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TOXICITY TESTING OF CHITOSAN OF PAPUYU FISH SCALE (*Anabas testudineus*) TOWARD BHK-21 FIBROBLAST CELLS

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ABSTRACT
Background*: Pulp capping is a treatment that aims to maintain the viability of the pulp tissue. Calcium hydroxide (Ca(OH)2 is a material that is often used for pulp capping procedures. However, this material does not provide a good adaptation to dentin. Therefore, an alternative material that is more biocompatible is needed, one of which is by using papuyu fish scale chitosan. papuyu fish as an alternative material must be proven to have no toxic effects.* ***Purpose****: to analyze whether the chitosan of papuyu fish scales (Anabas testudineus) is toxic to BHK-21 fibroblast cells using the MTT assay method.* ***Methods****: This study was a pure experimental study with a post-test-only design with a control group design consisting of 12 groups. The group given the chitosan treatment of papuyu fish scales consisted of 10 groups with concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% with 2 control groups namely control cells. and media control.* ***Results****: The results showed that the chitosan of papuyu fish scales did not have a toxic effect on BHK-21 fibroblast cells because the percentage value of cell viability of the entire treatment group was > 60% and the IC50 value was > 0.1%, which was* 5.405 %. ***Conclusion****: There was no toxic effect after administration of papuyu fish scales chitosan (Anabas testudineus) with concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% on cells BHK-21 fibroblasts.*

Keywords: *BHK-21 fibroblast cells, Papuyu fish scale chitosan, Toxicity test*
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INTRODUCTION

Pulp capping is a treatment carried out in cases of caries that aims to maintain the viability of the pulp tissue by stimulating the formation of a dentine bridge. The formation of a dentin bridge is one of the intrinsic responses to an exposed pulp. Pulp capping failure is generally due to a gap known as microleakage. The presence of microleakage results in bacterial contamination. Microleakage is a phenomenon of non-bonding or detachment that occurs between restorative materials and tooth structures, namely enamel and dentin. The material that is often used for pulp capping is Kalcium hydroxide (Ca(OH)2). In the long term, pulp capping materials such as calcium hydroxide are unstable in restorations. This happens because when the calcium hydroxide material is in contact with the dental fluid, it will experience water absorption. Calcium hydroxide does not provide good adaptation to dentin because calcium hydroxide does not have good adhesion properties causing poor sealing. Therefore, alternative materials are needed that can replace calcium hydroxide in pulp capping treatments, one way to get these alternative materials is to use natural ingredients. Several studies have proven that chitosan has excellent chemical and biological qualities so that it can be widely used in industry and in the health sector. [1,2,3]

Betok fish or papuyu fish (Banjar) (Anabas testudineus) is a fish that lives in swampy waters in Kalimantan. Papuyu fish scale chitosan have a degree of deacetylation of 97.48 % which is superior to shrimp chitosan.

The chitosan of papuyu fish scales contains NH2- which is able to act as an antioxidant that can reduce the activity of free radicals such as hydrogen peroxide. Therefore, chitosan can be used as a material in dentistry. [4,5,6] Materials used in humans must be tested for toxicity because it is the initial stage of evaluating a ingredient. The test medium recommended for use is Baby Hamster Kidney 21 (BHK-21). Method commonly used for toxicity testing is MTT assay method. MTT assay is very sensitive to evaluate cell viability. Toxicity test parameter is the value of Inhibitory Concentration 50 (IC50) which is the concentration value that inhibits growth in cells from the cell population and proves the potential toxicity of a compound to cells. [4,5,7]

Based on the above background, research on the toxicity test of haruan fish scales chitosan on BHK-21 fibroblast cells has been carried out with concentrations of 25%, 50%, 75% and 100%. Based on IC50, chitosan of Haruan fish scales was proven to have no toxic effect on BHK-21 fibroblast cells. However, there has been no research on the toxicity test of papuyu fish scales (Anabas testudineus). Chitosan made from different animal scales will of course have different chitosan qualities. This makes researchers interested in conducting research on the toxicity test of papuyu fish scales (Anabas testudineus) with the same concentration of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% against BHK-21 fibroblast cells in vitro.

