# Biocompatibility Test of Ceramic Materials in Zebrafish Development

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**Abstract.** The use of dental implants, which be implanted directly into bone is one of the major advances in modern dentistry. One of the dental implants that have recently been proposed is bio-ceramic. One of the prerequisites for the use of bioceramic is biocompatibility, which means being able to interact and connect with living tissue and the physiological environment without causes any adverse effect on the tissue. Zebrafish (*Danio rerio*) are known as model organisms that have similarities with humans such as the composition of bones, bone cells, and molecular signaling. The purpose of this study was to determine the effect of various bio-ceramic concentrations exposure to the structure of zebrafish bone tissue. The research was conducted in the laboratory of animal structure and development, Faculty of Biology, Gadjah Mada University. Fertilized zebrafish embryos were chosen and exposed to specific concertation of a ceramic material solution on the well plate, as follows 250µg, 500µg, and 750µg for a period of 72 hpf. One-way ANOVA test was used to assess the significance (P<0,05) of the control treatment results and various bio-ceramic concentrations. The results showed no effect of bio-ceramics (CHA-Ag) on survival rate, hatching rate, and heartbeat rate per minute at all exposures. However, some embryos and larvae at the age of 24-72 hpf concentration of bio-ceramics exposure 750µg, indicating were morphological changes such as egg yolk deformation, malformations of the spine, tail, and caudal fin, and stunted body or eye growth.

**Keywords:** biocompatible, bone, ceramics, dental implants, zebrafish.

## 1 Introduction

The use of dental implants, which be implanted directly into bone is one of the major advances in modern dentistry. Dental implants can provide better comfortability for patients in the healing function after tooth loss [1]. Implants are recognized as having the ability to heal and maintain epithelial and connective tissue around the implanted area. Functionally, dental implants rely heavily on osseointegration around the implant [2]. The characteristics required for dental implants are biocompatibility and functionality in tissues [3].

Some of the materials that have been studied for use as dental implant materials are metals, alloys, ceramics, polymer-based materials, glass, and carbon. However, ceramics are considered a promising material in the future. Ceramic materials are used as implant biomaterials because they have the same bone mineral components, biocompatibility, and osseointegration with human tissue. Bioceramics are ceramic materials commonly used in the manufacture of implants

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and orthopedic application devices which function to repair and replace diseased and injured parts in the human body [4].

Ceramics material for medical purposes is referred to as bioceramics, containing inorganic or non-metallic compositions in the form of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (HA). HA can support cell migration and angiogenesis resulting in the formation of new bone during tissue repair [5]. HA is biocompatible, osteoconductive, non-toxic, non-inflammatory, and is not an immunological agent. Also, HA is bioactive or can bond chemically with living tissue in humans [6]. HA-related material is carbonate hydroxyapatite $Ca_{10-x}Na_x(PO_4)_{6-x}(CO_3)_x(OH)_2$ (CHA) which is believed to be more promising because it has a more accurate chemical composition approaching one of the inorganic parts of bone tissue and has a fairly high biosorption rate [7]. A recent study conducted a combination of CHA with Ag, the mixture shows an antibacterial also non-toxic effect [8].

The bioceramics used in this study were made from silver-coated coralline hydroxyapatite (CHA-Ag). This material has antibacterial and biological properties because it has major components such as calcium, phosphorus, oxygen, carbon, and silver. The presence of antibacterial properties is caused by positively charged $Ag^+$ which can bind and stick to negatively charged bacterial surfaces due to Kulun's gravitational force. After damaging the bacterial cell wall, $Ag^+$ then enters the cell and immediately reacts with lysine, arginine, and other chemical compounds, causing the active protein to freeze and the activity of the bacterial metabolic enzymes to decrease. Compared to the structure of CHA, the new material, CHA-Ag is believed to have the advantage of being able to maintain the 3-D porous structure so that it has benefits for the structure and composition of bone conduction [8].

