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Bioviability of Beta-Tricalcium Phosphate Nanoencapsulation from Synthesis of *Anadara granosa* Shells on Fibroblast Cell Line BHK-21 Cell Culture

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Abstract: The purpose of this research is to measure the toxicity of beta-tricalcium phosphate (β-TCP) nanoencapsulation resulting from 18 hours of hydrothermal process on *Anadara granosa* (*A. granosa*) shell and 3 hours of sintering. The encapsulation process was carried out to reduce the side and toxic effect, as well as to inhibit the speed of calcium solubility, which can prevent the tunnel effects. The result of cell viability data that analysed using the one-way ANOVA statistical test showed there was no significant difference between the *A. granosa* shell encapsulation processes with treatment groups of 1, 2, 3, 4, 5, and 6 hours. The highest viability occurred in treatment group 2, with the *A. granosa* shell encapsulation stirred process of 2 hours. Therefore, the *A. granosa* clam shell nanoencapsulation was proved to be non-toxic and can be used in dentistry therapy.

Keywords: Encapsulation, synthesis, blood shells, *Anadara granosa*, fibroblast, cytotoxicity, cell culture

1. Introduction

The current development of restoration materials involves the utilisation of natural resources. Good restoration materials must have good physical and mechanical natures and good biocompatibility if they are to be clinically applied. An ideal dental material must not only be inert and non-toxic to pulp but must also be bioactive toward tissues by stimulating migration, proliferation and differentiation of osteogenic cells [1]. Pulp capping treatment is defined as the materials used to make a protective layer on open dental pulp to allow the tissue to recover and maintain its normal function and vitality [1]. One commonly used material for pulp capping is calcium hydroxide ($Ca(OH)_2$), which can terminate microorganisms because of its high pH and can stimulate fibroblast formation and the growth of tertiary dentin to be re-mineralised [2], [3]. Several studies also showed that $Ca(OH)_2$ has deficiencies that can cause inflammation and necrosis on a pulp surface after capping due to its inability to adapt to dentin. This results in a microfiltration/tunnel effect and degradation; the formulated dentin bridge tends to be frail because of its high solubility, and this may lead to treatment failure [2-4].

The shell of *A. granosa* is 98.68% of calcium carbonate ($CaCO_3$) [5]. Calcium is an essential mineral for bone and dental growth in the re-mineralisation process [6]. The *A. granosa* shell can be synthesized into hydroxyapatite (72%), beta-tricalcium phosphate (β-TCP) (21%) and $CaOH_2$ (6%) via the application of the hydrothermal method for 12 hours at a temperature of 900 ºC followed by sintering for 3 hours [7]; β-TCP has a chemical component that is close to bone and dental structure [8]. The encapsulation process is an effort to reduce side effects and toxic effects, obstructing

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the rapid calcium solubility that prevents the tunnel effect from occurring [9]. Encapsulation is a micro-sized coating system whereby a polymer is formed around a material absorbed in the core of the system. A good microencapsulation system can prevent aggregation for a long time during storage and over the lifetime of the product. The success of the encapsulation process depends on the type of polymer and the preparation method used. The polymer used must, in terms of main requirements, be biodegradable, biocompatible and non-immunogenic, because it aims to avoid toxicity in its application to the body [10], [11]. The encapsulation process uses the ionic gelation system, which is the easiest and most widely used system. The polymer used is sodium alginate because it has muco-adhesive properties that increase drug absorption and has ideal properties for a polymer, namely, it is biocompatible, biodegradable, non-toxic and inexpensive [12]. Ionic gelation is a method of cross-linking polyelectrolytes and their multivalent ion pairs. The ionic gelation process is followed by the formation of a polyelectrolyte complexation with the opposite polyelectrolyte. The process of forming a cross-linked bond can increase the mechanical strength of the particles formed [12]. Based on the findings above, it is necessary to find material alternatives for direct pulp capping that can be used in dentistry and will allow optimization of dentin regeneration so that the inflammation process and the risk of necrosis can be minimized.

2. Experimental

This research used the number of viable fibroblasts on cell culture after a treatment as parameters. Treatment groups in this research were divided based on the stirrer process during encapsulation as shown in Table 1.

