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Short Communication

Saltwater Fish Powder for Amelogenesis in Zebrafish Larvae (*Danio rerio*)

Sandy Christiono[1]*, Seno Pradopo[2], Islamy Rahma Hutami[3], Novia Indasari[4], Arlina Nurhapsari[5], Yayun Siti Rochmah[6], Zurairah Ibrahim[7], Savira Nurazky Yuniar[4], and Suparmi Suparmi[8]

[1]Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Islam Sultan Agung. Indonesia
[2]Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Islam Sultan Agung. Indonesia
[3]Department Orthodontic, Faculty of Dentistry, Universitas Islam Sultan Agung. Indonesia
[4]Faculty of Dentistry, Universitas Islam Sultan Agung. Indonesia
[5]Departemen Conservative, Faculty of Dentistry, Universitas Islam Sultan Agung. Indonesia
[6]Departemen of Oral and Maxillofacial Surgery, Faculty of Dentistry, Universitas Islam Sultan Agung. Indonesia
[7]Departemen Orthodontic, Faculty of Dentistry. Universitas Sains Islam Malaysia. Malaysia
[8]Departement of Biology, Faculty of Medicine Universitas Islam Sultan Agung. Indonesia

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*) Corresponding author:
E-mail: sandy@unissula.ac.id

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Abstract

Many nutrients found in saltwater fish are thought to promote amelogenesis in ameloblast cells during tooth development. The aim of the study is to determine the effect of LC_{50} toxicity on saltwater fish powder on zebrafish embryos. The experimental research method was a post-test-only control group design, consisting of eight groups, namely the negative control and the internal group using embryo media, the positive control using 3,4-dichloroanillin, and the treatment group 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml, and 4000 µg /ml using 384 zebrafish embryos. Saltwater fish powder is diluted and observed for 96 hours, controlled every 24 hours. There were no dead zebrafish embryos from various concentrations, negative control, and internal control. The toxicity test of saltwater fish powder from the lowest concentration of 125 µg/ml to the highest concentration of 4000 µg/ml showed no signs of zebrafish embryo mortality nor developmental abnormalities. It can be concluded that saltwater fish powder is non-toxic.

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1. Introduction

Amelogenesis is an enamel-forming process of the teeth by ameloblasts of epithelial origin. This process begins shortly after dentinogenesis (dentin formation) at about 15 weeks intrauterine (Setiawan *et al*., 2022). Amelogenesis is a complex process that involves several stages, including enamel matrix deposition, maturation, and mineralization of the enamel matrix. During the late bell stage, the cells of the internal enamel epithelium will differentiate into enamel-forming cells known as ameloblasts. A number of life cycles are experienced by these cells, including; presecretory, secretory, transition, maturation, and post-maturation stages. In the presence of calcium, enamel mineralization will begin following the secretion of organic matrices (Sitosari *et al*., 2019). Lack of calcium during odontogenesis causes defects in enamel such as hypoplasia and hypomineralization (Collignon *et al*., 2022; Sitosari *et al*., 2019). Calcium deficiency could disturb amelogenesis and osteogenesis, it has been shown that children with calcium deficiency exhibit enamel hypoplasia (Mubaraki, 2019). In addition to helping promote mineralization, calcium administration is crucial for developing of enamel matrix crystals. Calcium has a role in the mineralization process and ameloblast cell development, which will eventually affect the enamel matrix's density (Obtel *et al*., 2022).

The saltwater fish powder comes from sardines (*Sardinella fimbriata*), peperek (*Leiognathu splendens*), and cobs (*Euthynnus affinis*), which are rich sources of Omega-3, calcium, and vitamin D. It helps to increase the density of tooth enamel through the apposition and calcification stages, which also affect bone mineral metabolism (Christiono *et al*., 2021). Saltwater fish powder also helps to optimize collagen and non-organic formation at the stage of tooth development (Christiono *et al*., 2022, 2023a). Pregnant women are the intended target for this saltwater fish powder nutritional supplement. Increasing calcium intake during pregnancy will also increase the absorption of calcium in the blood. It will stimulate the expression of calcitonin which plays a role in increasing the activity of odontoblasts and ameloblasts during the process of tooth formation in the fetus (Christiono *et al*., 2023b). Since pregnant women require a lot of nutrition during the first trimester, it can be consumed during this period. Furthermore, saltwater fish powder can aid in the development of teeth and bones (Christiono *et al*., 2023b; Ho *et al*., 2023). Pregnant women are more vulnerable to supplements and medications because taking a drug without knowing the level of toxicity will be detrimental for the fetus. A toxicity test is a clinical trial conducted before a product is used by humans (Bauer *et al*., 2021).