Preclinical in vivo studies in clinically relevant animal models is a fundamental step in research. The use of larger-sized animals has a limited cost, care, and complex ethical issues [9]. The in vivo model using the Zebrafish (*Danio rerio*) has recently undergone a development. Zebrafish and humans have similarities in genes homologous, genome, anatomy, physiological, cardiovascular, nervous, and digestive systems. Zebrafish can be used to evaluate the in vivo toxicity of degraded particles from newly developed biodegradable metal implants which can accelerate the material development process to clinical application, and avoid the ethical considerations associated with using mammals as a research tool [10]. In this paper, we identify the biocompatibility properties of ceramic material silver-coated coralline hydroxyapatite (CHA-Ag) using zebrafish as an animal model.

## 2 Materials and methods

### 2.1 Places of Research

The research was conducted at the Laboratory of Animal Structure and Development, Faculty of Biology, Gadjah Mada University in October-November 2020.

### 2.2 Zebrafish maintenance and egg collection

The animal used in this study was the Zebrafish (Danio rerio) Wild Type from University of Leiden. Zebrafish were maintained in standard conditions, on a cycle of 14-10 light-darks with a temperature of 27-28.5 and dissolved oxygen around 6-8. The fish were kept until they were ready to spawn. The spawning was arranged females and males ratio of 2:3. The eggs were collected and selected to determine the normal zygote, which were used in the experiment.

### 2.3 Preparations of ceramic materials

The ceramic material used in this study is made of CHA-Ag. The medium used was egg water solution. The ceramic material was prepared with three concentrations of 0.05 gr/ml, 0.1 gr/ml., and 0.15 gr/ml in egg water, respectively.

### 2.4 The embryo exposure

The 120 selected embryos were grouped into four treatments: control, CHA-Ag of 250µg/200ml, 500µg/200ml, and 750µg/200ml. with three replication each. The ceramic material solution was added to each well plate treatment, and 10 embryos were placed in each plate hole. The period of fish exposure time on the ceramic solution was 72 hours. Biocompatibility assessment were evaluated base on parameters as follows: Hatching rate, survival rate, morphological characteristics, and physiological changes of the fish, which be compared with the control group.

### 2.5 Post-exposure maintenances

The larva was reared at 20x30 cm aquarium. Water quality management was carried out by maintaining water following standard for zebrafish media. Debris and dirt were regularly removed to maintain water quality.

### 2.6 Preparations

One month of old larvae were sacrificed and processed for bone staining preparations Alizarin Red S. Alcian Blue (ARAB).

### 2.7 Data collections

Control and treated embryo were observed with a Leica DM750 microscope to determine their morphological shape, muscle contraction, and heart rate. Embryo development was observed every 6 hours. Morphological and physiological observations of larvae were carried out once a day by observing each change and compared with the control group. Morphological parameters include pigmentation, body size ratio, and organogenesis, and organ completeness in the embryo. Physiological parameters include swimming activity, feed response, and swimming ability.

### 2.8 Statistical analyses

Data were statistically analysed with IBM SPSS Statistics 21. One-way ANOVA test was used to determine differences between treatment groups of various concentrations of ceramic material. Data were presented as mean standard deviation and were considered significant if $P < 0.05$.

## 3. Results and Discussion

### 3.1 Effect of the ceramic material on embryo survival rate

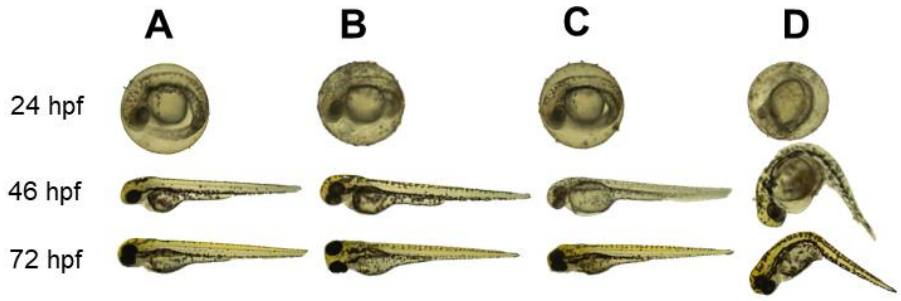


**Figure 1**. The phenotip of fish embryo and larvae which were exposed to CHA-Ag for the period of 72 hours. **A** control, **B** concentration of 250 µg, **C** 500 µg, and **D** 750 µg.

