circulation and kidney function. Specifically, it obstructs glomerular formation and electrolyte reabsorption, disrupting the osmotic balance in the fish’s body (Cedron et al., 2020). Exposure to...
paracetamol also induces ROS, which can disrupt gene regulation of pax2a, sim1, and wt1 homologs, preventing proper nephron and kidney development and function (Hill et al., 2003; Kang et al., 2020). Research by Kitipaspallop et al. (2021) has shown that pericardial edema can also result from oxidative stress and inflammation, which are related to the expression of tumor necrosis factor-alpha (TNF-α) and interleukin-1b. These three genes are also associated with apoptosis and hematopoiesis. The expression of Tal1 and gata1 influences the hematopoiesis process, where Tal1 is a transcription factor that regulates Spilb and mpx hematopoietic stem cells, while gata1 regulates erythroid cells. The discovery of pericardial edema and blood clots in zebrafish, an animal model, is also found in the human body due to the consumption of analgesic drugs in inappropriate dosages. The formation of edema and blood clots can affect heart function, leading to myocardial infarctions, cardiac dysfunction, cardiac arrhythmias, and even heart failure due to an acetaminophen overdose (KhabazianZadeh et al., 2019).

### Eyes Pigmentation Abnormalities

A grading system was implemented to assess the eye pigment abnormalities. The

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**Figure 6.** Representative images of pericardial edema and blood clot induced in zebrafish embryos exposed to different paracetamol concentrations.

*Note.* Bc = Blood clot; Ed = Edema
grading score ranged from 0 to 4, where 0 corresponded to dark black eyes with pigment levels above 90%, 1 for black eyes with pigment levels ranging from 63–90%, 2 for dark gray eyes with pigment levels from 36–62%, 3 for gray eyes with pigment levels between 10–36%, and 4 for light gray eyes with pigment levels less than 10%. Exposure to 3 mM and 5 mM concentrations of paracetamol resulted in a significant decrease in the formation of eye pigment. At 72 hpf, embryos exposed to 3 mM paracetamol displayed gray eyes with a score of 3, while those exposed to 5 mM paracetamol exhibited light gray eyes with a score of 4.

In zebrafish, pigmentation is due to the presence of melanocytes, iridophores, and xanthophores, pigmentation cells derived from NC cells (Cedron et al., 2020). Melanocytes produce black pigment at the embryonic age of 24 hpf, and research shows that exposure to acetaminophen hinders the formation of black pigment along the spinal column and head to eyes (Cedron et al., 2020). In this study, the percentage of eye pigmentation was measured, and it was found that exposure to paracetamol caused hypopigmentation. The statistical analysis results indicated significant differences in each treatment ($p<0.05$). Additionally, Tukey’s HSD analysis revealed that paracetamol concentrations of 3 and 5 mM significantly affected eye pigmentation compared to controls (Figure 7).

The black spots in the zebrafish eye and body pigmentation can be observed at 24 hpf and are more prominent at 48 and 72 hpf in the control groups. However, exposure to paracetamol resulted in hypopigmentation or incomplete pigment formation, causing the larvae to appear transparent (Figure 8). The analysis of eye pigmentation was conducted by measuring the percentage of blackness in the eyes with Image J. At 24 hpf, the control group had an eye pigmentation of 4.72%, while the 3- and 5-mM paracetamol exposure groups had 1.72 and 0.88% eye pigmentation, respectively. At 48 hpf, the control group’s eye pigmentation increased sharply to 66.78%, while the paracetamol exposure groups did not show a significant increase, with 3.68 and 2.40% eye pigmentation at 3 and 5 mM, respectively. At 72 hpf, the control group’s eyes were darker, with a percentage of 93.26%, whereas the paracetamol exposure groups at 3 and 5 mM were 18.43 and 9.69%, respectively.