With the European Union's restrictions on toxicity tests using vertebrates and the issue of the Organization for Economic Co-operation and Development test guideline 236, the fish embryo test (FET) has become a promising alternative to the acute fish toxicity test (AFT), with zebrafish embryos being used the most (Su *et al*., 2021). The LC_{50} toxicity test is a test used to determine the concentration that causes 50% of the embryo of zebrafish to die. The lower the LC_{50} value of sample can be regarded more hazardous, vice versa (Sijabat *et al*., 2023). Zebrafish (*Danio rerio*) is an animal that can be used to become an embryotoxic research model. LC_{50} is the value that shows the concentration of toxic compounds causing organism mortality up to 50%. LC_{50} focuses on the total mortality of tested animals rather than the specific damage to the organs (Rasyid *et al*., 2020). Zebrafish (*D. rerio*) can show the same toxic exposure effect on humans. The advantages of using zebrafish embryos (*D. rerio*) were that they are easy to obtain and observe, they had 70% of DNA that was homologous to humans and could show the same toxic exposure as humans (Bauer *et al*., 2021; Sellathory *et al*., 2019).

Saltwater fish powder has been shown to provide a number of advantages in earlier research, but its potential toxicity is yet unknown. Advances in the biological sciences have led to an ongoing paradigm shift in toxicity testing based on the expanded application of high-throughput in vitro screening and in silico methods to assess potential health risks of environmental agents (Krewski *et al*., 2020). This study aimed to determine the toxic value of saltwater fish powder to zebrafish (*D. rerio*) embryos. In order to provide guidelines, this study is necessary to estimate the risk of using saltwater fish powder or its exposure to humans. On the basis of the foregoing explanation, studies on the toxicity of saltfish powder LC_{50} in relation to the in vitro process of enamel amelogenesis within zebrafish embryos are urgently required.

2. Materials and Methods

2.1 Materials

The instruments used in the toxicity test procedure are aquarium tools, incubators, pH meters, thermometers, oxygen meters, multi-well plates, an 80-fold binocular microscope, Petri cups, beaker glasses, micropipettes, aerators, filter paper, spawn traps, and sonicators. The sample-taking technique was done using Simple Random Sampling. The sampling in this study was determined on the basis of the large determination of the samples according to the Fish Embryo Acute Toxicity (FET) test ('OECD', 2013). The size

of the sample used for each treatment group is 20 embryos of zebrafish (*D. Rerio*). The treatment group for each concentration was given 20 zebrafish embryos, and the treatment was given saltwater fish powder and embryo media and then sonified so that the lautrants mixed flatly. The negative control group was given 20 embryo zebra fish by being treated with embryo media, namely, that contained sterile RO water, methylene blue, and sterile E3 (NaCl, KCl, CaCl, $MgS0_4$), and a positive control group was given 20 embryos with the treatment of 3.4 DSA, and internal control group was given 4 zebrafish embryos with the treatment of sterile RO water, methylene blue, and sterile E3 (NaCl, KCl, CaCl, $MgS0_4$). This toxicity test research was done twice. The first study required 192 zebrafish embryos, with the total sample count used in this research was 384.

2.1.1 Ethical approval

This experimental research was performed on the basis of ethical clearance approval number 222/B.1-KEPK/SA-FKG/IX/2020 by the ethics committee of the faculty of dentistry, Sultan Agung Islamic University, Indonesia.