Fish embryos were monitored for 72 hours of the total exposure period. The embryo morphology in each treatment group is shown in Figure 1. Specifically, compared to the morphology of the control group, the head was smaller, and the tail was bent after CHA-Ag treatment (figure 1 D). Despite the development defect and morphological abnormalities, which clearly observed in the embryo, the heart rate in the embryo and larvae still actively continued to occur.

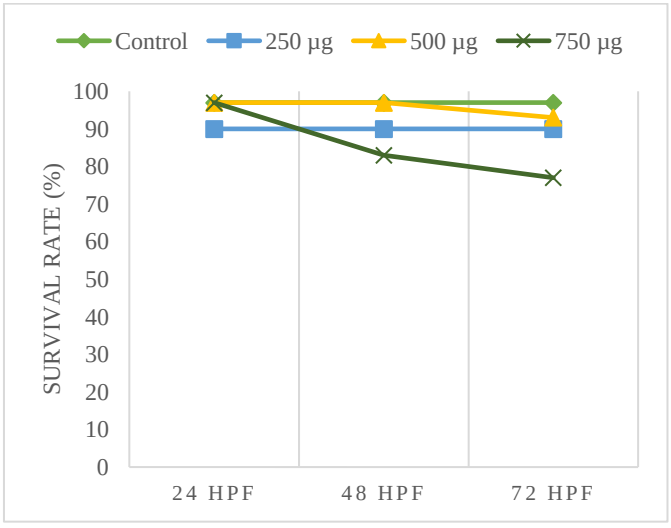


**Figure 2.** graph of zebrafish embryos survival rate (%) of control and which were exposed to CHA-Ag concentrations of 250 µg, 500 µg, and 750 µg for 72 hours.

The effect of CHA-Ag on embryos presented in figure 2 showed the number of survivals at 24 hpf of each treatment. The number of embryo survival on control 96%, concentrations of 250µg was 90%, 500µg was 97%, and 750µg was 97% respectively. The Prolong period

exposure of 48 and 72 hpf showed the tendency of lower survivability of larvae. Moreover, lower survivability percentage was showed by the embryos and larvae treated with 750µg for the period of 48 and 72 hpf of 83% and of 77%.

**Table 1.** The effect of CHA-Ag concentration on zebrafish embryos survival rate at 72hpf.

| Treatment | Average (%) |
|---|---|
| Control | 97[a] |
| CHA-Ag 250µg | 90[a] |
| CHA-Ag 500µg | 93[a] |
| CHA-Ag 750µg | 77[a] |

Exposure of embryo with CHA-Ag for period of 72 hours showed that the lowest survival rate of zebrafish embryos and larvae occurred on the treatment concentration of 750 µg with 77% of embryo survived. This corresponds to the presence of several embryos/larvae which were deformed during development due to high concentrations of ceramic material (Figure 1). However, the analysis result showed that survival percentage of control embryos and exposure to CHA-Ag were not significantly different ($p < 0.05$).

### 3.2 Effect of the ceramic material on embryo hatching rate

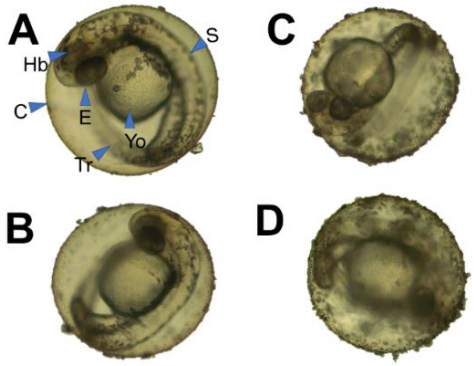


**Figure 3.** Zebrafish embryos exposed to ceramic material at 24 hpf. **A** control embryo, **B** the embryo at concentration of 250 µg, **C** 500 µg, and **D** 750 µg. Hindbrain (Hb), Corion (C), Eye (E), Tail region (Tr), Yolk (Yo), Somite (S).

