ORIGINAL ARTICLE

Particle Size Analyses and Cytotoxicity of Nanoencapsulation of β-Tricalcium Phosphate from Synthesis of *Anadara granosa* Shell as Pulp Capping Material

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ABSTRACT

Introduction: Anadara Granosa (AG) clam shells hydrothermally treated for 18 hours and sintering for 3 hours will produce *β*-TCP which contains calcium, used to help the process of reparative dentin formation. Nano encapsulation prosess is carried out to prevent excessive calcium absorption. Objective: To investigate cytotoxicity of nano encapsulation of β-TCP from synthesized Anadara granosa shell. **Materials and methods:** This research was conducted using post-test-only control group design. BHK-21 fibroblast cell culture in 96 wells was divided into two groups, namely the encapsulation group with a stirring time of 7(P1) and 8(P2)hours. The particles resulting from the encapsulation process were measured using particle size analyzer (PSA), MTT assay with color absorbance response converted into percent cell death with a formula. The cell viability data was analyzed using the T-test and see if there is a change in particle size in each group. **Results:** Two nano encapsulation P1(370 nm) and P2 (385 nm) were determined to be examined for citotoxicity. The cytotoxicity test showed significant difference between groups P(1) (0.31±0.28) and P(2)(0.41±0.38), where both showed no toxic ingredients. The highest viability occurred in the 8 hours encapsulation treatment group. **Conclusion:** Encapsulation of β-TCP for 8 hour (385 nm) was not cytotoxic to BHK-21 cell fibroblast cell line culture.

Keywords: Anadara granosa, Beta-tricalcium phosphate (*β*-TCP), Fibroblasts, Cytotoxicity, Cell culture.

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INTRODUCTION

The development of restorative materials continues to this day with the aim of producing restorative materials that have improved physical and mechanical properties and are biocompatible and good for clinical application. Several in vitro studies have found that the placement of restorative materials in dentin can be potentially toxic and can damage the pulp, therefore the biocompatibility of restorative materials is important so that they can be used as restorative materials for vital teeth [1].

Treatment of pulp tissue so that it remains vital is called pulp capping [2, 3, 4]. Pulp capping treatment according to the American Association of Endodontists (AAE) is a dental pulp treatment procedure using dental material placed over traumatized pulp to stimulate the formation of reparative dentin, prevent bacterial invasion, and maintain pulp vitality [5, 6, 7, 8]. A good pulp capping material must have ideal properties, namely that it can stimulate the formation of reparative dentin, can maintain pulp vitality, can release fluoride to prevent secondary caries, is bactericidal or bacteriostatic, can adhere well to dentin and restorative materials, is sterile, radiopaque, antibacterial. [8]

The material that until now is still the gold standard for pulp capping materials is Calcium hydroxide because it has the ability to stimulate tertiary dentinogenesis, coupled with its antibacterial properties, low cytotoxicity [9, 10, 11]. Several long-term studies have proven that Calcium hydroxide is less adaptable to dentin, stimulates odontoblast differentiation consistently, cytotoxic in cells, and high pH causes calcium hydroxide to dissolve easily resulting in tunnel defects [12, 13]. This results in hydraulic pressure outwards which causes exudation of the pulp liquid, so that the calcium hydroxide layer will be damaged, as a result of bacterial invasion and pulp inflammation. [8, 14, 15].

Conversely, there are several studies that state that the quality of dentin bridges is better if the overburden or material applied is thicker, it minimizes leakage. This controversy prompted researchers to explore other materials that could potentially serve as pulp covering

materials. Another alternative direct pulp caping material that can be used in the field of dentistry by utilizing basic materials from the animal environment, one of which is the anadara granosa (AG) shell, which has the largest calcium content of 98% [16, 17]. Calcium contains minerals essential for the growth of bones and teeth in the remineralization process [18, 19]. AG shells can be synthesized into hydroxy apatite (15%), β-tricalcium phosphate (β-TCP) (79%), and $CaOH_2$ (6%) through hydrothermal method for 18 hours at 200^0C and sintering for 3 hours at 900^0C [20]. β-tricalcium phosphate (β-TCP) can be used as a potential bone substitute because it is biocompatible, bioresorbable, and osteoconductivity, has an interconnected structure so that new cells can move and new blood vessels can form. [21, 22]. β-tricalcium phosphate (β-TCP) can release calcium. Calcium released from pulp capping material plays an important role in maintaining the viability and function of human dental pulp, and increased extracellular calcium concentrations can promote odontogenic/ osteogenic differentiation and mineralization of hDPCs. Calcium-based pulp capping materials can promote the entry of calcium from the extracellular space via CaSR. [23] The use of nanoparticle technology as a drug delivery system in the field of dentistry began to be used, tissue engineering principles, material particle size can affect biological effects, namely the smaller the particle size, the more surface area, so that the interaction of the material and surrounding tissue increases [24].