RESEARCH METHODS

This research was preceded by an ethics session to obtain ethical feasibility from the Ethics Committee of the Faculty of Dentistry, University of Lambung Mangkurat No. 006/ KEPKG-FKGULM/EC/III/2022. This study used a true experimental method with a posttest-only design with control group design to test the toxicity of papuyu fish scale chitosan on fibroblasts using the MTT assay method of baby Hamster Kidney-21 (BHK-21). The population in study used BHK-21 fibroblast cells which could be obtained from the results of culturing at the Central Laboratory of Veterinary Farma Surabaya. The sample used in this study consisted of 12 groups with 10 treatment groups and 2 control groups. The treatment group consisted of concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100%. The control group consisted of control cells and control media. Each treatment has 3 repetitions with calculations using the Federer formula and obtained a total sample of 36 samples. Papuyu fish scales made at the Biochemistry Laboratory of the Faculty of Medicine, ULM.

Making Papuyu Fish Scales Chitosan

2 kg of papuyu fish scales were obtained from fish scale waste at the Kuripan market, Banjarmasin then the papuyu fish scale waste was cleaned and washed with clean water. Then dried, then in a blender. Then put into a 3.5% (w/v) NaOH solution and then heated for 2 hours at a temperature of 650C while stirring using a magnetic stirrer. Then this mixture is cooled and filtered with a cloth. Then washed using aquadest. Then dried in an oven at 650C for 24 hours. [6]

Deproteination: In the deproteination stage, it begins with the isolation of chitin from Fish scales that have been blended are then washed. The scale is put into a 1000 ml beaker and then the immersion process is carried out using boiling sodium hydroxide in a ratio (4% w/v) for one hour. NaOH solution as much as 4% with a ratio of powder and solution 1:4 w/v. Then the sample was boiled with a sodium hydroxide, then the fish scales were allowed to stand for 30 minutes at room temperature and filtered the sample residue. Furthermore, the sample was washed repeatedly using distilled water until a neutral pH was obtained. then the sample residue was filtered and put into the oven to be dried for 24 hours at 50^{o}C.[8,9]

Demineralization: at the demineralization stage using 1% HCl with a ratio of 1:4 w/v between powder and solution for the removal of minerals (especially calcium carbonate) immersion was carried out for 24 hours. Furthermore, the sample residue was filtered and washed repeatedly using distilled water until a neutral pH was obtained. Then the neutral sample was put into an oven for 24 hoursat 50°C and re-weighed the demineralized sample and recorded again. To convert chitin into chitosan, it must go through a deacetylation process. [8.,9]

Chitin test: in the chitin test stage, the Van Wesslink color reaction can be used. Then the chitin is reacted using a 1% I2-KI solution so that a yellow-brown color will appear, then 1 M H2SO4 is added so that it can turn purplish red or red violet. The positive reaction for the presence of chitin can be seen from the color change from yellow-brown to purplish red. [10]

Deacetylation: In the deacetylation stage, 50% NaOH solution is added using powder and solution in a ratio of 1:4 w/v and boiled for 2 hours at 100 ° on a hot plate. Then the sample was allowed to stand for 30 minutes at room temperature. Then, the sample was washed repeatedly by using cold NaOH 50% and filtering the residue. then the sample was washed using distilled water repeatedly until it got a neutral pH and then filtered until retain the solid substance, namely chitosan. The sample was then dried in an oven for 24 hours at a temperature of 50°C and re-weighed the deacetylated sample. Chitosan will be in the form of a white powder obtained from deacetylation.[8,9]