2.2 Methods

2.2.1 Experimental design

This research is in an experimental laboratory with a post-test-only control design. Consisting of eight treatment groups, namely the experimental research method of post-test only control group design, composed of eight groups of negative controls with embryo media, internal groups with embryo media, positive control using 3,4-dichloroanilin, treatment groups 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml, 4000 µg/ml, and 4000 µg/ml. The treatments were carried out using solutions made from saltwater fish powder for the treatment groups. After that, they spawn zebrafish with a ratio of 2:1 (6 females and 3 males). After the selection of a 5.25-hour zebrafish embryo with a complete and transparent amnion pouch, the toxicity test was done according to the FET test according to Guidelines for the Testing of Chemicals No. 236 OECD. It took 96 hours of observations, with observations controlled every 24 hours, and then proceeded with the probit test to determine the LC_{50} (OECD, 2019).

2.2.1.1 Dissolution of saltwater fish powder

First, 4000 mg of saltwater fish powder are weighed, and then the particles are dissolved in embryo medium until the 1000 mL limit is reached. Sonification is subsequently carried out until the solution combines, and this procedure begins the dilution process. Afterwards, using the equation $V_1N_1=V_2N_2$, the initial solution are diluted at concentrations of 125, 250, 500, 1000, and 2000 µg/ml. For LC_{50} concentration calculations, the sixth group of concentration values from the above type of dilution are utilized. 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml, and 4000 µg/ml represent the concentrations that are used to determine the value of LC_{50}.

2.2.1.2 Zebrafish spawning

The comparison of the number of female zebrafish and male zebrafish when spawning is 2:1. The spawning of the zebrafish consists of 9 zebrafish (6 females and 3 males). The ready spawning zebrafish is about 6-24 months old (Santacà *et al*., 2022). Zebrafish are placed in aquariums at temperatures of 26°C and pH of 6.5-8.5. Spawning can be done after 2-3 days. Female zebrafish can lay 50-80 eggs per day. Fertilization of zebrafish is affected by the bright dark phase of 24 hours, so that male zebrafish are aroused to detect female gonad hormones. Installed automatic lights near the aquarium with a 10-hour dark cycle, 14-hour bright cycle, and oxygenation. In addition, under the aquarium is given a spawning trap. Spawning traps are used to collect the results of fertilizing adult zebrafish (OECD, 2019).

2.2.2 Biochemical test

The toxicity test of saltwater fish powder was done using the FET test based on Guidelines for The Testing of Chemicals No. 236 of the OECD.

2.2.2.1 Sample making

The selected zebrafish embryo is then transferred onto each multi-water plate using a micropipette. Starting from the treatment group with various concentrations, negative control, positive control, and internal groups. The concentrations of saltwater fish powder used are 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml, and 4000 µg/ml. Each concentration uses 20 zebra fish embryos for the test group. The positive and negative control groups used 20 zebrafish embryos and the internal control group used 4 zebrafish embryos. Each treatment requires 24 multi-life plates, 20 for concentration treatment and 4 for internal groups.

2.2.2.2 Treatment control and observation

The study was conducted for 96 hours, con-

trolled and observed every 24 hours. The treatment group uses multiple-layered plates with varying concentrations of saltwater fish powder, embryo medium added at each concentration, and sonification to mix the solution. Each treatment takes 6 days, namely 1 day for the embryo breeding of zebrafish, 1 day for treatment, and 4 days for observation.

2.2.3. LC_{50} probit regression analysis

Probit regression analysis is used to analyse binomial response variables, including the distribution between the number of responses and the concentration.

3. Results and Discussion

This study aimed to determine the embryotoxicity effects of saltwater fish powder (SFP) at concentrations of 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml, and 4000 µg/ml compared to the control and positive control of 3,4-dichloroanilin. Figure 1 showed all parameters of zebrafish embryo toxicity (ZET), including coagulation, disruption of somites, failure of the tail to detach, and absence of a heartbeat in embryos. The toxicity test on zebrafish embryos using saltwater fish powder showed coagulation only occurred in the positive control group (Figure 2). The lowest tested concentration of 125 µg/ml and the highest concentration of 4000 µg/ml did not cause any coagulation in the zebrafish embryos during the 96-hour observation period.

The observed indicator was no release of the tail-bud from the yolk. The tail-bud is the posterior end of the embryo's tail. The yolk is the large, round egg yolk part of the embryo. Tail-bud release from the yolk starts at 18 hours of age. Exposure to 1000 µg/ml saltwater fish powder for 48 hours results in somite formation (Figure 4). No separation of the tail-bud from the yolk was seen with any concentration of saltwater fish powder (Figures 5, 6).