The hatching rate is one of the important indicator to estimate the developmental progress of zebrafish embryo. The normal hatching period for zebrafish embryos is between 48 to 96 hpf [11]. The zebrafish embryo is surrounded by chorion layer during the pre-hatching stage (figure 3). The chorion is a layer that surrounds and protects the zebrafish embryo as a barrier from external stimuli. The presence of chorion can affect the level of chemical contact with the

embryos and their subsequent biological responses [12]. According to [13] this layer serves as a protection for the embryo which blocks the accumulation of particles due to surface charge interactions. The zebrafish chorion has pores 0.5–0.7 µm in diameter required for the transport of oxygen and nutrients to the embryo. so the chemicals that can enter the chorion are only smaller than the pores[14].

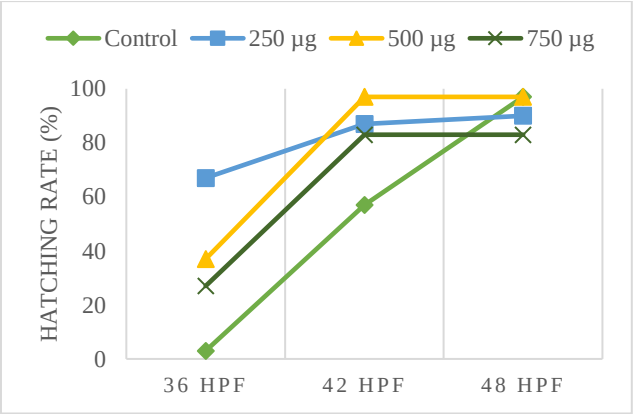


**Figure 4.** Hatching rates (%) of zebrafish embryos were exposed to CHA-Ag concentrations of 250 µg, 500 µg, and 750 µg for 48 hours.

Embryos were exposed to ceramic materials until they become larvae for 72 hours. The embryo hatchability of 36 hpf, the control was only 3%, the CHA-Ag concentration treatment was 250 µg 60%, 500 µg 37%, and 750 µg 27%. Whereas in the treatment of 42 hpf the hatchability of the embryos in the control treatment increased rapidly by 57%, the treatment concentration of CHA-Ag 250 µg 87%, 500 µg 97%, and 750 µg 83%. At 48 hpf control treatment 97%, CHA-Ag treatment 250 µg 90%, 500 µg 97%, and 750 µg 83%. all embryos incubate at 48hpf. Figure 4 shows.

**Table 2.** Effect of ceramic material (CHA-Ag) on hatching rate of zebrafish embryos at 48 hpf.

| Treatment | Average (%) |
|---|---|
| Control | 97[a] |
| CHA-Ag 250µg | 90[a] |
| CHA-Ag 500µg | 97[a] |
| CHA-Ag 750µg | 83[a] |

The statistical analysis was carried out in Table 2, it showed that the treatment of all concentrations of ceramic materials had not significantly different ($P < 0.05$) with control at 48 hpf. All embryos hatched at 36 - 48 hpf, this indicates that the embryo was developing normally.

This follows the statement [15] Zebrafish embryos begin to hatch at 48 hpf and finish hatching at 72 hpf in normal condition.

### 3.3 Effect of the ceramic material on the heartbeat rate (per minutes)

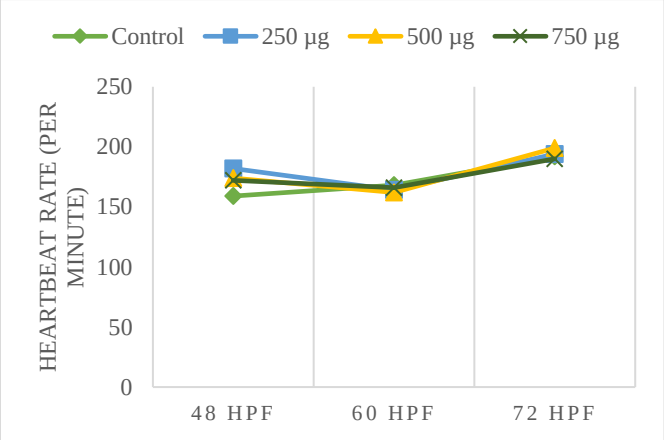


**Figure 5.** Heartbeat rate (per minutes) of embryos and larvae were exposed to CHA-Ag concentrations of 250 µg, 500 µg, and 750 µg for 72 hours.

