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Original Article

BIOCOMPATIBILITY OF NANO-HYDROXYAPATITE GRAFT FROM UNAM SNAIL (*VOLEGALEA COCHLIDIUM*) SHELL ON NIH 3T3 FIBROBLAST

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ABSTRACT

Objective: Periodontitis may cause progressive damage to the periodontal tissue; thus, treatment such as bone grafts is often needed to restore tissue function. One of the synthetic bone graft materials used is hydroxyapatite. Hydroxyapatite can be found in nature, such as egg shells, coral, and shells of mussels and snails. Unam snail (*Volegalea cochlidium*) is a shelled animal with a high calcium carbonate level as a calcium source in hydroxyapatite. Despite the fact that numerous studies have demonstrated the benefits of using materials sourced from natural resources, discussions continue about their biocompatibility concerning cytotoxic reactions.

Methods: Nanoparticles of hydroxyapatite (nHAP) are produced by the sol-gel process. The 3T3 fibroblast cells were cultured on Dulbecco's Modified Eagle Medium (DMEM), and its viability was measured using the 2,5-diphenyl-2H-tetrazolium bromide (MTT) test method and repeated four times.

Results: The mean viability of NIH 3T3 fibroblast cells in different nHAP concentrations, respectively, 95.9±1.69% (1.6875 mg/ml), 92.9±0.82% (0.8437 mg/ml), 78.3±0.98% (3.375 mg/ml) and 38.3±0.9% (6.75 mg/ml). ANOVA test showed a significant difference between concentrations with $p<0.001$. The IC_{50} value of nHAP to the proportion of viability of NIH 3T3 fibroblast cells was 5.81 mg/ml.

Discussion: With an IC_{50} of 5.81 mg/ml, indicating moderate cytotoxicity, cell viability was over 90% at lower concentrations. The results validate Volegalea cochlidium as a new and feasible biogenic source of nano-hydroxyapatite.

Conclusion: Derived from Volegalea cochlidium, nano-hydroxyapatite (nHAP) exhibits concentration-dependent biocompatibility, with safe viability levels found below the IC_{50} threshold of 3.49 mg/ml. The increasing concentration of nano-hydroxyapatite (nHAP) tends to decrease the viability of 3T3 fibroblast cells.

Keywords: Biocompatibility, Hydroxyapatite, Nanoparticles, NIH 3T3

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INTRODUCTION

Bone graft materials are classified into two main categories: materials derived from nature and synthetic. Bone grafts are derived from natural materials, including autograft, allograft, xenograft, and photogenic. Materials from nature are still used to treat periodontitis, but they have drawbacks. Autografts require surgery in other areas and may cause postoperative pain and wound-healing complications. Allografts require special care and storage. The application of xenograft and photogenic materials can be exposed to viruses or bacteria through donor and plasma examinations and require inactivation of the protein [1, 2]. Through this material, immunological reactions may also occur, causing the host graft reaction. The synthetic materials include hydroxyapatite, β-tricalcium phosphate, biphasic calcium phosphate ceramic, calcium phosphate cement, bioglass, calcium sulfate, and polymer, which are made in various forms with varying physicochemical properties to complement the limitations of natural material [2, 3].

Among other synthetic materials, hydroxyapatite, which contains calcium phosphate molecules, is considered the most biocompatible and has a high affinity for biopolymers to bind the bone [4]. Hydroxyapatite is the dominant inorganic component in the hard tissues of the human body [5]. The most recent advancement in nano-sized hydroxyapatite (nanohydroxyapatite) demonstrates enhanced biomechanical characteristics, resemblance to natural bone, and accelerated response to external stimuli, improved spatial distribution and release of bioactive molecules, including growth factors, and enhanced osteoregenerative properties. Furthermore, the nanostructure enhances the material's absorption, promoting more efficient adhesion, proliferation, and differentiation of osteogenetic progenitor cells. Nanohydroxyapatite has better sintering properties and density, increasing strength and other mechanical properties [4, 5]. Raw materials to produce hydroxyapatite can be found in nature, such as limestone, eggshells, coral, and shells of mussels and snails [6, 7].