Another observed indicator under the microscope was the absence of a heartbeat. The heartbeat of zebrafish embryos is visibly observable, starting at 48 hours (Figure 7). There was no alteration or absence of heartbeat seen with any concentration of saltwater fish powder. In summary, the lowest and highest test

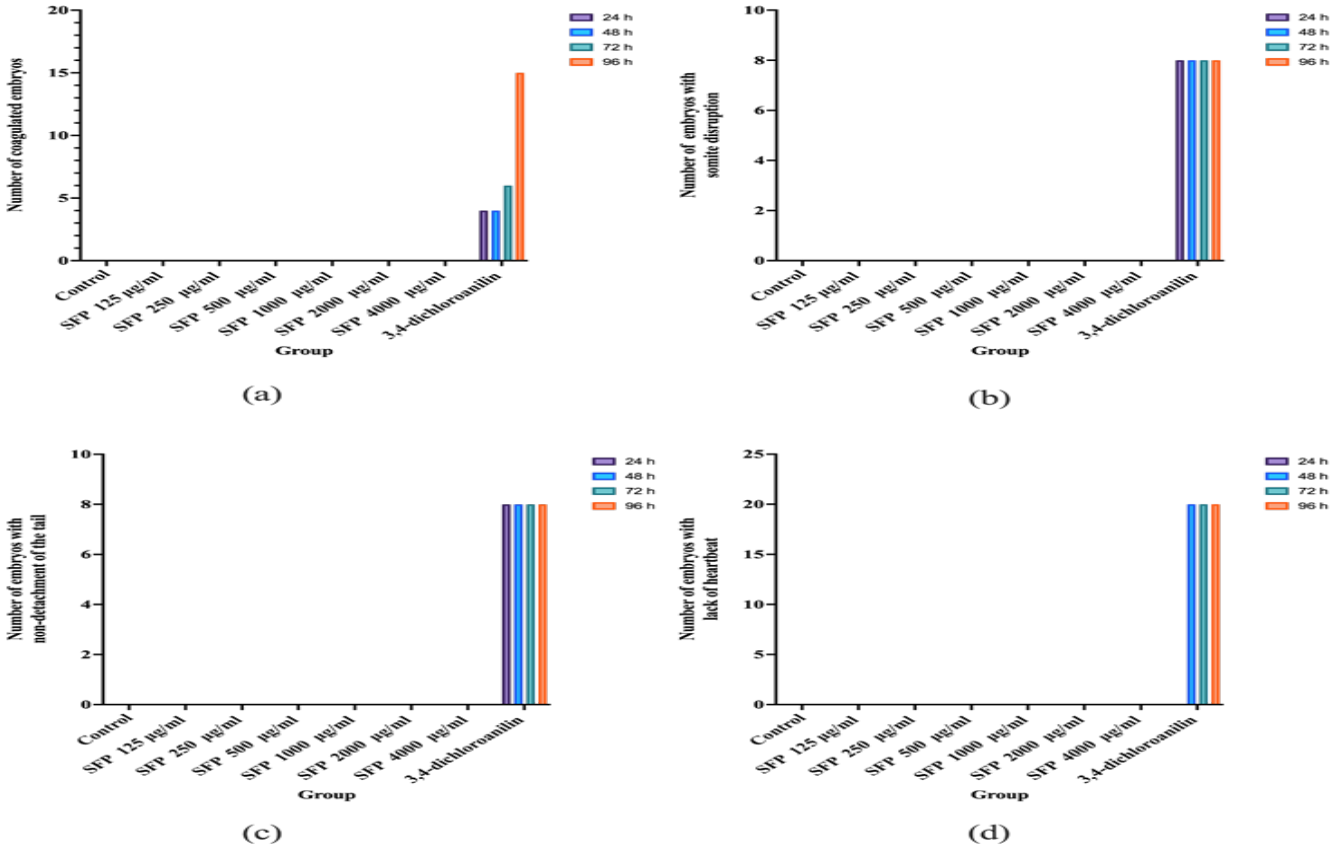

Figure 1. The embryotoxicity effects of saltwater fish powder (SFP) at concentrations of 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml, and 4000 µg/ml compared to the control and positive control of 3,4-dichloroanilin with parameters (a) coagulation, (b) disruption of somites, (c) failure of the tail to detach, and (d) absence of a heartbeat of embryos after 24, 48, 72, and 96 hours of exposure.