This study’s results suggest that paracetamol impacts pigmentation, as

![Figure 7. Eye pigmentation in zebrafish exposed to different concentrations of paracetamol](image-url)
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Evidenced by hypopigmentation. Cedron et al. (2020) found that exposure to acetaminophen in zebrafish significantly reduced melanocyte levels at ages 48, 72, and 96 hpf. Similarly, Wrześniok et al. (2016) reported that cell cultures showed hypopigmentation due to defects in melanin synthesis and cell survival. Nogueira et al. (2019) discovered that paracetamol caused a decrease in melanocytes and induced oxidative stress and epigenetic modification.

Heartbeat Abnormalities

Abnormalities in the hearts of zebrafish embryos can include edema and blood clots, causing disruptions to the heartbeat and blood circulation. A study by Xia et al. (2017) found that acetaminophen caused a decrease in the average heart rate per minute. Statistical analysis showed significant differences between treatments \(p<0.05\). Tukey’s HSD analysis revealed that paracetamol concentrations of 3 and 5 mM significantly affected heartbeat abnormalities compared to the control group, with the concentration of 5 mM having a greater effect.

At 48 hpf, the control group had a normal heart rate of 137 beats/min, while those exposed to 3- and 5-mM paracetamol had 67.90 and 62.65 beats/min rates, respectively. The decrease in heart rate was more pronounced at 72 hpf, with exposure to 3- and 5-mM paracetamol resulting

Figure 8. Abnormalities in eye pigmentation in zebrafish embryos exposed to different concentrations of paracetamol
in rates of 61.68 and 13.99 beats/min, respectively (Figure 9). The metabolism of vitamin A is affected by both the deficiency or excess of retinoic acid (RA) and N,N-diethylaminobenzaldehyde (DEAB) inhibitors, and this can have an impact on heart development by altering retinoid metabolic pathways (Jarque et al., 2020).

**Abnormalities in Spontaneous Movement**

This study analyzes spontaneous swimming activity as it is the first movement in the development of zebrafish, resulting from the development of muscle motoneurons, triggering hatching or emergence (Xia et al., 2017). The statistical analysis showed significant differences in each treatment ($p<0.05$). Furthermore, Tukey’s HSD analysis revealed that 3- and 5-mM paracetamol concentrations significantly affected spontaneous movement compared to the control (Figure 10). The observation of spontaneous movements was conducted until 48 hpf because, by 72 hpf, all embryos had hatched. There is a notable difference in the observation of 48 hpf, where spontaneous movements were still observed at 3 mM paracetamol exposure, at a rate of 7.5%, and the rate is even higher at 5 mM exposure, reaching 67.5%.

Paracetamol exposure has been found to induce diverse abnormalities in zebrafish, an important model organism for studying freshwater animals. While paracetamol has been detected in high concentrations in seawater (Koagouw et al., 2021) and drinking water (Al-Kaf et al., 2017), the possibility of its presence in freshwater environments raises concerns about the potential deleterious effects on the growth and development of aquatic fauna. Findings from this study may serve as valuable evidence or a reference point for investigating the prevalence of paracetamol in freshwater systems and inform the implementation of waste management practices, particularly regarding medicinal
waste, to mitigate the risk of environmental contamination.

Lastly, assessing the impact of paracetamol contamination on the aquatic environment also requires knowledge of the amount of paracetamol residue in various parts of the fish body. Future research should prioritize collecting residue data of paracetamol in different organs, such as gills, liver, muscle, and skin, to address this knowledge gap. Additionally, the study could focus on different fish species and their geographic locations to develop a comprehensive understanding of residue level variations. Also, further research could explore the effect of paracetamol contamination in non-fish aquatic organisms. The findings of such research could help develop better policies and guidelines for ensuring the protection of aquatic ecosystems.

CONCLUSION
Exposure to paracetamol at concentrations of 3 and 5 mM has been shown to impact the development of zebrafish embryos and larvae significantly. The negative effects on the egg hatchability, survival rate, spontaneous movement, spinal development, pericardial edema, blood clots, eye hypopigmentation, and heart rate become more pronounced with increasing concentrations and prolonged exposure time.

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