Fibroblast Cell Preparation BHK-21

Fibroblast cells were derived from BHK-21 cell culture in the form of a cell line using Eagle's media and 10% FBS and then implanted in a flask/roux bottle. The fibroblast cells were incubated using a CO2 incubator at a temperature of C for 24 hours. Fibroblast cells were propagated until fibroblast cells could adhere and fill the walls of the flask/roux bottle. After the cells were full, the Eagle's and FBS media solutions were placed in a flask/roux bottle. Then the The flask/roux was washed using PBS 3 times repeatedly, this aims to remove the remaining serum. Next, ml of Trypsine versene is added to release the cells on the bottle wall, this aims to separate the bonds between cells so they don't clump together, at this stage this can be done by patting the bottle until the flask/roux bottle wall is clean. Then the cells can be transferred to a 96-well microplate according to the number of samples and controls using a multichannel micropipette. [11]

Papuyu Fish Scales Chitosan Toxicity Test

Prepared BHK-21 fibroblast cells in a 96-well microplate (column 1 is control cell (KS), column 2 is media control (KM), while the other 4 columns are cell columns treated with chitosan samples of papuyu fish scales (Anabas testudineus) according to the specified concentration). BHK-21 fibroblast cells were treated with chitosan of papuyu fish scales (Anabas testudineus) according to the concentration. Cells were incubated to 24 hours using CO2 incubator. then incubation, The sample was cleaned and the Eagle and FBS media solutions were added to the collecting bottle. To remove residual serum, washing using PBS 3 times was repeated, to remove residual serum. Then, 10 L of yellow MTT reagent was added to the well. Fibroblast cells were incubated for 4 hours using a CO2 incubator. After incubation, the MTT solution was removed and the reaction between MTT and cells was completed by giving a DMSO stopper. Then The microplate was shifted or shaken for 5 or 10 minutes to stop the oxidation that could occur in each cell resulting in the release of formazan. Next, a 96-well microplate with a wavelength of 620 nm was inserted into the ELISA reader to process cell viability readings. The ELISA reader was turned off and then recorded the absorbance data. percentage of cell viability and IC50 analysis was calculated by SPSS (probit analysis). After the toxicity test was carried out, the BHK-21.11 fibroblast cell culture was removed. Then calculated the percentage of cell viability and IC50 analysis with SPSS for windows to obtain the value of the toxicity test.

RESULTS

The results of the toxicity test of chitosan papuyu fish scales from MTT staining on Microplate 96 Well which had been treated with chitosan of papuyu fish scales with concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% , and 100%. ELISA reader was used to obtaining the absorbance value of chitosan from papuyu fish scales against BHK-21 fibroblast cells.

Table 1. Percentage of Viability of BHK-21 Fibroblast Cells

Chitosan concentration of papuyu fish scales	Viability
10%	85%
20%	1%
30%	99%
40%	00%
50%	00%
60%	00%
70%	00%
80%	00%
90%	00%
100%	00%

The table above shows the viability values of BHK-21 fibroblast cells that have been treated with papuyu fish scale chitosan. If the percentage of viability > 60%, it means that the extract is not toxic. The percentage of viability of the papuyu fish scales chitosan obtained was

used to calculate the IC50 value using SPSS for windows and the IC50 value was 5,405% which means that the papuyu fish scale chitosan has no toxic effect on BHK-21fibroblast cells.

DISCUSSION

Based on the results of the research on the toxicity test of chitosan of papuyu fish scales, it showed that the chitosan of papuyu fish scales (Anabas tesdudineus) with concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% did not have a toxic effect on BHK-221 fibroblast cells because it showed a percentage of cell viability >60% and was assessed as 0.1%. Cell viability was assessed based on the color absorption of the enzymatic reaction of the MTT reagent against living cells. The IC50 value can be used to determine whether the chitosan of papuyu fish scales is toxic to fibroblast cells BHK-21.[12] The principle of the MTT method is a colorimetric measurement based on the formation of purple insoluble MTT formazan salts from the reduction reaction of tetrazolium which is soluble in water by producing yellow solution. Tetrazolium succinate which is included in the respiratory chain in the mitochondria of living cells forms purple formazan crystals and is insoluble in water. The formation of the purple color that occurs is equivalent to the number of living cells so that when the intensity of purple increases, the number of living cells also increases. [13,14] Chitosan has an amine functional group (-NH2) which is positively charged and highly reactive, so that it can bind to the bacterial cell wall. which is negatively charged. This bond will cause denaturation of the cell wall which can change the permeability resulting in disruption of the flow of intracellular components resulting in cell death. [15,16]

