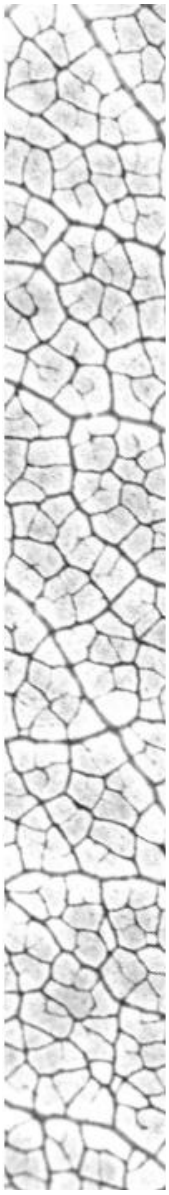

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Toxicity test of shrimp shell (*Litopenaeus Vannamei*) chitosan as bone graft scaffold on BHK-21 fibroblast cell cultures

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Abstract

Background: Chitosan is a material that is biocompatible, biodegradable, bioadhesive, and nontoxic when it comes to human cells. Chitosan has potential to be used as an antimicrobial and antioxidant material. Chitosan's biocompatibility still depends on its origin, method of manufacture, and degree of deacetylation. To be used as an alternative scaffold material for bone augmentation that is safe to use in the field of dentistry, the authors are interested in conducting toxicity tests on BHK-21 fibroblast cell cultures.
Aim: To determine the toxicity of chitosan from shrimp shells (Litopenaeus Vannamei) on BHK-21 fibroblast cell cultures.
Method: This type of research is a laboratory experiment with a Post Test Only Control Group Design. Treatment was conducted by administration of chitosan from shrimp shells (Litopenaeus Vannamei) with a concentration of 100%, 75%, 50%, and 25% on the BHK 21 fibroblast cell cultures.
Results: The percentage of remaining fibroblast cells at concentrations of 100%, 75%, 50%, and 25% respectively were 51%, 53%, 54%, and 56%. Toxicity results were obtained by using the MTT assay technique after 24 hours. Optical density absorbance values describe the viability of living cells and the readings were conducted by using ELISA readers.
Conclusion: The results of the toxicity test of shrimp shells (Litopenaeus Vannamei) chitosan did not show toxic effects on BHK-21 fibroblast cells.

Keywords: shrimp shell chitosan, toxicity, BHK-21 fibroblast cells

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INTRODUCTION

Most studies suggest that carries and periodontal disease are the main causes of tooth loss. Tooth loss can be caused by caries, periodontal disease, severe attrition, and trauma such as falls, exercise, and traffic accidents. Disease factors such as caries and periodontal disease that cause tooth loss are associated with increasing age (Esan *et al.*, 2004, Rahajeng and Tuminah, 2009, Tian *et al.*, 2016)

Periodontitis is inflammation of the periodontium which damages the supporting tissues of the teeth, loss of the periodontal ligament and causes damage, and resorption of alveolar bone (Annamalai *et al.*, 2014). The factors involved in the process of bone damage in periodontitis are bacteria and hosts (Younes *et al.*, 2012; Younes *et al.*, 2014). Periodontitis results in damage to the tooth supporting tissue which if not treated, it will cause bone damage which can result in the tooth being removed from the socket (Newman *et al.*, 2011; Trung *et al.*, 2020)

The process of bone healing can be accelerated by using a substitute material or material, namely the bone graft. Bone grafts can be taken from bones in other places and substituted into bone tissue that has a defect. Bone graft material that is quite often used is xenograft which uses bone substitution material taken from animals (van Gaalen *et al.*, 2008; Duan *et al.*, 2017)

Chitosan is a material that has biocompatibility, biodegradability, bioadesi, and nontoxicity in human cells. Chitosan is very potential to be used as an antimicrobial material, because it contains the enzyme lysosim and the aminopolysacharida group which can inhibit microbial growth and the efficiency of chitosan inhibition against bacteria depends on the concentration of chitosan dissolution. The ability to suppress bacterial growth is caused by chitosan having a positively charged polycation that can inhibit bacterial and mold growth. A substance taken from crustacean shells such

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as crabs and shrimp obtained by deacetylation of chitin (Sarwono, 2010; Uragami, Yamamoto and Miyata, 2003; Goa, 2015).