Table 1 - The group experiment based on its stirrer time

Group	Stirrer Time
P1	1 hour
P2	2 hour
P3	3 hour
P4	4 hour
P5	5 hour
P6	6 hour

The materials used were *A. granosa* shell, narium alginate, calcium chloride ($CaCl_2$), sterile distilled water, 96% ethanol, BHK-21 fibroblast cell culture, RPMI media, trypinsine versene, 10% foetal bovine serum (FBS), phosphate buffer solution (PBS), dimethyl sulfoxide (DMSO) and MTT [3- (4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide]. The *A. granosa* shell was prepared in accordance with the hydrothermal sample preparation process: the cleaned A. *granosa* shells were ground into powder and sifted using a 200-mesh sifter; 10 g *A. granosa* powder was then mixed with 100 ml Aqua Dest and 6.9 g $NH_4H_2PO_4$, stirred in the magnetic stirrer for 30 minutes until homogeneous and then put into the reactor to be heated in an electrical oven at 200 °C temperature for 18 hours. After this, a cleaning using Aqua Dest with a magnetic stirrer was conducted until it reached pH 7 [13].

A final cleaning was conducted with methanol to limit HA particle agglomeration during sample drying. The samples were then dried in an electrical oven at 50 °C for 3 hours and sintered at 900 °C for a further 3 hours to eliminate dirt and intensify sample crystallization to create a TCP-active substance. Once β-TCP powder was obtained, the encapsulation process was conducted with polymer natrium alginate and $CaCl_2$ as the cross-linker [13]. The encapsulation process samples were divided into groups based on their stirrer processes [13]. A 50 ml Aqua Dest and 0.5 g β-TCP powder were mixed with a magnetic stirrer until homogenous; 0.5 g natrium alginate was then added, and a magnetic stirrer was applied until the result was homogenous. Then, 0.5 g $CaCl_2$ was dissolved in 25 ml Aqua Dest until homogenous and dripped into a solution with natrium alginate until homogenous. After all materials were mixed and homogenous, the samples were clustered into six test groups in accordance with stirring lengths: P_1 for one hour of stirring, P_2 for two, P_3 stirrer for three, P_4 for four, P_5 for five and P_6 for six. Once the stirring was done, each sample group was centrifuged at 2500 rpm speed for six minutes. The filtrates were thrown away, and the sediments were kept. The obtained sediments were stored in a freezer for one night and frozen-dried for 12 hours [13].

The fibroblast cells used in this research were BHK-21 fibroblast cells stems that were bred naturally in the medium of Rosewell Park Memorial Institute 1640 (RPMI-1640). Stem cell cultures were melted in sterile Aqua Dest at 37 °C and stirred in a centrifuge at 50 rpm for 5 minutes. That sediment was taken and suspended with RPMI medium and 10% foetal bovine serum; 36 ml RPMI medium was then poured into a bottle containing 4 ml of serum, resulting in 40 ml mixed RPMI medium and serum. Suspended cell sediment was then planted in a sterile Roux bottle and incubated at 37 °C with 5% CO_2 until monolayer cells were formed (about two days), to be examined under a microscope. The cells then were prepared to be moved into microplates. The medium in the big Roux bottle, containing BHK-21 cells, was then thrown away and washed with 15 ml PBS 3–5 times. The Roux bottle was filled with 1 ml versene trypsin. Cells in the bottle looked clustered and were homogenized with 10 ml RPMI medium. The homogenous cells were then put into microplates 96 well with 2×10^5 density for 24 hours in an incubator at 37 °C and 5% CO_2 flow [14-16]. The incubated fibroblast cells in the microplates were treated with *A. granosa* shell

encapsulation sample with stirring times previously set for 1, 2, 3, 4, 5, and 6 hours. Two test variables used in this research were cell control and samples with various stirring treatments. Cells were then incubated for 24 hours in an incubator at 37 °C and 5% CO_2 [14-16]. Before readings using ELISA Reader were conducted, the media were thrown away, and each well was filled with 10 µl MTT and incubated for 4 hours; next, 50 µl DMSO was put in each well and shaken up with a shaker tool. Readings were conducted by putting microplates into ELISA Reader with wavelength of 620 nm and measuring the absorbance. The amount of absorbance showed the number of cells viable in the medium culture. Later, the reading results were converted into percentages [14-16].