The production of mussels and snails in Indonesia increases yearly; in 2010, the production reached 34.929 tons [8, 9]. The North Sumatra region contributes to its marine resources. Based on the development of fishery production, in 2011, Deli Serdang Regency produced 203, 322, 400 tons of marine products [10]. Until now, shells of mussels and snails have only been used as handicraft materials, even though these shells have a high calcium carbonate composition as a source of calcium in hydroxyapatite synthesis. Unam snail (*Volegalea cochlidium*) is a group of mollusks from the marine gastropod class. Unam snails have a hard shell and contain a high concentration of calcium carbonate [6, 11, 12]. The conical, thick, and heavy shell consists of a 95-99.9% calcium carbonate crystal structure interspersed with 0.1-5% organic protein components. The more complicated the shell, the higher the calcium content [12].

Several research methods have been reported to synthesize hydroxyapatite: precipitation, sol-gel, dry, micro-emulsion, hydrothermal, and other combination methods. A sol-gel method synthesizes hydroxyapatite, producing nanoparticles with a large surface area through a low combustion temperature. Cell viability test is a part of the toxicity test used to evaluate the effect of material on cell culture biologically [13, 14]. Viability testing was carried out to measure the impact of the material in terms of cell number and growth, cell membrane integrity, biochemical activity,

and genetic material [15]. One of the viability tests carried out is the column metric test [15, 16]. MTT test (3-(4,5-dimethylthiazol)-2,5-diphenyl-tetrazolium bromide) is a simple column metric test to measure the number of living and proliferating cells developed by Mosmann and Cole (1983). The test was carried out by converting MTT into formazan crystals in the living cells studied, where the results showed mitochondrial function in these cells [17].

Fibroblast cells are connective tissue in the periodontal ligament and produce collagen fibers in the tissue healing process. The ability of fibroblast cells to overgrow in wound tissue and live alone make fibroblast cells easily cultured and used for research [16]. Research on the biocompatibility of hydroxyapatite against a cell with different raw materials and concentrations has been reported. A study conducted by Kartono *et al.* used blood clam shell extract (*Anadara granosa*) on BHK-21 fibroblast cells. It stated that the highest number of living fibroblast cells was at a concentration of 0.8437 mg/ml [18]. A similar study by Nastiti *et al.* showed that blood clam shell extract against mesenchymal stem cells had the highest viability at a 6.75 mg/ml concentration [19].

Based on the background, few studies use bone graft material for hydroxyapatite nanoparticles, especially those from the Unam snail shell (*Volegalea cochlidium*). *Volegalea cochlidium* may demonstrate favorable biocompatibility, comparable to or exceeding that of other mollusk-based sources due to its high calcium carbonate content and its plentiful presence in North Sumatra, Indonesia, which makes it a promising and sustainable biogenic precursor for hydroxyapatite synthesis. The study aimed to evaluate the viability of National Institutes of Health 3 d transfer, 3 x 10^5 inoculum (NIH 3T3) fibroblast cells on hydroxyapatite nanoparticles contained in the shell of the Unam snail (*Volegalea cochlidium*) synthesized by the sol-gel method.

MATERIALS AND METHODS

The study was approved by the Ethics Committee of Universitas Sumatera Utara (394/KEP/USU/2021). This present study is an experimental laboratory investigation that employs a post-test-only control group design using Unam snails (*Volegalea coccidium*) and NIH 3T3 fibroblast cell culture. The Unam snail species is identified by the Zoological Laboratory, Indonesian Institute of Sciences, Cibinong. The NIH 3T3 fibroblast cells were obtained from PT. Prodia Stemcell Indonesia (Jakarta, Indonesia). Fibroblast cells were taken from NIH 3T3 cell lines fibroblast culture (3-day transfer, inoculum $3x10^5$ cells). The inclusion criteria of Unam snail shells were ≥ 5 cm long, intact shell structures and morphology, and in good condition, shells were taken from the exact location. The sample devided into 3 groups with.

Material preparation

One kilogram of Unam snail shells was cleaned and dried until there was no smell. The clean shell was rewashed using Aquadest. The shell was crushed using a mortar, calcined at 1000 °C for 2 h with a furnace machine, put in a desiccator, and cooled to room temperature. Forty grams of CaO (calcium oxide) Unam snail shells powder was mixed in the HNO_3 2M solution (12.83 ml of 65% HNO_3 solution dissolved in 100 ml of aquadest) to form a mixture of $Ca(NO_3)_2$. The mixture was stirred and filtered using filter paper until the $Ca(NO_3)_2$ precipitate remained. The residue was dissolved into 100 ml of aquadest and filtered using Whatman 42 filter paper to obtain $Ca(NO_3)_2$ filtrate.