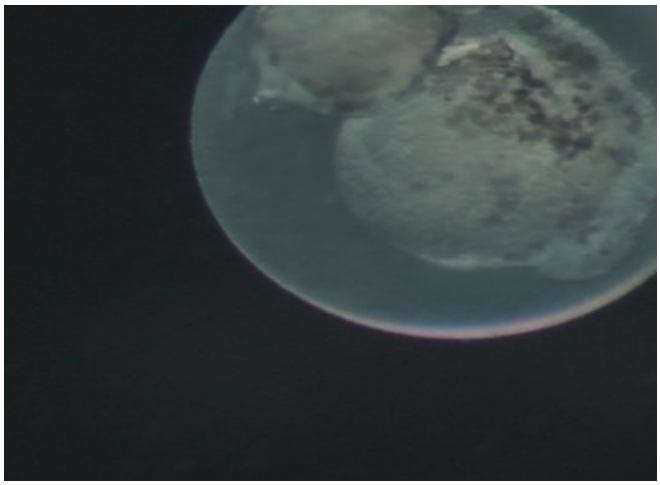

Figure 2. Coagulation shown in the positive control group

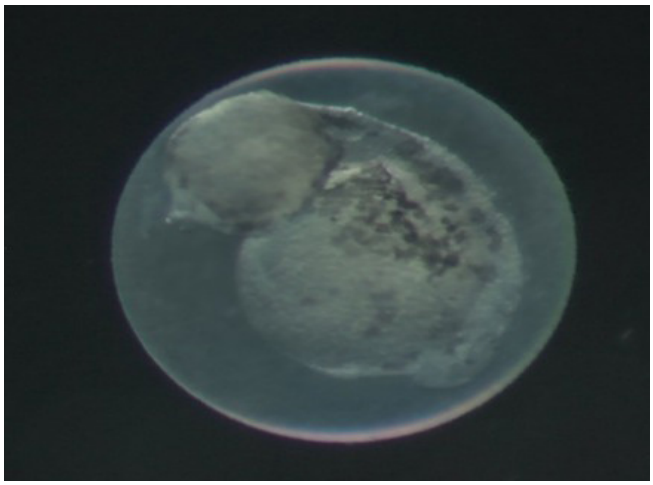

Figure 3. No somite formation occurred on positive control for 48 hours

Figure 4. Image of somite formation on exposure to 1000 µg/ml saltwater fish powder for 48 hours

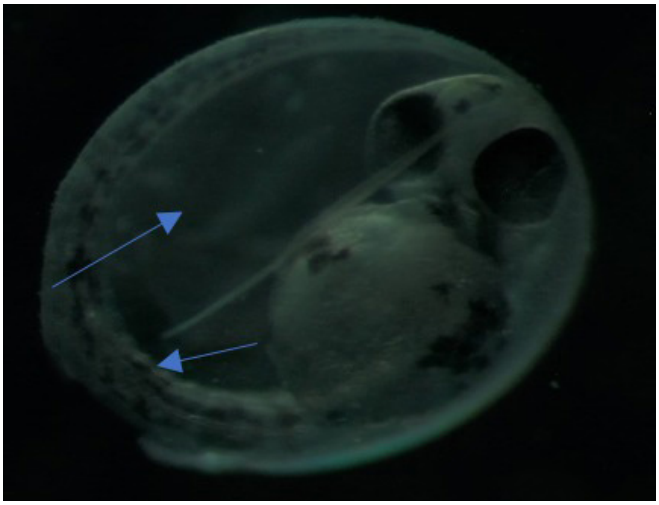

Figure 5. Image of tail-bud detachment (a) of yolk (b) embryo zebra fish exposure to saltwaterfish powder concentration 2000 µg/ml for 48 hours