Shrimp is an important commodity for Indonesian fishery products. In general, the shrimp is exported in the form of pasteurized meat. By-product of shrimp processing is in the form of skin waste and head. (Syah Putri, Kusumaningrum and Tristiana, 2020) Shrimp waste is very abundant and has not been utilized properly and efficiently, even most of it is a waste that also pollutes the environment. One alternative effort to utilize shrimp waste in order to have value and usability which is a high economic value product is processing shrimp waste into chitosan. One of the types of shrimp that can be treated as chitosan is *Litopenaeus Vannamei*. Thus, this shrimp waste can be used as raw material for producing chitin, chitosan, and high value derivatives (Suptijah, Jacoeb and Rachmania, 2011)

Amine groups contained in chitosan have toxic properties to cells, but the emergence of toxic responses is influenced by the dose and concentration of exposure to chemicals given. One condition that materials used in dentistry should not be toxic, not irritating, and must have biocompatibility properties or the material produced should not have a detrimental effect on the biological environment, both locally and systemically. For this reason, research is needed to test the toxicity of a material as a standard screening material used in dentistry (Brunner *et al.*, 2006; Heidari *et al.*, 2018)

One method to test the toxicity value of a material is the MTT method *(Methyltiazolyldiphenyl-tetrazolium bromide)* Assay. This test is based on the ability of living cells to reduce 3- [4,5-dimethylthiazol-2yl] -2,5-diphenyl tetrazolium bromide (MTT) salts which are yellow and dissolve into purple and insoluble formazan deposits. The research sample used in this research was fibroblast cells (BHK cell culture 21). Fibroblast cells are the most important cells and the largest component of the pulp, periodontal ligament, and gingiva. Test results using BHK21 can still be used as a basis for accurate testing (Meizarini, 2006)

Based on the background above, it is necessary to test the toxicity of chitosan from shrimp shell material *(Litopenaeus Vannamei)* to BHK-21 fibroblast cells which is useful to determine scaffold material in bone grafts that are not toxic and do not cause tissue damage.

MATERIALS AND METHODS

This type of research is a laboratory experimental study with a research design, The Post Test Only Control Group Design. Treatment was conducted by giving shrimp shell chitosan (Litopenaeus Vannamei) to BHK-21 fibroblast cell culture (Baby Hamster Kidney). The subject of this research was chitosan made from the shell of Lithopenaeus Vannamei shrimp. The location of this research was in the Pharma Veterinary Center Laboratory (PUSVETMA) Surabaya, Surabaya Research and Industrial Consultation Laboratory, and the Laboratory of the Faculty of Fisheries and Marine Universitas Airlangga, Surabaya.

The material used in this research was chitosan originating from *Litopenaeus Vannamei* shrimp shells obtained from the Faculty of Fisheries and Marine Universitas Airlangga Surabaya which had undergone chitin deacetylation.

The first stage (Hebeish *et al.*, 2014), preparation for making chitosan powder by deacetylation, chitin added 50% NaOH dissolved in 1000 ml of aquades, heated for 4 hours at 80 °C while stirring, then filtered and washed to neutral pH or close to pH 7.

The second stage (Fitriani, Soetojo and Subiwahjudi, 2019), making chitosan suspension by means of chitosan powder obtained was made into suspension by mixing 2 grams of chitosan powder with 100 ml of 2% acetic acid to obtain a chitosan suspension of 2% (w / v).

Third stage (Fitriani, Soetojo and Subiwahjudi, 2019) preparation of BHK-21 fibroblast cell cultures, fibroblast cells were taken from BHK-21 cell culture (Baby Hamster Kidney-21) in the form of cell-line planted in Roux bottles. After full culture was harvested using a solution of Trypsine Veresene. The crops were planted in Eagles medium containing 10% Fetal Bovine Serum incubated for 24 hours at 37 °C. Cells were transferred into small bottles (Roux) and bred. Cells were cultured in each well containing cells and Eagles media as much as 50□l.

The fourth stage (Fitriani, Soetojo and Subiwahjudi, 2019), BHK-21 fibroblast cells that have been distributed in wells were divided into 7 treatment groups, namely group I as cell control, group II as media control, group III exposed to chitosan with 100% concentration, group IV exposed to chitosan with concentration of 75%, group V was exposed to chitosan with a concentration of 50%, and group VI was exposed to chitosan at a concentration of 25%. Each treatment has 7 replicas / well.□l

The fifth stage (Fitriani, Soetojo and Subiwahjudi, 2019), the reading by means of MTT added directly to the plate containing as much as 25□l culture medium was incubated for 4 hours at 37 °C. Then, each of the wells is added DMSO (Dimethylsufoxide) as much as 50□l. The plates are stirred mechanically with Plate Shaker until the formazan crystals were dissolved for 5 minutes. Furthermore, formazan read the absorbance by spectrophotometry with ELISA reader with a wavelength of 620 nm. The thicker the color, the higher the absorbance value and the more the number of cells.

Data analysis was continued by using the Games-Howell multiple-comparison Test to compare differences in each concentration group and the control group.

Conflict of Interest: There is no conflict of interest.

Source of Funding: This study is self-funded.

