The Effect of Carbonate Hydroxyapatite (CHA) Dental Implant Material on the Early Development of Zebrafish Embryos *(Danio rerio)*

Sandi Fransisco Pratama[1,] Ika Dewi Ana[2] and Bambang Retnoaji[1,*]

[1]*Department of Biology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Senolowo, Sinduadi, Kec. Mlati, Kabupaten Sleman, Daerah Istimewa Yogyakarta, Indonesia. 92113*
[2]*Faculty of Dentistry, Universitas Gadjah Mada, Jl. Teknika Selatan, Senolowo, Sinduadi, Kec. Mlati, Kabupaten Sleman, Daerah Istimewa Yogyakarta, Indonesia. 92113*
*Coresponding author. Email: bambang.retnoaji@ugm.ac.id

ABSTRACT

Carbonate Hydroxyapatite (CHA) is one of the bioceramic materials that can be used as dental implants. As dental implant material, the biocompatibility of CHA is an important prerequisite factor. This study aims to determine the biocompatibility of CHA on zebrafish embryos. The zebrafish embryos treatment was carried out on 3 hours post-fertilization (hpf) embryos aged. The embryos were exposed to CHA with concentrations of 15 µg/ml, 125 µg/ml, 500 µg/ml, and 2000 µg/ml, respectively. Biocompatibility assessment was carried out by measuring the hatching rate, survival, morphological changes, and embryo physiological performance, respectively. The hatching rate was determined as the number of embryos that hatched at 48, 72, and 96 hpf. The survival rate was determined by the number of live embryos at 24, 48, 72, and 96 hpf, respectively. Moreover, the morphological change was observed on the shape of the embryo's heart and yolk, as well as the tail-yolk detachment as an indicator of a normal body wall occurrence, respectively. Moreover, the physiological performance was decided base on the heartbeat rate performance of the embryos. The result showed no effect of CHA on the hatching and survival rate as well as embryo morphology in all treatments. All embryos heart laterality was normally formed, and the tail-yolk detachment occurred at the period of 24 hpf, which indicated normal development of the embryo. The heartbeat rate showed no differences between CHA treatment compared to control, where the embryo heartbeat rate in all treatments was more than 200/minute, indicating normal embryos development. In conclusion, the CHA treatment did not cause any measurable development defect on zebrafish embryos.

Keywords: Biocompatibility, CHA, Dental, Embryo, Hatching, Zebrafish

1. INTRODUCTION

Bioceramics are a type of material that is used to fix or replace bone tissue that has been damaged [1]. Alumina, zirconia, bioactive glass, glass ceramics, hydroxyapatite, absorbable calcium phosphate, and other materials are examples of bioceramics [2].

Bioceramics in dentistry has developed rapidly. Bioceramics show many clinical applications, such as pulp wrapping materials, root tip fillers, and the appearance of permanent dentin [3]. One of the bioceramics which are widely used in dentistry is hydroxyapatite. Hydroxyapatite is calcium phosphate-containing hydroxide, a member of the mineral group that makes up bones.

However, the biological apatite constituent of bones and teeth is slightly different from the structure of the constituent hydroxyapatite. Bones and teeth contain some additional elements which are not present in hydroxyapatite. One of them is carbonate (CO_3^{2-}) which is about 5-8% in bone [4]. Carbonate hydroxyapatite (CHA) has a structure similar to the biological apatite in bone [5].

The structure of the hydroxyapatite [Ca10 (PO4) 6 (OH) 2] allows for broad, nonstoichiometric atomic substitution between the Ca, P positions, and the anion channels. The difference between hydroxyapatite and carbonate apatite molecules is the addition of a carbonate group. Type A apatite carbonate has a carbonate group on the hydroxyl side, while type B apatite carbonate has a carbonate group on the phosphate side. The natural content of bone is type B apatite carbonate [6].

The use of carbonate hydroxyapatite as a dental implant material is placed at a location in direct contact with the periapical tissues. So that the non-toxic characteristic and biocompatibility are very important [7]. Biocompatibility can be defined as the ability of a material to have a suitable application functionality and host response [8].

The need for biocompatible materials in dentistry requires toxicity assay. Zebrafish has proven to be a promising animal model for toxicity screening. The toxicity of the dental material in zebrafish can be easily correlated with humans because it has genetic and physiological similarities [9]. Moreover, zebrafish embryonic development also similar to mammals [10]. This study aims to determine the effect of carbonate hydroxyapatite (CHA) on the early development of zebrafish embryos.