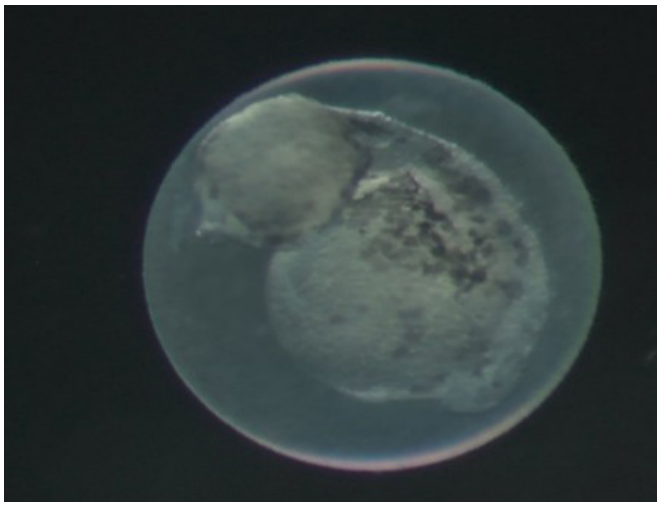

Figure 6. No tail-bud detachment on zebrafish embryo on positive control for 48 hours

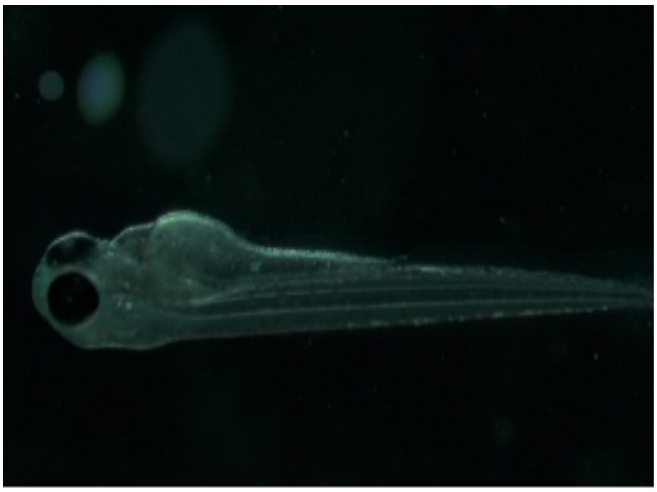

Figure 7. Heart embryo zebra fish visible on exposure to sea fish powder concentrations of 250 µg/ml

ed concentrations of saltwater fish powder, as well as all concentrations in between, did not cause any coagulation, detachment of the tail-bud, or changes in heartbeat rhythm in zebrafish embryos during 96 hours of observation compared to the control group with 3,4-dichloroanilin (Figure 8).

3.1 Toxicity Potential

The researchers did not find any dead zebrafish embryos at any tested concentration, so the data could not be processed by the LC_{50} probit regression analysis. The results showed that concentrations from the lowest 125 µg/ml to the highest 4000 µg/ml did not exhibit any toxic signs of saltwater fish powder. There was no coagulation, lack of somite formation, detachment of the tail-bud from the yolk, or altered embryonic heartbeat rhythm. The toxicity (LC_{50}) of Hg(II) for the freshwater fish *Oreochromis niloticus* was found to be in the amount of 0.1435 mg-Hg(II)/L. After exposure to Hg(II), the fish showed pathological changes, namely pale gills, anaemic eyes, and whitish body color. Histopathologically, exposure to Hg also affected the organs of the gills, liver, and hepatopancreas (Suhendrayatna *et al*., 2019; Tsai *et al*., 2023; Washburn *et al*., 2018). Research conducted by Santos et al. In this study, a number of developments are evaluated in the exposed zebrafish. The MPs used in the present study are in the range of 1-5 µm; therefore, the smallest particles have a size within the range of the zebrafish chorionic pores (300 nm-1 µm) (Santos *et al*., 2020). The study used saltwater fish dried into a powder so that the content of mercury and Hg was considered lower than fresh fish.