The formation of chemical reaction was: M HNO_3+CaO → $Ca(NO_3)_2$ $2H_2O$

Hydroxyapatite synthesis

The hydroxyapatite was synthesized using the sol-gel method. A total of 8.52 gs of powdered disodium hydrogen phosphate (Na_2HPO_4 98%) was dissolved into 100 ml of aqua dest until it reached 0.6 M. 100 ml of $Ca(NO_3)_2$ solution was added slowly using a magnetic stirrer for 30 min to reach the Ca/P ratio 1.67. To stimulate the reactivity of the precursor, the mixture was agitated for 15 min after its formation. The solution was allowed to stand at room temperature for 24 h, subjected to reflux in an oil bath for 16 h at 70 °C, and then evaporated in a water bath for 15 h at 100 °C. The resultant gel was dehydrated in an oven and pulverized with a mortar and pestle. The powder underwent sintering at a constant temperature of 800 °C. The hydroxyapatite powder was ground with a ball mill for 30 min and then sieved using a 200 mesh sieve. Then, the particle size distribution of the synthesized nano-hydroxyapatite was carrried using a laser diffraction particle size analyzer (Analysette 22 NanoTec, Fritsch GmbH, Germany).

Harvesting (Towing) procedure and NIH 3T3 fibroblast cell counting

The NIH 3T3 fibroblast cells were obtained from PT. Prodia Stemcell Indonesia (Jakarta, Indonesia). Cell line authentication was confirmed by the provider through short tandem repeat (STR) profiling to ensure cell identity. Routine mycoplasma screening was conducted using PCR-based assays, and only mycoplasma-free cells were used for experimentation. The NIH 3T3 cell cultures were stored in a chamber at-80 °C or in liquid nitrogen. At the harvest time, NIH 3T3 cells were examined to determine if at least 80% were confluent (growing evenly) and not contaminated. Harvesting was done by removing the liquid medium in the disk culture with repeated aspiration using a micropipette. The disk culture was rinsed with phosphate buffer saline into the medium to clean the remaining dead cells. A total of 400 µl of trypsin EDTA 0.25% was poured slowly into the culture disk and put in a CO_2 incubator for 4 min to break the bonds between cells and the medium; 5 ml DMEM was added, and cells were removed one by one with up and down method. The NIH 3T3 cells were counted using a hemocytometer. Eight thousand cells/well were used with 8 ml of DMEM to test the viability. After counting, the cells were transferred into conical tubes and stored in a 5% CO_2 incubator for 24 h at 37 °C.

Viability testing

To make the Unam snail stock solution, 400 mg of the sample was dissolved in 100 ml of dimethyl sulfoxide (DMSO), resulting in a 4 mg/ml concentration. The sample without a medium was introduced into the tube, while a DMSO 2% medium was established as the control variable. The initial solution was partitioned and diluted (0.4 mg/ml in each sample) based on the calculated concentration required for each sample. The first tube contains sample I at a concentration of 6.75 mg/ml, the second tube contains sample II at a concentration of 3.375 mg/ml, the third tube contains sample III at a concentration of 1.6875 mg/ml, and the fourth tube contains sample IV at a concentration of 0.843 mg/ml. A volume of 100 microlitres from each tube was introduced into the well plate using a separate tip for each sample. All specimens were placed in a 5% CO2 incubator at 37 °C for 24 h. Cellular observations were conducted 24 h later. The solution test and its comparison were discarded, 10 ml of MTT (Microculture Tetrazolium Salt) was added with a 5 mg/ml concentration to each well plate. Re-incubated in 5% CO_2 incubator for 4 h. Living cells will bind to MTT to form formazan fibers (formazan crystals). The formazan crystals were dissolved with a 10% SDS stopper with 0.01 HCl to stop the MTT reaction, and the plate was wrapped and left overnight at room temperature. The cells were observed using a microplate reader and were read at 595 nm. Viability was expressed by comparing the absorbance value of the treatment group exposed to the test material with the control group (sample without test material) using a formula from *in vitro* technologies.

IC_{50} value

In this study, the procedure for testing the IC50 value was similar to cell viability testing. The IC_{50} value was determined by regression analysis between the viability percentage and the determined nHAP concentration.

Statistical analysis

Data collected from observations were analyzed. A normality test was applied to evaluate the distribution. A one-way ANOVA test (ANOVA) was carried out to compare the viability of each concentration. Furthermore, a post-hoc using LSD (Least Significant Difference) test was utilized to see the difference in viability between all groups.