Graph showed that the embryo and larva heart rate of 48 hpf, the control was 159 beats/minute, concentration of 250 µg 180 beats/minute, 500 µg 174 beats/minute, and 750 µg 172 beats/minute, while the 60 hpf embryo showed that the control treatment heart rate was 168 beats/minute, concentrations 250 µg 164 beats/minute, 500 µg 162 beats/minute, and 750 µg 166 beats/minute, and the 72 hpf embryos heart rate in control 192 beats/minute, concentrations of 250 µg 194 beats/minute, 500 µg 199 beats/minute, and 750 µg 190 beats/minute. Decreased heart rate at 60 hpf and an increase of 72 hpf is indicated because the activity of the cardiomyocytes begins to peel off the ventricular wall to initiate trabeculation and complete at 72 hpf [16]. According to [17] the zebrafish heart rate has increases with embryo development to ensure perfusion in all developing embryonic tissues. in a study [18] the abnormal phenomenon in zebrafish exposed to the chemicals diphenoconazole and cyhalofop-butyl had a teratogenic effect and caused a weakened heart rate.

**Table 3.** Effect of ceramic material (CHA-Ag) on the heartbeat of embryos and zebrafish larvae 72 hpf.

| Treatment | Average |
|---|---|
| Control | 192[a] |
| CHA-Ag 250µg | 194[a] |
| CHA-Ag 500µg | 199[a] |
| CHA-Ag 750µg | 190[a] |

Table 3 shows that the results of the analysis showed that the heart rate ratio of embryos and zebrafish larvae was not significantly different ($P < 0.05$) between the control treatment with

the ceramic material treatment of all concentrations. In general, the normal heart rate of the embryo and larvae of the zebrafish 72 hpf is around 120-180 bpm [19].

### 3.4 Effect of the ceramic material on the morphology of zebrafish larvae

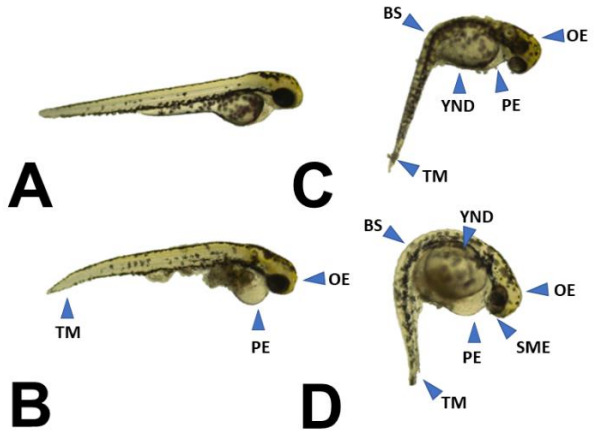


**Figure 6.** Comparison morphology of the larvae which were exposed to CHA-Ag for the period of 66 hpf. **A** control, **B**, **C**, and **D** concentration of 750 µg. Tail Malformation (TM), Yolk Not Depleted (YND), Pericardial Edema (PE) Ocular Edema (OE), Bent Spine (BS), Submandibular Edema (SME).

For the assessment of the toxic properties of ceramic materials in zebrafish, qualitative morphological observations were to determine the changes in zebrafish morphology because of the concentration of CHA-Ag and treatment time. After being treated with various concentrations of CHA-Ag, zebrafish embryos can be assessed based on the severity of their morphological defects. In Figure 6 zebrafish aged 66 hpf, part **A** shows no visible toxic effects and **D** shows severe defects and is not fully developed. Sections **B** and **C** indicate defects that are not severe or moderate.