The choice of nanoparticle manufacturing method depends on the physicochemical characteristics of the polymer and the properties of the drug/material used [25]. The method of making nanoparticles in this study was carried out by the ionotropic gelation method with aerosolization techniques where this method has the advantage that the active substance can be encapsulated without using organic solvents and high temperatures that can damage the active substance. This ionotropic gelation method is a simple, fast, cost-effective method [26, 27]. The advantage of encapsulation is that it can release the active material gradually, therefore the selection of the type of polymer must be appropriate if it is not right it can affect the ability to increase the absorption effect, increase the penetration of the active substance and be out of control. Natural polymers that are often used as nanoparticle matrices are alginates [27, 28]. The use of nanoparticle technology as a drug delivery system in the field of dentistry began to be used, tissue engineering principles, material particle size can affect biological effects, namely the smaller the particle size, the more surface area, so that the interaction of the material and surrounding tissue increases [29]. One of the advantages of nanoparticles is the ability to penetrate intercellular spaces that can only be penetrated by colloidal particle size [30], the ability to penetrate higher cell walls, either through diffusion or opsonification, and their flexibility to be combined with various other technologies. The choice of nanoparticle manufacturing method is determined by several formulation factors and related technologies, including the desired particle size range, processes that do not affect the stability of pharmaceutical active ingredients, the release profile must be reproducible, and there are no toxic ingredients in the final product [31, 32]. Based on the background of the above problem, the researcher felt the need to conduct a toxicity test of β-tricalcium phosphate (β-TCP) as a pulp capping material in vitro.

MATERIALS AND METHODS

This research was classified as true experimental laboratory research, using Randomized Post Test Only Control Group Design. The cytotoxicity test sample is fibroblast cells (BHK-21) by cell culture method, which is divided into 2 groups where each group consists of 6 samples. Where P(1): β-tricalcium phosphate (β-TCP) encapsulation 7 hours, P(2): β-tricalcium phosphate (β-TCP) encapsulation 8 hours.

This study was conducted to determine the cytotoxicity of nanoencapsulation β-tricalcium phosphate (β-TCP) from Anadara granosa shell synthesis against BHK-21 fibroblast cell culture. The computation of the cytoxicity test was done by counting the number of viable fibroblast cells, using the T-test statistical with a significance level of 95% (p = 0.05) on SPSS Version 16.0.

The research data were analyzed descriptively. The optical density (OD) score was converted by the formula of cell viability percentage and the resulting average percentage in each treatment group. Control media is assumed to have a cell viability percentage score of 0%, while control cells are assumed to have a cell viability percentage score of 100%. Formula to determine the percentage of cell life (Fig. 1).

$$\text{Cell Viability (\%)} = \frac{\text{OD Treatment} + \text{OD Media}}{\text{OD Cell} + \text{OD Media}} \times 100\%$$

Fig. 1: Formula to determine the percentage of cell life.

The calculation results are said to be non-toxic if the percentage of the number of living fibroblast cells > 50%, but if the percentage of living fibroblast cells < 50%, then the test material is declared toxic [35].

Synthesization of beta-TCP

Blood clam shells (Anadara granosa) after cleaning, crushed using mortar and pastle until the results are obtained in the form of powder. Furthermore, a hydrothermal process was carried out at a temperature of 200oC for 18 hours, then sintered with a temperature of 900°C for 3 hours to obtain β-tricalcium phosphate (β-TCP). [20]

Nanoencapsulation of beta-TCP

After proses Synthesization of beta-TCP , the process of making encapsulation with the Ionotropic Gelation