RESULTS

The hydroxyapatite synthesis result showed nano-sized particles with an average particle size of 0.09041 µm or 90.41 nm. The average proportion of NIH 3T3 fibroblast cell viability are highest at an nHAP concentration of 1.6875 mg/ml, counting>95%. The result indicates that with a concentration of 1.6875 mg/ml, nHAP stimulates the optimum growth of fibroblast cells and are not toxic. Similar viability results can be seen at 0.8437 mg/ml (92.9%). The highest concentration of 6.75 mg/ml signifies the lowest viability of 38%, showing the concentration of 6.75 cannot stimulate the optimum growth of fibroblast cells and can be stated toxic (table 1).

Fig. 1 shows the microscopic appearance of fibroblast cells after treatment. Very dense formazan fiber bonds are seen at concentrations of 1.6875 mg/ml and 0.8437 mg/ml, indicating the number of fibroblast cells that lived after the 24 h MTT test. The higher the concentration, the fewer formazan fiber bundles are seen.

The calculation of IC_{50} was determined by regression analysis of each viability proportion and nHAP concentration. Based on the graph (fig. 2), the correlation coefficient (R^2) is close to 1 (0.957 or 95.7%), presenting a solid correlation between the concentration and viability. In addition, the regression equation is y = (-9.931)x+107.7, showing a negative association between the two variables. This means that increasing the concentration of nHAP will decrease the viability proportion. The IC_{50} value was 5.81 mg/ml, indicating the maximum concentration of nHAP that stimulates 3T3 fibroblast cell proliferation by 50%.

The ANOVA test result shows a significant difference between concentrations with p<0.001 (table 2.). The post-hoc LSD test result are presented in table 3. The result showed a significant difference between concentration and viability of 3T3 fibroblast cells in all groups (p<0.05).

Table 1: The average viability of fibroblast cells in different concentrations of nHAP

nHAP concentration (mg/ml)	Viability proportion (Mean±SD)
0.8437	92.9±1.82
16,875	95.9±1.69
3,375	78.3±0.98
6.75	38.3±0.94

nHAP= Nanoparticles of hydroxyapatite; SD= standard deviation

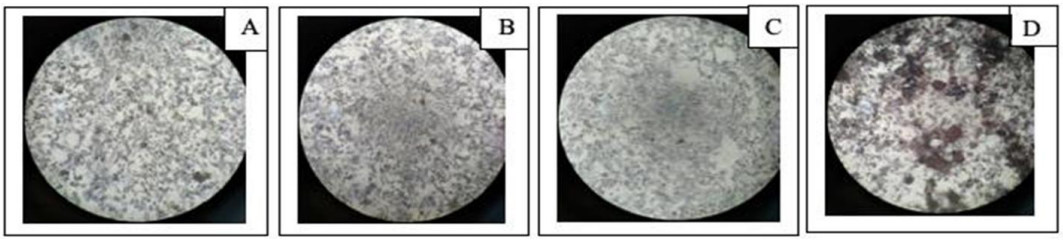

Fig. 1: Microscopic fibroblast cells with different nHAP concentrations. (A) 0,8437 mg/ml; (B) 1,6875 mg/ml; (C) 3,375 mg/ml; dan (D) 6,75 mg/ml. Very dense formazan fiber bonds are seen in A and B, indicating the number of fibroblast cells lived after 24 h MTT test. The higher the concentration, the fewer formazan fiber bundles are seen