2. MATERIALS AND METHODS

This study was an experimental assay with a control group and a carbonated hydroxyapatite (CHA) treatment group. The research was conducted at the Laboratory of Animal Structure and Development, Faculty of Biology, Universitas Gadjah Mada (UGM) in October 2020.

2.1. Maintenance of Fish and Preparation of Zebrafish Embryos

Zebrafish Wild Type adults were maintained in aquariums (30 cm x 30 cm). The zebrafishes were maintained at standard conditions, at 14:10 light: dark cycle, temperature $27\text{-}28.5^0C$, and dissolved oxygen (DO) at 6-8. The fish were fed commercial fish under the brand name Takari®. Feeding was done three times a day. Embryos are obtained by spawning males and females who were placed in a spawning aquarium. The ratio of females to males is 2: 1. The spawning procedure was carried out in a photoperiod of 14 hours light and 10 hours dark. Fertilized eggs were collected for research.

2.2. Exposure of Zebrafish Embryos to Carbonate Hydroxyapatite (CHA)

The CHA which was used in the study were provided by Faculty of Dentistry, UGM. CHA treatment on fish embryo was carried out following Makkar et al., (2018) [9]. Three (3) hours post-fertilization (*hpf*) old zebrafish embryos were selected based on normal morphology and cleavage performance and used for toxicity assay. The embryos were maintained in 15 wellplates, ten embryos in each well and were treated with CHA with different concentrations. Wellplates were exposed to a 14:10 hour light: dark photoperiod at 28^0C. The treatment settings are as follows:

- Zebrafish embryo in egg water.
- Zebrafish embryo in egg water and CHA concentration of 15 µg/mL.
- Zebrafish embryo in egg water and CHA concentration of 125 µg/mL.
- Zebrafish embryo in egg water and CHA concentration of 500 µg/mL.
- Zebrafish embryo in egg water and CHA concentration of 2000 µg/mL.

2.3. Survival Rate and Hatching Rate of Zebrafish Embryo

Survival was determined by the number of embryos at 24, 48, 72, and 96 *hpf*. The survival assessment was carried out comparing the number of embryos at period of observation with the number of embryos at the beginning of the treatment (3 *hpf*). The hatching rate was expressed as the number of embryos that hatched at 72, 80 and 96 *hpf*. Hatching assessment was carried out by comparing the embryos hatched with the number of embryos at the observation period.

2.4. Morphological Change and Heartbeat Rate.

Morphological observations were carried out by observing any morphological changes through stereo microscope observations. Yolk morphology and the tail-yolk detachment were observed as standard parameters for normal embryo development. The embryo heartbeat rate of each CHA treatment was calculated and compared with that control treatment.

2.5. Data Analysis

Data analysis was carried by both out quantitative and qualitative. Quantitative data were obtained from the assessment of egg hatching, embryo survival, and heart rate. Statistical analysis was performed using IBM SPSS 2020 [11]. One-way ANOVA was used to assess

the significance of the effect of treatment on outcomes. Qualitative data were in the form of yolk morphology and the tail-yolk detachment. Morphological changes were observed directly using a stereomicroscope.

3. RESULT AND DISCUSSION

3.1. Exposure of Zebrafish Embryos to Carbonate Hydroxyapatite (CHA).

Exposure of zebrafish embryos to CHA was carried out at 70% epibolic development stage (**Figure 1**). According to Kimmel et al (1995) [12], in zebrafish embryos, the epibolic stage begins in the embryo age 4 *hpf* and enters 70% epiboly in the embryo aged 8 *hpf*. However, this may vary, depending on the conditions of the embryo and the environment.

The epibolic stage is sensitive to embryo development. Exposure to toxic substances at this stage causes morphological malformations in embryos and larvae [13]. Observation showed no effect of CHA on early embryo development (24 *hpf*).
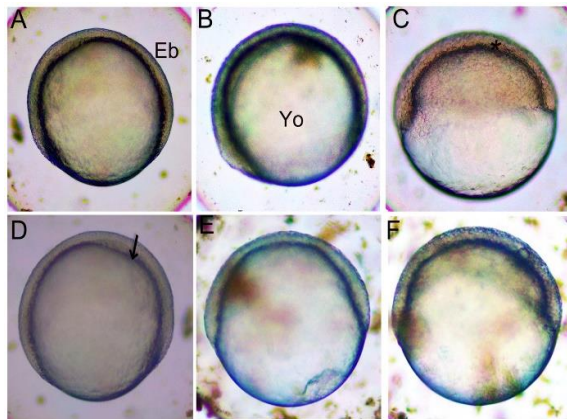

Figure 1. Zebrafish embryos 70% epiboly developmental stages were treated with CHA with different concentrations (A: Control, B: 15 µg/mL, C: 125 µg/mL, D: control 2, at the epibolic stage of 70%, a prechordal plate was seen (arrow), E: µg/mL, F: 2000 µg/mL). Yo (Yolk), Eb (Embryo).