Method with Aerosolization Technique using Sodium alginate polymer and CaCl2 crosslinker is carried out. [20] β-tricalcium phosphate (β-TCP) powder is put into a beaker glass as much as 0.5 grams dissolved into 50 ml of aquades stirred until evenly distributed, then added 0.5 grams of sodium alginate powder is stirred using a magnetic stirrer until no alginate bubbles are visible, then drip 0.5 gr $CaCl_2$ solution dissolved into aquades as much as 25 ml until finished. Next, the solution has been mixed with $CaCl_2$ in the stirrer for 7 hours and 8 hours. Next, each solution is taken as much as 6 ml to be inserted into the centrifuge tube and adjust the rotation for 6 minutes at a speed of 1000 rpm. After that, the precipitate separated with liquid is filtered using filter paper. Centrifuge is used to separate solids that are quite small in size. Filtering is carried out so that the volume of water in the sediment is slightly reduced before weight measurements are carried out using digital scales. The precipitate that has been obtained is put into a tube for freezing for 12 hours with a temperature of -20°C. Furthermore, after freezing this precipitate will be placed under vacuum. This allows the frozen solvent in the precipitate to evaporate without going through the liquid phase, a process commonly known as sublimation. After that, the precipitate is delivered heat to accelerate sublimation. A low-temperature condenser removes the solvent that evaporates in the vacuum chamber by converting it back to the solid phase. This shows that heat transfer occurs by conduction (conduction), so that the resulting precipitate will be more stable without changing the aroma and color.

Particle size analysis

Then a PSA Test is carried out. [20]. Nano-encapsulated beta-TCP weighing 1 gram was dissolved with 8 ml of distilled water, then sonicated for 10 minutes to make the sample homogeneous. The sample was put into a plastic cuvette, then inserted into the VASCO-Particle Size Analyser Tool to see the particle size of the sample. [20]

Cytotoxicity test

Preparation for making culture. All work is done in laminar flow. Roux vials containing BHK-21 stem cells were first thawed at 37°C. The BHK-21 stem cells are then rotated centrifuges to remove the remaining old media containing preservatives. The fibroblast cells in the large Roux were transferred into 4 bottles of small Roux and each filled with 20 ml of Eagle media containing 10% serum bovine, 0.02 ml streptomycin, 0.06 ml fungison then covered with aluminum foil and tied with rubber. Put in an incubator with a temperature of 37^0C for 24 hours. Cells that have been confluent (full) are removed Eagle media, washed with a 15 ml phosphate buffer saline solution. This is meant to dispose of the remaining serum in the bottle. Washing is carried out 2 times to achieve a clear result. Given trypsin versene 0.25% as much as 1 ml. Versene to release cells from the walls of bottles, while trypsin to separate bonds between cells. After shaking it to flatten, then trypsin versene is left to keep the surface of the tissue wet. Left for 5-10 minutes until the cells are removed from the bond between the cells with the walls of the bottle. The remaining Roux cells were added 20 ml of Eagle media and 10% bovine serum and shaken until all cells were detached from the vial wall and from intercellular bonds. Cells are ready to be transferred to the microplate, put in the incubator until the cells are full, then a sample test is carried out.

Sample Preparation: With guttap point encapsulation β-tricalcium phosphate (β-TCP) from each group was taken and dripped weighing 0.01g at each microplate hole. To test the encapsulation of β-TCP concentration 1:1 16 holes microplate, For encapsulation of β-TCP with alginate concentration 2:1 16 holes microplate. The prepared cells are divided into microplate holes that have been given sample material. After 3 minutes added MTT, put in the incubator for 4 hours. Added DMSO, dishaker then read with elisa reader with wavelength 630. The results of this study were carried out normality test with Kolmogorov Smirnov Test statistical test and homogeneity with Levene's Test statistical test. Then an independent T-test was carried out to determine any significant differences between the treatment groups.

RESULTS

The research that has been done showing the average percentage of cell death (Table I). The higher the percentage of cell viability of a contribution, the more cells that live in the well. In Table I it appears that group P(2) has a percentage value of cell viability greater than group P(1). In Table II show Average and standard absorbance values, this is indicates that there has been cell proliferation in BHK-21 fibroblast cells after treatment. In this study, the results of living fibroblast cells > 50% with the highest percentage of viability in the 8 hours encapsulation group. Normality and homogeneity tests showed that the data were normally distributed and homogeneous. (Table III). The difference in meaning between treatment groups can be determined by T test analysis with a degree of meaning $p<0.05$. The results of the T-Test, test obtained a result of 0.004 which means that there is a significant difference between groups P(1) and P(2).