3.2 Calcium and Omega 3

Based on previous research, saltwater fish powder contains calcium and omega 3. Omega 3 contains linolenic acid, eicosatrienoic acid, arachidonic acid, EPA, and DHA (Christiono *et al*., 2022). The content of saltwater fish powder is inferred to be non-toxic with no active compounds causing embryonic death. The highest concentration in the experiment is 4000 µg/ml. According to OECD toxicity test guidelines, 4000 µg/ml is the highest recommended concentration for zebrafish embryo toxicity testing, and this concentration showed no toxicity (von Hellfeld *et al*., 2020). The study involved the examination of carotenoid-rich crude extracts derived from Cantaloupe melon, which were subsequently nanoencapsulated and subjected to studies using larval and adult zebrafish. The absence of detrimental alterations in optomotor response, anxiety-like behavior, locomotion, and sociability underscores the neuroprotective nature of these substances (Pais *et al*., 2023).

Fish is widely recognized as a significant dietary source of omega-3 fatty acids, which have been

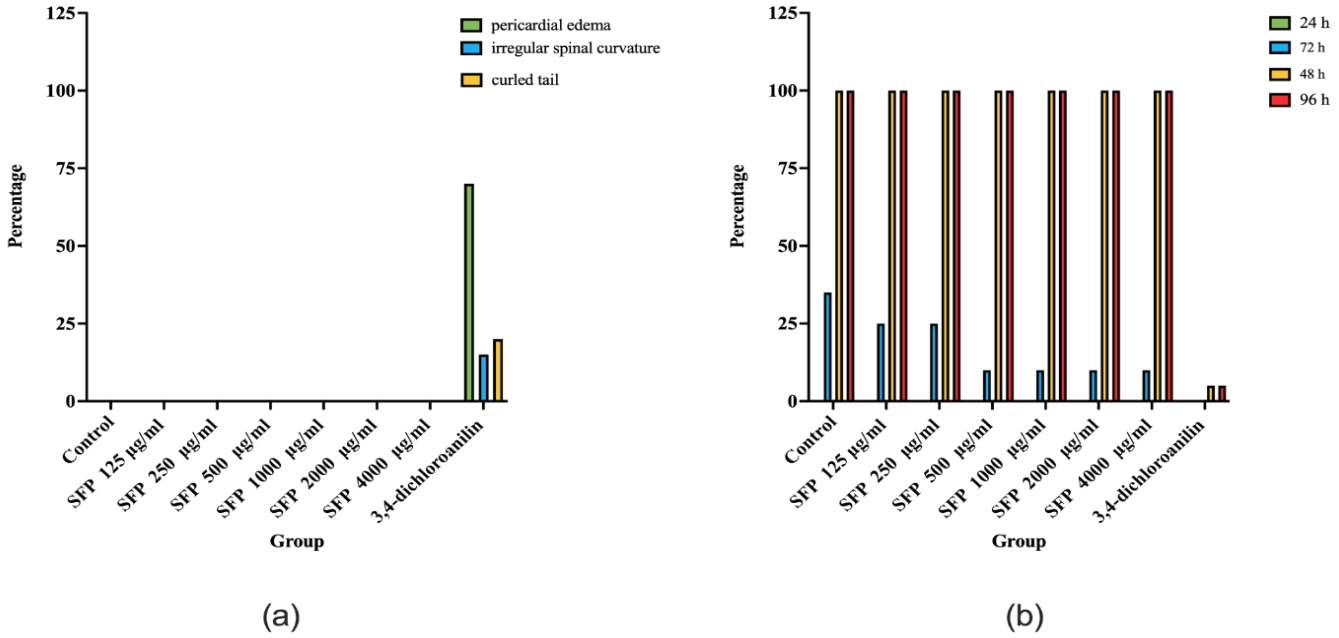

Figure 8. The embryotoxicity effects of saltwater fish powder (SFP) at concentrations of 125 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 2000 µg/ml, and 4000 µg/ml compared to the control and positive control of 3,4-dichloroanilin with parameters (a) The percentages of embryos with pericardial edema, irregular spinal curvature, and curled tail after 96 h exposure (b) the percentages of hatching embryos after 24, 48, 72, and 96 hours of exposure.