3.2. Effect of CHA on Survival Rate and Hatching Rate of Zebrafish Embryos.

The toxic effect of CHA was assessed in zebrafish embryos. One of the parameters of assessment is the survival rate. As shown in Figure 2, observations at 24 *hpf*, 48 *hpf*, 72 *hpf* and 96 *hpf* show embryo survivability above 90%. Compared with controls, the survival rate did not differ significantly (p: 0.01 <0.05) on CHA exposure. CHA treatment did no affect the decreasing survival rate of the zebrafish embryo.

Embryo hatching was observed for 48 hpf, 72 hpf and 96 hpf embryos (Figure 3.). Observations at 48 hpf showed that the percentage of embryos that hatched in the control treatment was 63%, µg/mL was 47%, 125 µg/mL was 42%, 500 µg/mL was 30%, and 2000 µg/mL was 48%.

While observations on 72 hpf of control embryos and 2000 ug/mL had hatched 100%. At 96 hpf control, 15 µg/mL, 500 µg/mL, and 2000 µg/mL had hatched 100%. Meanwhile, in the treatment of 125 µg/mL was 97% hatched.

According to Kimmel et al (1995) [12], zebrafish embryos hatch at the age of 48 *hpf*. The results of this study indicated a delay in hatching in some embryos. Hatching delays occurred in the control and CHA treatment groups. So that the cause of delay is not CHA treatment.

Although there was a delay in hatching, the statistical analysis showed that there was no difference between the control group and the CHA treatment. CHA treatment did not affect hatching of zebrafish embryos (p: 0.01 <0.05).
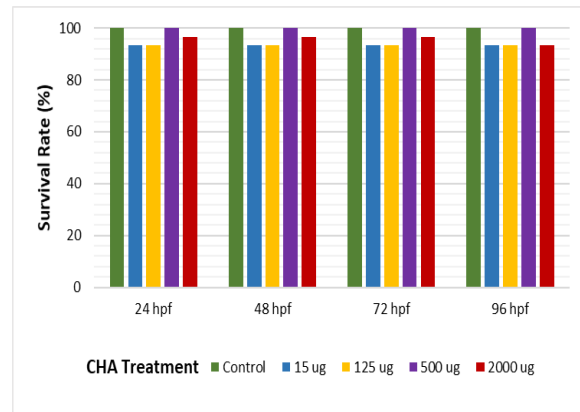

Figure 2. Survivability rate of zebrafish embryo exposed to different concentrations of CHA at different hours postfertilization (*hpf*). All measurements were made in triplicate, and values are presented as means of the three independent trials. ANOVA analysis (p: 0.01 <0.05) showed no significant change in control and treated embryos at all hours postfertilization (*hpf*).
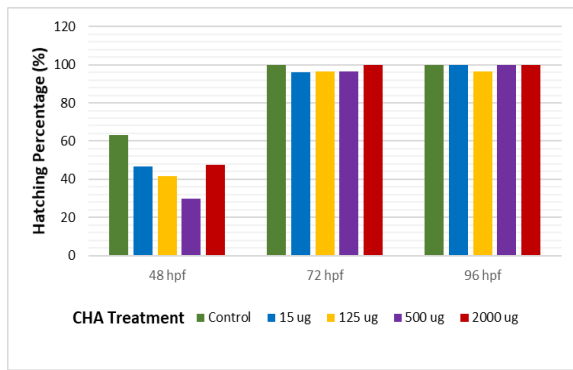
Figure 3. Hatching rate of zebrafish embryo exposed to different concentrations of CHA at different hours postfertilization (*hpf*). All measurements were made in triplicate, and values are presented as means of the three independent trials. ANOVA analysis (p: 0.01 <0.05) showed no significant change in control and treated embryos at all hours postfertilization (*hpf*).