Developmental disorders can be characterized by a non-depleted or deformed egg yolk; malformations of the spine, tail, and caudal fin; formation around the pericardial sac or yolk; delay of hatching; stunted body or eye growth; damaged and opaque tissue; chorionic excretions that are characterless; and edema in the body cavity, pericardial sac, or yolk sac region [20]. According to [21] developmental abnormalities and teratological effects that occurred in zebrafish embryos and larvae after exposure to chemicals resulted in reduced heart rate, reduced blood circulation, notochord deformities, pericardial edema abnormalities, and yolk sac deformity in the early stages of zebrafish life.

### 3.5 Zebrafish larvae bones structure

Zebrafish is an ideal alternative vertebrate model for studying and skeletal malformations [22]. Based on this, we observed the bone structure using the Alizarin Red S. Alcian Blue (ARAB) staining method on zebrafish aged 30 days after being exposed to ceramic material for 72 hours. The Alizarin Red S. Alcian Blue (ARAB) Staining method was used following the standard method [23].

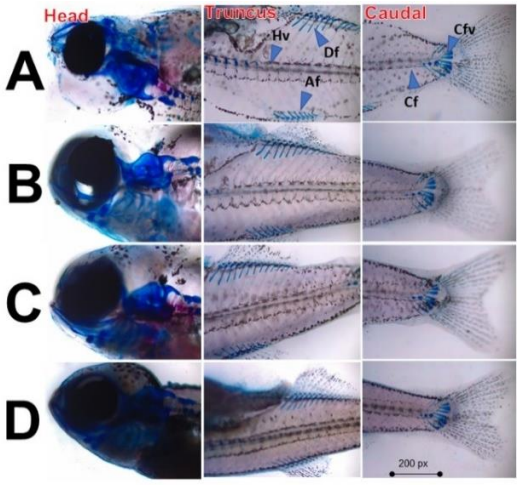


**Figure 7.** Bone structure of the zebrafish 30 dpf. Alcian Blue-Alizarin Red stain. **A** control, **B** concentration of 250 µg, **C** 500 µg, and **D** 750 µg. Caudal fin vertebrae (Cfv), Caudal fin (Cf), Anal fin (Af), Dorsal fin (Df), Hemal vertebrae (Hv).

Figure 7 shows the skull part of the control and ceramic material treatment (CHA-Ag) all concentrated in blue which means cartilage, while red in the vertebrae indicates hard bone. Also, the visible parts are the caudal fin vertebrae, caudal fin, anal fin, dorsal fin, and hemal vertebrae. The staining results shown in Figure 7 identified normal bone structure and development. Generally, according to [24] many studies have focused on the components of the fish skeleton system including the skull, axial skeleton, ribs and fins, and intermuscular bones.

The results of this study provide an overview of CHA-Ag treatment in early zebrafish embryo development did not affect decreasing survival, hatching, and heart rate of zebrafish embryos. Statistical analysis showed that there was no difference between the control group and ceramic material treatment (CHA-Ag). The morphological observations showed that there were malformations and developmental disturbances in the CHA-Ag treatment with a concentration of 750 µg. At 48 hpf all embryos in the control and CHA-Ag exposure had hatched, and the heart rate at 72 hpf was above 140 beats/minute, which is an indicator of normal embryo development. The results of this study provide an overview of the use of zebrafish as animal models for testing the biocompatibility of ceramic materials (CHA-Ag) as dental implants.

## 4. Conclusion

Zebrafish has been developed in this study as a reliable in vivo screening model for the biocompatibility of ceramic materials. Ceramic material (CHA-Ag) had no significant effect ($P<0.05$) on survival rate, hatching rate, and heartbeats rate in zebrafish embryos and larvae. However, a concentration of 750 µg showed an effect on morphology and disruption in the development of embryos and larvae during 72 hours of exposure. This is characterized by changes in morphology and disruption in the development of embryos and larvae such as egg yolk deformation, spinal malformations, tail, caudal fins, body growth, and stunted eyes. Therefore, zebrafish can be used as a good animal model in testing the biocompatibility of

ceramic materials (CHA-Ag) as dental implants. Further studies on biomaterials using zebrafish embryos can be carried out dechorionated and HE & MAF staining of larvae to explore the toxicity mechanisms underlying the effects of chemical exposure. While further studies are required to corroborate these assumptions and to improve assessment of the biocompatibility of CHA-Ag. Dechorionated embryos and HE & MAF staining of larvae could be undertaken to explore the toxicity mechanisms underlying the effects of exposure to these chemicals.