Chitosan that interacts with cell membranes with a negative charge on the positive charge of amino groups will also form a layer that can inhibit the transformation of ion channels and inhibit the work of enzymes in cells so that cells lack nutrients which will cause cell death. In addition, because -NH2 also has a lone pair of electrons, this group can attract Ca2+ minerals found in the bacterial cell wall by forming coordinating covalent bonds. Gram-negative bacteria with lipopolysaccharide in their outer 20 layers have negative poles that are very sensitive to chitosan. The antibacterial activity of chitosan is due to the interaction between chitosan and the outer cell membrane of the bacteria. The antimicrobial mechanism of chitosan against bacteria occurs through two theories. The first theory is based on the presence of amine functional groups in chitosan which can form bonds with bacterial cell walls and cause leakage of intracellular constituents so that bacteria will lyse. The second theory states that starting with damaging the bacterial cell wall, chitosan performs intracellular binding, blocks mRNA, and inhibits protein synthesis. [16]

The concentration of chitosan also affects the antibacterial power, namely the higher the concentration of chitosan, the greater the inhibition of bacteria. However, up to a certain concentration, the antibacterial activity of chitosan actually decreases. This is possible due to the lower viscosity of the solution. In addition to the concentration factor, the degree of deacetylation (DD) of chitosan also provides differences in antibacterial activity. The greater the DD of chitosan, the greater the antibacterial activity. This is because the greater the DD of chitosan, the greater the number of positively charged amine groups formed so that the opportunity for interaction with negatively charged bacterial cells is also greater. [16,17]

The results of the study showed that the higher the concentration of chitosan in papuyu fish scales, the darker the purple color produced, which means the viability of fibroblast cells increased along with the addition of the concentration of chitosan in papuyu fish scales. The more MTT reactions that are exposed to living cells, the more formazan salt or tetrazolium succinate which will produce dehydrogenase enzymes that are purple in color and insoluble in water in living cells. The purple color of this dehydrogenase enzyme is the reference to determine cell viability. [14.18]

Papuyu fish scale chitosan with concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% has antioxidants that are unable to completely inhibit or stop free radical activity. resulted in a decrease in the viability of fibroblast cells according to the given concentration. This is similar to the research of Saputra and Kroesnadi (2020) that chitosan has toxic content but the level of toxicity depends on the source of chitosan and its concentration level. The toxicity of chitosan can be determined by looking at the degree of deacetylation if more than 35% indicates low toxicity, whereas if the degree of deacetylation is below 35% the toxicity depends on the dose of chitosan used. [19]

The chitosan of papuyu fish scales has met the standards set by SNI, even the quality of

papuyu fish scales is better than the quality of chitosan set by SNI and chitosan derived from haruan fish scales. This is evidenced by the degree of deacetylation (DD) in papuyu fish scale chitosan, which is 97.40%, while the DD in Haruan fish scale chitosan is 85.25% and the standard DD set by SNI is 75%.[19,20] According to Duhan et al (2021) stated that the degree of deacetylation of chitosan affects the effectiveness of chitosan, the higher the degree of deacetylation of chitosan, the purer the chitosan. BHK-21.[21] The IC50 value obtained in This research can be used as a reference for future research.. Based on the discussion above, it can be concluded that there is no toxic effect on chitosan of papuyu fish scales (Anabas testudineus) with concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% against BHK-21 fibroblast cells.

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