3.3. Morphological Changes of Zebrafish Embryo

Morphological changes were observed in the yolk shape of the embryo and the tail-yolk detachment, respectively as indicators of normal development. Observations were made on 24 hpf embryos (Figure 4.). The yolk is normal in shape and there are no malformations at the yolk. In the 24 hpf embryo, there was the tail-yolk detachment in the control embryo and CHA treatment.
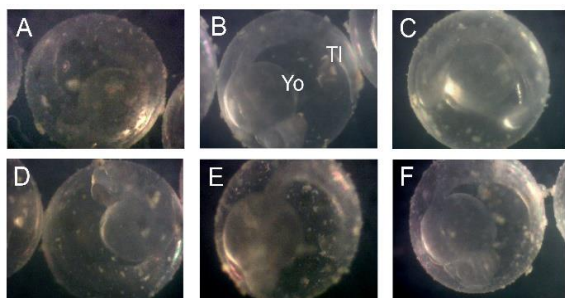

Figure 4. Zebrafish embryos 24 hours postfertilization *(hpf)*. There was a detachment of the tail from the yolk. The tail-yolk detachment is an indicator of a normal body wall occurrence. (A: Control, B: 15 ug / mL, C: 125 ug / mL, D: control 2, E: 500 ug / mL, F: 2000 ug / mL). Yo (Yolk), Tl (Tail)

The 10-24 hpf period is the segmentation stage. At this stage various morphogenetic movements occur, the somites develop, various primary organs such as the heart begin to appear, and there is an elongation of the tail buds. This period is also called

the "tail bud period", where the tail is released from the embryo and lengthens [12]. The tail-yolk detachment in all CHA treatments at 24 hpf indicated that the embryos were developing normally.

3.4. Heartbeat rate performance of the embryos.

In zebrafish embryos that developed normally at $26 \pm 1°C$, the heart rate was seen after 48 hpf [14]. The effect of CHA on heart rate performance was observed at 48 hpf and 72 hpf (**Figure 5.**). Heart rate is counted per minute. 48 hpf embryo observation showed heart rate in control treatment 220 beats / minute, 15 µg/mL 217 beats / minute, 125 µg/mL 205 beats / minute, 500 µg/mL 204 beats / minute, and 2000 µg/mL 179 beats / minute.
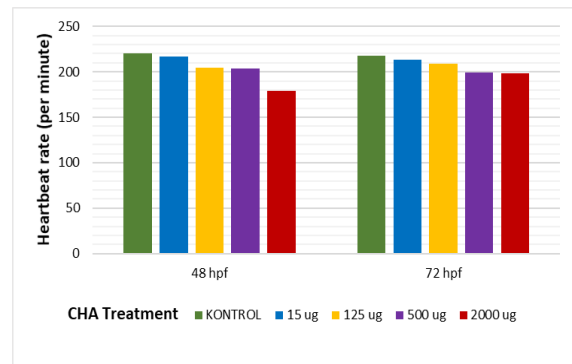

Figure 5. Heartbeat rate performance of the embryos. ANOVA analysis (P <0.05) showed no significant change in control and treated embryos at all hours postfertilization (*hpf*).

Meanwhile, the embryo observation of 72 hpf showed that the heart rate in the control treatment was 218 beats / minute, 15 µg/mL 213 beats / minute, 125 µg/mL 209 beats / minute, 500 µg/mL 199 beats / minute, and 2000 µg/mL 198 beats / minute. These observations indicated that the embryo was developing normally. A normally developed embryo will show a heart rate of at least 80 beats per minute [14]. Meanwhile Zhu *et al*. [15], in their study stated that a normal embryo produces a heart rate of 140-160 beats/minute [15].

Embryos possess a low antioxidant defense capability during the early stages of organogenesis and are much more vulnerable to the teratogenic effects of oxidative compounds. The development and function of zebrafish may therefore be affected by a disequilibrium between reactive oxygen species (ROS) and embryonic antioxidant protection, giving rise to several different congenital defects such as cardiac edema, growth delay, decrease in pigmentation, and even death [16].

CHA 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 CHA treatment group and the control group. Meanwhile, morphological observations show that there is no malformation in the embryonic yolk. In embryos aged 24 *hpf* there has also been a release of the tail-yolk detachment, which is an indicator of normal embryo development.

The results of this study provide an overview of the use of zebrafish as an animal model for testing the toxicity of dental implant materials. In addition, this study also provides information on the biocompatibility of CHA against zebrafish embryos.