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## References

[1] B. Zhao *et al.*, "Soft tissue integration versus early biofilm formation on different dental implant materials," *Dent. Mater.*, vol. 30, no. 7, pp. 716–727, 2014, doi: 10.1016/j.dental.2014.04.001.

[2] S. Najeeb, M. S. Zafar, Z. Khurshid, and F. Siddiqui, "Applications of polyetheretherketone (PEEK) in oral implantology and prosthodontics," *J. Prosthodont. Res.*, vol. 60, no. 1, pp. 12–19, 2016, doi: 10.1016/j.jpor.2015.10.001.

[3] D. Duraccio, F. Mussano, and M. G. Faga, "Biomaterials for dental implants: current and future trends," *J. Mater. Sci.*, vol. 50, no. 14, pp. 4779–4812, 2015, doi: 10.1007/s10853-015-9056-3.

[4] D. Shekhawat, A. Singh, M. K. Banerjee, T. Singh, and A. Patnaik, "Bioceramic composites for orthopaedic applications: A comprehensive review of mechanical, biological, and microstructural properties," *Ceram. Int.*, no. August, 2020, doi: 10.1016/j.ceramint.2020.09.214.

[5] R. N. Granito, A. C. M. Renno, H. Yamamura, M. C. de Almeida, P. L. M. Ruiz, and D. A. Ribeiro, "Hydroxyapatite from fish for bone tissue engineering: A promising approach," *Int. J. Mol. Cell. Med.*, vol. 7, no. 2, pp. 80–90, 2018, doi: 10.22088/IJMCM.BUMS.7.2.80.

[6] S. Syamsuddin, "Sintesis dan Karakterisasi Biokeramik Tulang sebagai Bahan Implant dengan Metode Sintering," *J. INTEK*, vol. 4, no. 2, p. 84, 2017, doi: 10.31963/intek.v4i2.147.

[7] Y. Y. Filippov, E. S. Klimashina, A. B. Ankudinov, and V. I. Putlayev, "Carbonate substituted hydroxyapatite (CHA) powder consolidated at 450°C," *J. Phys. Conf. Ser.*, vol. 291, no. 1, 2011, doi: 10.1088/1742-6596/291/1/012036.

[8] Y. Zhang *et al.*, "Antibacterial and biological properties of silver-loaded coralline hydroxyapatite," *Front. Mater. Sci. China*, vol. 4, no. 4, pp. 359–365, 2010, doi: 10.1007/s11706-010-0112-2.

[9] G. Brunello, S. Panda, L. Schiavon, S. Sivolella, L. Biasetto, and M. Del Fabbro, "The impact of bioceramic scaffolds on bone regeneration in preclinical in vivo studies: A systematic review," *Materials (Basel).*, vol. 13, no. 7, pp. 1–26, 2020, doi: 10.3390/ma13071500.

[10] S. Y. Pu, N. Hamid, Y. W. Ren, and D. S. Pei, "Effects of phthalate acid esters on zebrafish larvae: Development and skeletal morphogenesis," *Chemosphere*, vol. 246, p. 125808, 2020, doi: 10.1016/j.chemosphere.2019.125808.

[11] H. Al-Kandari *et al.*, "Ecotoxicological assessment of thermally- and hydrogen-reduced graphene oxide/tio2 photocatalytic nanocomposites using the zebrafish embryo model," *Nanomaterials*, vol. 9, no. 4, pp. 1–15, 2019, doi: 10.3390/nano9040488.