Table 1. Optical Density Value

Treatment	OD Value (%)
100%	51%
75%	53%
50%	54%
25%	56%
Media Control	0%
Cell Control	100%

Table 2. ANOVA *Welch* Test

Robust Test of Equality of Means				
	Statistic[a]	df1	df2	Sig.
Welch	278.622	5	16.551	.000

Df1 = Denominator Variable
Df2 = Numerical Variable
Sig. = Significance

Table 3. Games-Howell multiple-comparison Test

Treatment	100%	75%	50%	25%	Media	Cell
100%	-	0.074	0.04			
75%	0.074	-	0.680	0.103		
50%	0.04	0.680	-	0.766		
25%		0.103	0.766	-		
Media					-	
Cell						-

Ethical Clearance: This study was approved by Ethical Commission of Health Research Faculty of Dental Medicine Universitas Airlangga.

RESULTS

Based on the results of observations and readings of the absorbance values of the toxicity test through the ELISA reader which was divided into treatment groups with concentrations of 100%, 75%, 50%, 25% the results of the percentage of living cells were obtained as follows:

Table 1 shows the percentage of life of fibroblast cells after being treated with different concentrations of chitosan, the results of the percentage of live fibroblast cells were obtained after chitosan treatment, at a concentration of 100% with 51% percentage of live cells, at a concentration of 75% with a percentage of living cells 53%, at a concentration of 50% with 54% percentage of live cells, and at a concentration of 25% with a percentage of live cells 56%.

From the data from the research results in **Table 1** above, data processing can be conducted to see the mean and standard intersections of the number of life of fibroblast cells after being treated with chitosan with each concentration.

Based on **Table 2** obtained p-value of 0.000, where the p-value <0.05. Based on these results, it can be said that the results of the treatment carried out gave a significant difference because p-value <0.05.

Data analysis was continued by using the Games-Howell multiple-comparison Test to compare differences in each concentration group and the control group. Games-Howell Multiple-comparison Test uses the null hypothesis criteria there is no difference in the average between the two treatments and the alternative hypothesis there is an average difference between the two treatments with the criteria H0 rejected, if the p-value <α (= 0.05).

Based on **Table 3**, there are significant differences between the control group and the treatment group which means that there is an effect of the administration of chitosan in the treatment group on the number of fibroblast cells. Whereas in the treatment group, there was no significant difference between treatment groups which meant that all treatments in the chitosan concentration group had the same effect, namely reducing the number of living BHK-21 fibroblast cells.

DISCUSSION

The results of the study of the concentration of chitosan on BHK-21 fibroblast cells using MTT assay showed a mean percentage of live fibroblast cells at a concentration of 25% with a percentage of live cells of 56%, 50% concentration with 53% percentage of living cells, 75% concentration with percentage cells life is 52% and the lowest percentage of living cells is at a concentration of 100% with a percentage of living cells of 51%. The results obtained chitosan with a concentration of 25% produces the highest cell viability while chitosan with a concentration of 100% produces the lowest cell viability. These results indicate the suitability of the theory which states that the toxicity of a material is directly proportional to exposure. Exposure to a material has a determining factor, namely the concentration of an ingredient. The higher the concentration of a material, the more potentially it becomes toxic (Rozman, Doull and Hayes Jr, 2010)

Cell viability is the possibility of cells to survive after exposure to chitosan materials. Therefore, chitosan has a toxic effect on cell viability. This happens because chitosan can suppress cell apoptosis by denaturing cell proteins in cell membranes that can cause changes in cell permeability. Furthermore, the cell membrane cannot maintain the components that are in the cell and disrupt the flow of material that comes out and enters the cell, ultimately resulting in cell death. (Schmalz and Arenholt-Bindslev, 2009)

The positive charge of the amino group in chitosan has the ability to interact with negatively charged cell membranes. Then it will be absorbed to form a kind of layer that inhibits the ion transformation channel of cells and inhibits the work of the enzyme, thus cells experience a lack of substance and nutrients to develop and result in cell death. (Balicka-Ramisz *et al.*, 2005 , Goy, Britto and Assis, 2009)

Amine groups contained in chitosan have toxic properties to cells, but the emergence of toxic responses is influenced by the dose and concentration of exposure to chemicals given. The results of this study also found that the greater the concentration of chitosan, the smaller the average value of cell absorbance and

viability. This happens because in chitosan with a large concentration more negative charge is attached to the cell wall which causes cell membranes to break faster and there is apoptosis. At the concentration of chitosan 25%, 50%, 75% and 100% have cell viability included in the non-toxic category and according to classification based on the percentage of viable cell numbers using CD50 parameters. (Hughes and Mehmet, 2004)

CONCLUSION

The results of the percentage of live cells obtained from the treatment group for each concentration had the same effect which was reducing the number of live BHK-21 fibroblasts. In this study, the average percentage of live fibroblast cells after chitosan treatment was above the toxicity parameters of a material. It can be concluded that the use of chitosan shrimp shell (Litopennaeus Vannamei) as a bone graft material is safe to use and non-toxic and which means the results of this study are in accordance with the hypothesis.

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