Table I: Cell viability percentage

Group	
P(1) 7 hour	P(2) 8 hour
109.92	113.57

Table II: Average and standard absorbance values

Group	Mean	SD
P(1)	.31533	.028395
P(2)	.41250	.038083

Table III: Normality test

Group Statistic		Shapiro-Wilk		
		df	Sig.	
OD	P(1) 7 Hours	.771	6	.031
	P(2) 8 Hours	.958	6	.800

DISCUSSION

In this study, the ionic gelation method was chosen for the nanoparticle manufacturing process because this method has a simple and easy-to-do process.[33] Tricalcium Phosphate is encapsulated using sodium alginate polymer in order to reduce the effect of external influences and reduce the degree of solubility of the core material, the addition of crosslinking agents in the form of calcium chloride during encapsulation in order to increase the efficiency of the encapsulation process. [34]

Sodium alginate was chosen as a polymer material in the nano-encapsulation process because it can be used without the use of organic solvents so that this can minimize toxic effects due to organic solvents and has biocompatible properties.

Calcium chloride is used as a crosslinker because sodium ions are monovalent and calcium is polyvalent so crosslinking is needed to stabilize the bond [35]. Calcium chloride in high content will make encapsulation more efficient where calcium chloride reduces the particle size of pyracycinamide where the ideal size of β-TCP material as a pulp capping material is 50-500 nm. [36].

The process of mixing ingredients is carried out using a Magnetic stirrer is a laboratory device that uses a magnetic field to rotate which causes very rapid stirring of the liquid. Magnetic stirrers generally have speeds between 100 rpm and 3000 rpm [37].

A decrease in particle size occurs when increasing the speed and duration of stirring [38]. With an increase in speed, the intensity of the solvent molecules becomes greater and makes the solvent molecules come into contact with the polymer, causing the intensity of the rotation speed on the magnetic stirrer to increase and making the particle size results smaller [39]. With a smaller particle size, Ca + ions will be increasingly trapped in the system so as to stimulate odontoblasts in making dentin bridges [40]

In the research of Aprilia et al (2020), the optimum stirring time on the ideal particle size of the synthesized beta-TCP material of Anadara granosa is 2 hours where the particle size obtained is closest to the ideal size for pulp capping material, so it is determined in this study that the stirring time of the material is 2 hours [20].

In this study, 7 and 8 hours of stirring time were used to see if there was a decrease in particle size to become smaller.

Cell proliferation and viability have been studied and developed by various methods. Cytotoxicity testing is a formal requirement in drug development efforts [41]. The easiest measurement of the degree of cytotoxicity is to use 96 well microplates. The use of these microplates allows for testing with multiple treatments on many samples simultaneously in a short period of time [42]. MTT assay is the first cell viability test developed for the 96 well microplates format suitable for HTS (High Throughput Screening) systems, which is a way to select and determine the potential of a microorganism in a large number and in a short time. The basic principle of MTT assay is to measure cellular activity based on the ability of cell metabolism to reduce soluble yellow tetrazolium dye to water-insoluble purple formazan products. The color change is used as a parameter in calculating cell viability with the MTT assay method [43]. The absorbance value (OD) of the dissolved formazan crystal can be measured using a spectrophotometer (ELISA reader or microplate reader) with a wavelength of 490 nm. Maximum absorption depends on the solvent used. This absorption occurs only if mitochondrial reductase enzymes are active. Therefore, conversion can be directly related to cell viability [43, 44]. The OD value read on the spectrophotometer is the value of the number of particles absorbed by fibroblast cells, which in this case are particles from Sonneratia alba leaf extract. The result of the absorbance value read on the spectrophotometer is then converted into the percentage of cell cytotoxicity. If the calculation of fibroblast cell bioviability > 50%, the test material is declared non-toxic, while if the percentage of fibroblast cell bioviability < 50%, the test material is toxic [42]. This study used fibroblast cell cultures treated using Sonneratia alba leaf extract as a research sample. Fibroblast cells are used because they are the most widely found and are the main cells of connective tissue located on the lamina propia of the oral cavity. Fibroblast cells are also easy to culture so they are often used as biological research subjects [42]. Cytotoxicity tests are tests used to analyse the cytotoxic effects of materials and medical devices on living organisms. [45, 46]. The cytotoxicity test in this study was carried out in vitro because in vitro research meets the standards according to ethical, practical and economical factors. Some of the advantages of in vitro research include a simpler, easier to control assessment parameter system, minimizing confounding variables and determining more specific toxicity mechanisms [47, 48]. Further research after this cytotoxicity test is conducted: Morphological assessment using histology or ultrastructural analysis of cells using SEM or TEM, cell viability and proliferation tests, cell function tests such as measuring the release of inflammatory markers, determining glutathione, heat-shock protein tests, and

apoptosis tests [46]

CONCLUSION

Encapsulation of β-TCP for 8 hour (385 nm) was not cytotoxic to BHK-21 cell fibroblast cell line culture.

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