3. Results and Discussions

This research was conducted to prove the nanoencapsulation cytoxicity of beta-tricalcium phosphate of *A. granosa* shell synthesis on BHK-21 fibroblast cell culture. The computation of the cytoxicity test was done by counting the number of viable fibroblast cells, using the ANOVA statistical test with a significance level of 95% ($p = 0.05$) on SPSS Version 16.0. The data resulting from the research were analyzed descriptively to achieve a distribution description and were summarized to get a clearer presentation of the results. Optical density (OD) scores were converted with a cell viability percentage formula and the resulting average percentage in each treatment group. The control medium was assumed to have a 0% cell viability percentage score, while the control cells were assumed to have a 100% cell viability percentage score. The cell viability formula is shown in Eq. (1):

$$\text{Cell viability} = \frac{\text{OD treatment - OD media control}}{\text{OD cell control - OD media control}} \times 100\% \quad (1)$$

The higher the cell viability percentage for a well, the more cells are viable in that well. Table 2 states that group treatments have higher cell viability percentages than the cell control group. It shows that cells were non-toxic on BHK-21 fibroblast cells after treatment was applied. The counting result is said to be non-toxic if the percentage of fibroblast cells that are viable is more than 50%. However, if the percentage of viable fibroblast cells is less than 50%, then the test material is considered toxic. In this research, more than 50% of fibroblast cells in all treatment groups being viable, with the highest viability percentage in Group P2.

Table 2 - Cell viability percentage

Group	Cell viability (%)
P1	95.93
P2	114.88
P3	111.75
P4	102.10
P5	109.92
P6	113.57

A normality test was used to check the normality of the data. It used Shapiro-Wilk testing. If the significance number is lower than 0.05, then it can be said that the data distribution is not normal; if the significance number is higher than 0.05, then it can be said that the data distribution is normal. In this study, all data were normally distributed because the result was higher than 0.05. The homogeneity test showed a significant result of $p > 0.05$, which means that the data are homogenous and can be continued on a one-way ANOVA parametric test. The ANOVA test (Table 3) is used to determine that there should be a difference in the cell viability numbers in each treatment group, separately and together.

Table 3 - ANOVA test results

	Sig.
Cell viability	0.032

Significant number equal to $0.032 < 0.05$, which means that there is a significant difference between the six researched groups. The meaning of the differences between the treatment groups can be determined with post hoc analysis with meaning degree of $p < 0.05$. Table 4 showed the post hoc result of tested groups. The sig score between Group P1 = $0.05 < 0.05$. This means that there is a significant difference between those two groups. Other group's sig score was $p > 0.05$, which means that there is no significant difference between those groups. Based on Niles [17], the cytoxicity test is a requirement that should be met in the effort of material development. The simplest way to measure the degree of cytoxicity is to use microplates 96 well that are suitable for HTS (high throughput screening) test, a way to select and find out microorganism potential in large numbers in a short time [18].

The basic principle of the MTT assay method is measuring cellular activity based on metabolic ability to reduce soluble yellow dye to a purple formazan product that is not soluble. The colour change is used as a parameter in counting cell viability with the MTT assay method [19-21]. The absorbance score of dissolved formazan crystal was measured using a spectrophotometer (ELISA reader or microplate reader) with a wavelength of 490nm. Maximum absorption depends on the solvent used and happens only when the mitochondria-reduced enzyme is active. This will cause conversion directly related to cell viability [18]. The OD score read on the spectrophotometer is the number of particles absorbed by the fibroblast cells. The result of the absorbance score read on the spectrophotometer is converted into a cell cytoxicity percentage. If the fibroblast cell bioviability calculation result is < 50%, then the test material is toxic [21]. Table 5 and Fig. 1 summarized the average and standard absorbance values for each groups.

Table 4 - Post hoc result (Gomes Howell)

Group	1	2	3	4	5	6	Control
1		0.050*	0.144	0.787	0.249	0.102	0.976
2			0.999	0.590	0.983	1.00	0.275
3				0.869	1.00	1.00	0.554
4					0.961	0.787	0.997
5						0.999	0.739
6							0.449
Control							

* significant difference

Table 5 - Average and standard absorbance values

Group	Mean	Std. Deviation
K -	0.28780	0.017964
P1	0.27580	0.04685
P2	0.32000	0.021190
P3	0.31300	0.011979
P4	0.29560	0.010900
P5	0.30880	0.08289
P6	0.31540	0.010188