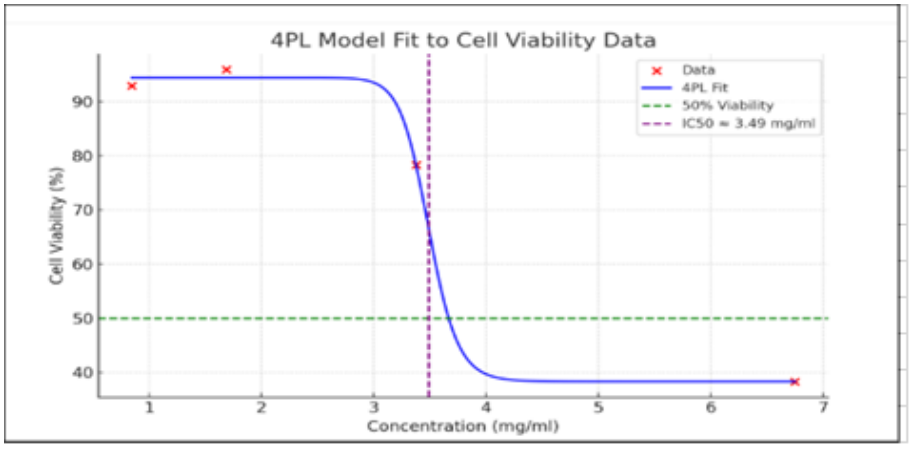

Fig. 2: Regression result of IC_{50} of nHAP concentration on fibroblast viability

Table 2: The mean difference of viability proportion of 3T3 fibroblast cell in different nHAP concentrations

nHAP concentration (mg/ml)	Viability proportion (Mean±SD)	P-Value
0.8437	92.9±1.82	0.001*
16,875	95.9±1.69	
3,375	78.3±0.98	
6.75	38.3±0.94	

*ANOVA test p-value<0.050 = significant between groups
nHAP= Nanoparticles of hydroxyapatite; SD= standard deviation

Table 3: LSD post hoc test on average proportion of viability of 3T3 fibroblast cells in different nHAP concentrations

nHAP Concentration (mg/ml)	0.8437	16,875	3,375	6.75
0.8437	-	0.029*	0.001*	0.001*
16,875	0.029*	-	0.001*	0.001*
3,375	0.001*	0.001*	-	0.001*
6.75	0.001*	0.001*	0.001*	-

* LSD *post hoc* test p-value<0.050 = significant between groups
nHAP= Nanoparticles of hydroxyapatite

DISCUSSION

The main ingredients in the synthesis of hydroxyapatite are calcium and phosphate. Calcium can be obtained from chemicals or natural materials such as limestone or inorganic materials such as bones, shells, coral, eggshells, and snails [9]. The sol-gel method to synthesize hydroxyapatite is carried out by mixing calcium and phosphate precursors in the solution phase, gelatinization (reflux and evaporation), drying, sintering, and refining to purify the hydroxyapatite powder [14]. The cell viability test is part of the toxicity test to evaluate the biological effects of material, measure the effect of a compound in experimental animals or treated cell cultures [19] and determine the response of cells to external factors [20]. The MTT test (2-(4, 5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H tetrazolium bromide) was used to test the viability due to fast, sensitive, and accurate for measuring cell activity, and it can be used on most cell types when the new substance is used. 3T3 fibroblast cell culture is often used for cell viability testing because of its applicative cell culture [20, 21]. This study aimed to determine the viability of NIH 3T3 fibroblast cells on nanohydroxyapatite and the value (IC50) of the Unam snail shell. This research is the first step in material testing adaptation to body tissues and a candidate treatment for periodontal disease.

The MTT test to determine the nHAP viability was carried out in several concentrations (0.8437 mg/ml, 1.6875 mg/ml, 3.375 mg/ml, and 6.75 mg/ml). (table 1) Similar tests were carried out by Kartono *et al.* using hydroxyapatite synthesized from clam shells (*Anadara granosa*) with a wider concentration range (54 mg/ml, 27 mg/ml, 13.5 mg/ml, 6.75 mg/ml, 3.375 mg/ml, 1.6875 mg/ml, 0.8437 mg/ml, 0.4218 mg/ml, 0.2109 mg/ml) [18]. The results showed that nHAP from Unam snail shells supported cell viability at lower concentrations, indicating good biocompatibility. However, several factors may influence cytotoxicity, including nanoparticle aggregation, pH shifts in the medium, and reactive oxygen species (ROS) generation, which can induce oxidative stress and apoptosis. These mechanisms highlight the importance of comprehensive evaluation in future applications. Maulidah *et al.* conducted a similar study using Haruan fish (*Channa striata*) with concentrations of 0.2109 mg/ml, 0.4218 mg/ml, 0.8437 mg/ml, 1.6875 mg/ml, 3.3375 mg/ml, and 6.75 mg/ml. ml, 13.2 mg/ml, 27 mg/ml and 54 mg/ml [23].