4. CONCLUSION

There was no effect of CHA treatment on the survival rate, hatching rate, and heart rate of zebrafish embryos. Assessment through yolk morphological observation and the tail detachment yolk showed no malformations or morphological defect on embryos exposed to CHA. This study shows that carbonate hydroxyapatite (CHA) has decent biocompatibility.

ACKNOWLEDGMENT

REFERENCES

[1] M. Roy, A. Bandyopadhyay, S. Bose, Ceramics in bone grafts and coated implants, Materials and Devices For Bone Disorders (2017) 265–314. https://doi.org/10.1016/B978-0-12-802792-9.00006-9

[2] S.S. Raghavendra, G.R. Jadhav, K.M. Gathani, P. Kotadia, Bioceramics in endodontics – a review, Journal of Instanbul University Faculty of Dentistry 51(3) (2017) 128–137. https://doi.org/10.17096/jiufd.63659

[3] G. Debelian, M. Trope, The use of premixed bioceramic materials in endodontics, Giornale Italiano di Endodonzia 30(2) (2016) 70–80. https://doi.org/10.1016/j.gien.2016.09.001

[4] J.C. Merry, I.R. Gibson, S.M. Best, W. Bonfield, Synthesis and characterization of carbonate hydroxyapatite, Journal of Materials Science: Materials in Medicine 9(12) (1998) 779–783. https://doi.org/10.1023/A:1008975507498

[5] Q. Liu, S. Huang, J. P. Matinlinna, Z. Chen, H. Pan, Insight into biological apatite: Physiochemical properties and preparation approaches, BioMed Research International (2013). https://doi.org/10.1155/2013/929748

[6] J. Legradi, N. el Abdellaoui, M. van Pomeren, J. Legler, Comparability of behavioural assays using zebrafish larvae to assess neurotoxicity, Environmental Science and Pollution Research, *22*(21) (2015) 16277–16289. https://doi.org/10.1007/s11356-014-3805-8

[7] D.M. Escobar-García, E. Aguirre-López, V. Méndez-González, A. Pozos-Guillén, Cytotoxicity and initial biocompatibility of endodontic biomaterials (MTA and biodentine ™) used as root-end filling materials, BioMed Research International (2016). https://doi.org/10.1155/2016/7926961

[8] D. Perrone, Book reviews, The Prison Journal 83(4) (2003) 483–486. https://doi.org/10.1177/0032885503256469

[9] H. Makkar, S.K. Verma, P.K. Panda, E. Jha, B. Das, K. Mukherjee, M. Suar, In Vivo molecular toxicity profile of dental bioceramics in embryonic Zebrafish (*Danio rerio*), Chemical Research in Toxicology 31(9) (2018) 914–923. https://doi.org/10.1021/acs.chemrestox.8b00129

[10] A. Hill, M. Jones, A. Dodd, H. Diekmann, A review of developmental toxicity screening using zebrafish larvae, Toxicology Letters 205 (2011) S115. https://doi.org/10.1016/j.toxlet.2011.05.413

[11] IBM Corp, IBM SPSS Statistics for Windows, Version 27.0, Armonk, IBM Corp, New York, 2020.

[12] C.B. Kimmel, W.W. Ballard, S.R. Kimmel, B. Ullmann, T.F. Schilling, Stages of embryonic development of the zebrafish, Developmental Dynamics 203(3) (1995) 253–310. https://doi.org/10.1002/aja.1002030302

[13] T.W. Sadler, Embriologi kedokteran langman 12th edition, A. Novrianti (Eds), EGC Jakarta, 2013. [In Bahasa Indonesia]

[14] OECD, Test no. 236: fish embryo acute toxicity (FET) test, OECD Guidelines for the testing of

chemicals, section 2, OECD Publishing, Paris, 2013. https://doi.org/10.1787/9789264203709-en.

[15] X. Zhu, L. Zhu, Y. Li, Z. Duan, W. Chen, P.J.J. Alvarez, Short communication developmental toxicity in Zebrafish (*Danio rerio*) embryos after exposure to manufactured nanomaterials : buckminsterfullerene aggregates (NC 60) and fullerol, Environmental Toxicology and Chemistry, *26*(5) (2007) 976–979. doi: https://doi.org/10.1897/06-583.1.

[16] S. Pujari-Palmer, X. Lu, M.K. Ott, The influence of hydroxyapatite nanoparticle morphology on embryonic development in a zebrafish exposure model, Nanomaterials 7(4) (2017). https://doi.org/10.3390/nano7040089