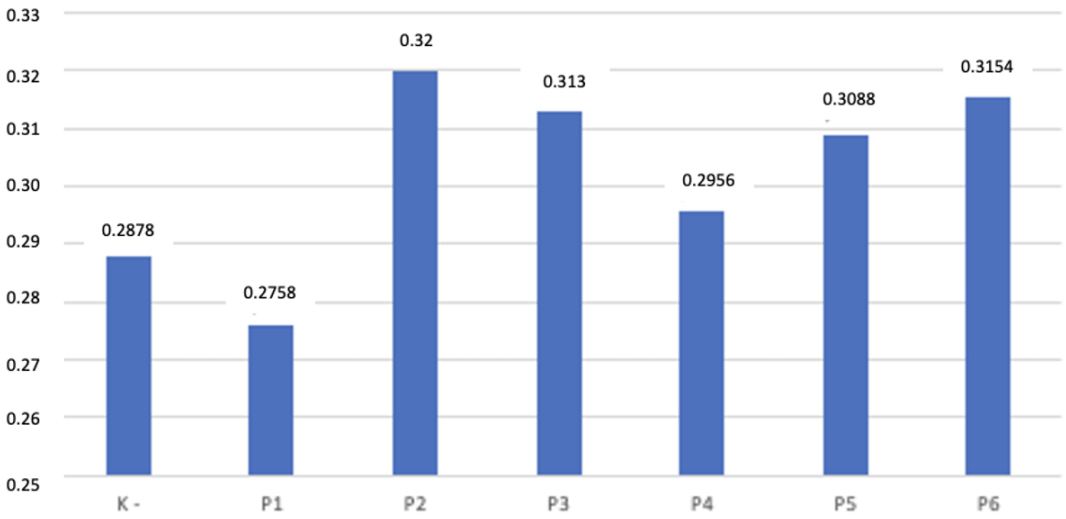

Fig. 1 - Bar chart of absorbance value, mean versus group

This research uses fibroblast cell culture with treatments that use beta-tricalcium phosphate encapsulation from *A. granosa* shell as a research sample. Fibroblast cell was used because it is easy to breed [22-24]. The cytoxicity test was conducted in vitro, which has benefits such as a simpler appraisal parameter system, ease of control, minimisation of confounding variables and a more specific toxicity mechanism definition [23], [25]. This research results show an increase in fibroblast cell viability in each treatment group. The percentage of cell viability in each P2 group is highest at 114.88%, showing that Group P2 has improvement at 14.88% compared to the cell control group (100%). Other groups show fibroblast cell bioviability > 50%, which means it is non-toxic. The ANOVA test results show a sig score = 0.032 < 0.05, which means there is a significant difference while with the post hoc formula the resulting difference

that occurred in Groups P1 and P2 in which the possibility is the stirrer process of 1 hour still unable to process a perfect encapsulation.

The *A. granosa* shell is mostly calcium [26]. Calcium is one of the second messengers that mediate cellular response for various stimuli such as proliferation, movement, secretion and cell neurotransmission [27]. Calcium is very important in the apoptosis process in physiology and pathophysiology [28], [29]. It is inserted into the cell through the diffusion process: ion movement from high to low concentration. Calcium channels in plasma membranes open and close randomly. The small size of the calcium particle makes it easy for it to get in through open calcium channels. On the other hand, if the calcium is big, this will make it hard for it to pass into an open calcium channel. The regulation of intracellular calcium is one of the complex processes that involve several calcium entrances and exits through cytoplasm. In general, calcium gets into cytoplasm after being released by endoplasm reticulum (SER) through the SER channel of calcium release or insertion of calcium through plasma membrane passing through the permeable calcium channel [27-29]. More inserted calcium will activate a number of enzymes with cellular effects, potentially causing damage. These enzymes include phospholipases, protease, endonuclease, and triphosphatases adenosine. The increase in calcium levels can also induce apoptosis with caspase direct activation and with escalation of mitochondria permeability [27-29].

Nastiti, et al. [30] research on hydroxyapatite bioviability of *A. granosa* shell extract against mesenchymal stem cells as alveol bone graft material concluded that *A. granosa* blood clam shell extract has the highest cell bioviability at a concentration of 6.75mg/ml and the lowest cell bioviability at a concentration of 54 mg/ml [30]. Based on the explanation above, it can be stated that the amount of intracellular calcium that enters has various roles, such as proliferation, development, contraction and secretion. If the absorbed amount exceeds the normal limit, it can cause cell death, in the form of both necrosis and apoptosis, in which calcium function has a role in cell viability through gene expression regulation [27-29].

4. Conclusion

Based on this research, it can be concluded that BTP microencapsulation of *A. granosa* shell is not toxic to cell line BHK-21 fibroblast cell culture.

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