This study showed that the number of living cells above 90% was found at concentrations of 0.8437 mg/ml and 1.6875 mg/ml, 92.9±1.82% and 95.9±1.69%, respectively. The percentage of viable fibroblast cells at a 3.375 mg/ml concentration was 78.3±0.98%, while the lowest fibroblast cell viability was found at 6.75 mg/ml. (table 2) Kartono *et al.* supported these results, in which the most living cells were present at lower concentrations, at 0.8437 mg/ml, 0.4218 mg/ml, followed by 0.2109 mg/ml with a consecutive proportion of 98.09±8.88%; 89.48±4.63%, and 91.04±3.74% [18] A study by Maulidah *et al.* also in line with the highest number of living cells found at concentrations of 0.2109 mg/ml, 0.4218 mg/ml, 0.8437 mg/ml, 85.75±3.04%; 82.2±2.69%, and 80.1±4.95%, respectively [23].

Unam shell nHAP concentration affects the viability of 3T3 fibroblast cells. With the higher nHAP concentration, there was a decrease in the number of living 3T3 fibroblast cells. This could be a result of several factors, and this condition might refer to the high calcium concentration. Calcium is one of the second messengers that mediate cellular receptors to various stimuli, such as proliferation, movement, secretion, and neurotransmission of cells. Calcium also has a role in the occurrence of apoptosis in cells, both physiologic and pathologic. Calcium concentration enters cells in large quantities; calcium will cause toxic effects on cells by promoting secretions of enzymes, such as phospholipases, proteases, endonucleases, and adenosine triphosphatases, that induce apoptosis [19]. Excessive calcium also causes inactivation of dehydrogenase enzymes in cells, causing a decrease in ATP levels in 3T3 fibroblast cells, damaging cell membranes, and apoptosis in fibroblast cells 3T3 [23].

The calcium concentration in the right amount can improve cell biocompatibility. Anjaneyulu *et al.*, using hydroxyapatite from snail shells against NIH-3T3 fibroblast cells, showed that hydroxyapatite could adhere and live on cells with more than 90% in NIH-3T3 fibroblast cells. The concentration of hydroxyapatite used ranged from 12.5 to 100 g/ml. This indicates that the appropriate concentration will not cause a toxic cell reaction [24]. The IC_{50} value was obtained from the regression equation between the viability percentage and the determined nHAP concentration in this study. Based on the study results, the IC_{50} value of nano-hydroxyapatite from Unam snails (*Volegalea cochlidium*) was 3.49 mg/ml. Higher IC_{50} was found in different materials, such as the horse snail (*Pleuroploca trapezium*) by Anand *et al.*, which showed a 4,021 mg/ml value [25]. This variation could be attributed to differences in synthesis methods, material purity, particle size, and crystallinity-all of which can influence cellular uptake and biological responses [Sergey, Harald]. In addition, outcomes regarding cell viability can differ based on the specific cell lines utilized; as an example, the sensitivity of fibroblasts varies from that of mesenchymal or epithelial cells [Eleonore]. It is crucial to keep the nHAP concentration below the IC50 level to reduce cytotoxicity and guarantee its safe use in biomedical applications, irrespective of these factors.

The results showed that the number of living cells is more than 50% in the concentration group below IC_{50} (3.49 mg/ml), so higher material concentration will result in lower cell viability. This study observed 3T3 fibroblast cells' viability 24 h after treatment. Based on the results of viability studies of 3T3 fibroblast cells lived at nHap concentrations of 6.75 mg/ml, below 50% and caused toxicity to fibroblast cells. Therefore, further research is needed, both *in vitro* tests on other cells with the right concentration and a longer observation time and *in vivo* tests on experimental animals to see the compatibility of the Unam snail shell hydroxyapatite nano as a candidate for bone graft material in periodontic fields.

CONCLUSION

Increasing the concentration of nano-hydroxyapatite (nHAP) tends to decrease the viability of 3T3 fibroblast cells. The number of live fibroblast cells as much as 50% was obtained at a nano hydroxyapatite (nHAP) concentration of 3.49 mg/ml (IC_{50}: 3.49 mg/ml).

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AUTHORS CONTRIBUTIONS

PW, FZ, OAH, IE, N, JM, JMS, DZA, RL: Researcher DMPS, AHN, SI, IE, PW: Researcher, Critically Revised the Manuscript. DMPS: Writing, Analysis, Interpretation. DMPS, AHN, SI, IE, PW: Conception and Data

Design. DMPS, AHN, SI, IE, PW: Analysis, Writing, and Performed the Experiments.

CONFLICT OF INTERESTS

Declare that there is no conflict of interest regarding the publications of this paper.

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