[12] C. M. Tran, H. Lee, B. Lee, J. S. Ra, and K. T. Kim, "Effects of the chorion on the developmental toxicity of organophosphate esters in zebrafish embryos," *J. Hazard. Mater.*, vol. 401, no. March 2020, p. 123389, 2021, doi: 10.1016/j.jhazmat.2020.123389.

[13] T. S. P. Rothenbücher, J. Ledin, D. Gibbs, H. Engqvist, C. Persson, and G. Hulsart-Billström, "Zebrafish embryo as a replacement model for initial biocompatibility studies of biomaterials and drug delivery systems," *Acta Biomater.*, vol. 100, pp. 235–243, 2019, doi: 10.1016/j.actbio.2019.09.038.

[14] L. Qiang *et al.*, “Silver nanoparticles in Zebrafish (Danio rerio) embryos: Uptake, growth and molecular responses,” *Int. J. Mol. Sci.*, vol. 21, no. 5, pp. 1–14, 2020, doi: 10.3390/ijms21051876.
[15] D. Huang, H. Li, Q. He, W. Yuan, Z. Chen, and H. Yang, “Developmental Toxicity of Diethylnitrosamine in Zebrafish Embryos/Juveniles Related to Excessive Oxidative Stress,” *Water. Air. Soil Pollut.*, vol. 229, no. 3, 2018, doi: 10.1007/s11270-018-3739-8.
[16] H. C. Yalcin, A. Amindari, J. T. Butcher, A. Althani, and M. Yacoub, “Heart function and hemodynamic analysis for zebrafish embryos,” *Dev. Dyn.*, vol. 246, no. 11, pp. 868–880, 2017, doi: 10.1002/dvdy.24497.
[17] E. De Luca *et al.*, “ZebraBeat : A flexible platform for the analysis of the cardiac rate in zebrafish embryos,” *Sci. Rep.*, vol. 4, pp. 1–13, 2014, doi: 10.1038/srep04898.
[18] Y. Yang, S. Qi, J. Chen, Y. Liu, M. Teng, and C. Wang, “Toxic Effects of Bromothalonil and Flutolanil on Multiple Developmental Stages in Zebrafish,” *Bull. Environ. Contam. Toxicol.*, vol. 97, no. 1, pp. 91–97, 2016, doi: 10.1007/s00128-016-1833-4.
[19] B. P. Sampurna, G. Audira, S. Juniardi, Y. H. Lai, and C. Der Hsiao, “A simple ImageJ-based method to measure cardiac rhythm in zebrafish embryos,” *Inventions*, vol. 3, no. 2, pp. 1–11, 2018, doi: 10.3390/inventions3020021.
[20] A. Raghunath and E. Perumal, “Analysis of lethality and malformations during zebrafish (Danio rerio) development,” *Methods Mol. Biol.*, vol. 1797, pp. 337–363, 2018, doi: 10.1007/978-1-4939-7883-0_18.
[21] C. Merola *et al.*, “Toxicological assessment and developmental abnormalities induced by butylparaben and ethylparaben exposure in zebrafish early-life stages,” *Environ. Toxicol. Pharmacol.*, vol. 80, no. September, 2020, doi: 10.1016/j.etap.2020.103504.
[22] A. Salvaggio *et al.*, “Toxic effects of zinc chloride on the bone development in Danio rerio (Hamilton, 1822),” *Front. Physiol.*, vol. 7, no. APR, pp. 1–6, 2016, doi: 10.3389/fphys.2016.00153.
[23] B. Retnoaji, R. Akiyama, T. Matta, Y. Bessho, and T. Matsui, “Retinoic acid controls proper head-to-trunk linkage in zebrafish by regulating an anteroposterior somitogenetic rate difference,” *Dev.*, vol. 141, no. 1, pp. 158–165, 2014, doi: 10.1242/dev.097568.
[24] C. Nie *et al.*, “Loss of scleraxis leads to distinct reduction of mineralized intermuscular bone in zebrafish,” *Aquac. Fish.*, no. January, pp. 0–1, 2020, doi: 10.1016/j.aaf.2020.04.006.