found to possess the capacity to mitigate the risk of developing cardiovascular disease (CvD) by modulating triglyceride levels. The correlation between elevated levels of triglycerides in blood plasma and susceptibility to cardiovascular disease is the underlying cause of this phenomenon (Christiono *et al*., 2021). Citrinin (CTN) is a mycotoxin that is commonly detected as a contaminant in a diverse range of food and feed grains, as well as fermented dietary supplements. This study demonstrates that the exposure of zebrafish embryos to CTN at concentrations ranging from 2 to 20 µM resulted in both poor neurodevelopment and disturbed behavioral patterns in the larvae. Additionally, the examination of the transcriptome and molecular characteristics of SH-SY5Y cells treated with CTN reveals that the neurotoxic effects of CTN at doses relevant to food consumption primarily stem from the disruption of neuronal differentiation and guidance of neuronal projections rather than being directly linked to mitochondrial dysfunction (de Oliveira Filho *et al*., 2017; Tsai *et al*., 2023).

3.3 Role of Endosulfan

The harmful effects of pollutants could be influenced by nutrients. For instance, the detrimental effects of pesticides on oxidative stress can be mitigated through the use of specific nutrients, including vitamin E, vitamin C, and selenium (Cano-Sancho *et al*., 2023). The use of vegetable-based substances as substitutes for saltwater items in aquaculture feed has been growing in popularity. The nutrient composition of diets is altered, resulting in the exposure of fish to novel pollutants, such as residues of agricultural pesticides (Santos *et al*., 2020).

Endosulfan (ESF) is an organochlorine insecticide with lipophilic properties, allowing it to dissolve in fats and oils. This characteristic contributes to its ability to stay in the environment over extended periods of time. Exposure to ESF elicits oxidative stress as a component of its harmful mechanism during the early developmental stages of zebrafish. This oxidative stress is believed to contribute to the reported ESF-induced morphological damage, behavioral alterations, and suppressed expression of antioxidant genes. The administration of Vitamin E and Omega 3 resulted in a decrease in behavioral abnormalities generated by ESF. Furthermore, this intervention provided partial protection against morphological damage. Additionally, the co-supplementation of these nutrients in chronic hemodialysis (HD) patients led to an improvement in the malnutrition-inflammation score (MIS) (Graterol Torres *et al*., 2022).

The study investigates the potential of saltwater fish powder, a source of nutrients like calcium, vitamin D, and omega-3 fatty acids, in promoting amelogenesis (enamel formation) during tooth development. This line of research could lead to the development of new nutritional supplements or interventions to improve dental health and prevent enamel defects. Overall, the novelty lies in the combination of using zebrafish embryos to assess the toxicity of a novel nutritional supplement (saltwater fish powder) aimed at promoting amelogenesis while also contributing to the understanding of nutrient-toxicant interactions and expanding the applications of zebrafish embryo models in nutritional research.

The study has limitations in its implementation: researchers do not observe the spectrum of toxic symptoms in the heart, liver, and nerves to determine the organ changes that occur at each concentration due to the restricted capacity of research support facilities, which limits the ability to evaluate toxic symptoms. The maximum zebrafish embryo concentration was tested, and no toxic effects seen. Furthermore, the conversion of zebrafish dosage to humans remains unknown.

4. Conclusion

This study highlight the use of the zebrafish embryo model and the FET test following OECD guidelines, the testing of saltwater fish powder as a novel supplement for amelogenesis, the range of concentrations evaluated, and the observational period to assess embryotoxicity endpoint. The toxicity test of saltwater fish powder from the lowest concentration of 125 µg/ml to the highest concentration of 4000 µg/ml showed no signs of zebrafish embryo mortality nor developmental abnormalities, including coagulation, somite disruptions, loss of tail detachment, and absence of the embryo's heartbeat, indicating saltwater fish powder has a lack of toxicity. Further acute toxicity testing in mice is needed to determine human-equivalent dosages from the zebrafish results. Additional studies on saltwater fish powder with nanoparticle sizes are also suggested.

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Authors' Contributions

All authors have contributed to the final manu-

script. The contribution of each author as follow, Sandy, Seno; conzeptualization and conceived of the presented idea, verified the experimental method and data analysis, planned the experimental design and technical design. Islamy, Novia, Arlina, Zurairah; drafted the manuscript, carried out the experiment, collected the data. Yayun, Suparmi, Savira; interpretation of the results and analysis, critical revision of the article, editor. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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