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Research Report

Toxicity testing of chitosan from tiger prawn shell waste on cell culture

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ABSTRACT

***Background**: A biomaterial used in oral cavity should not become toxic, irritant, carcinogenic, and allergenic. Chitosan represents a new biomaterial in dentistry. **Purpose**: To examine the toxicity of chitosan from tiger prawn shell waste on cell culture with MTT assay. **Methods**: Chitosan with concentration of 0.25%, 0.5%, 0.75% and 1% was used in this experiment. Each sample was immersed on eppendorf microtubes containing media culture. After 24 hours, the immersion of media culture was used to examine the toxicity effects on BHK-21 cell based on MTT assay method. The density of optic formazan indicates the number of living cells. All data were then statistically analyzed by one-way Anava. **Results**: The number of living cells in chitosan from tiger prawn shell waste was 93.16%; 85.07%; 78.48%; 75.66%. Thus, there was no significant difference among groups. **Conclusion**: Chitosan with 0.25%, 0.5%, 0.75% and 1% concentrations from tiger prawn shell waste were not toxic for BHK-21 cell culture when using parameter CD_{50}.*

***Key words**: toxicity, chitosan, MTT assay*

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INTRODUCTION

Chitosan is a distillation product of chitin. Nowadays, chitosan is still considered as harmful waste for the environment. More than 10^9–10^{10} tons of chitosan are predictably produced every year. According to statistical data, countries which have shell producing industries produce this waste about 5,200 tons. However, product of chitin derivatives, chitosan oligomer, is the most expensive product in world market today.[1]

For instance, the use of chitosan in medical fields known as biomaterial nowadays can be used in many clinical applications. The reason is because chitosan has some special characters such as good biocompatibility, nontoxic and non bioactive, biodegradable, non allergenic and non carcinogenic.[2,3] The result of medical research on chitosan, moreover, shows that its commercial products can be used as skin regeneration for burn wounds or necrotic ulcers, as wound bandage, as surgical thread, and as membrane barrier for preventing abnormal ligament formation. The combination of chitosan and other materials cannot only be used as drug delivery system, oral vaccine carrier, and scaffold for tissue engineering, but can also react with living tissues during implementation procedure.[3]

Furthermore, in dentistry, the other use of chitosan, especially 0.5% *phosphorylated chitosan,* as mouthwash can significantly can reduce the early plague formation.[4] The combination of chitosan and chlorhexidine in reducing the plague formation is better than chlorhexidine without chitosan.[5] In endodontic field, chitosan can also be used as dressing material which has anti-inflammation effect for root path in periapical lesion since chitosan can stimulate fibroblastic cell to release chemotatic inflammatory cytokines, especially interleukin 8 (IL-8).[6,7]

Chitin and chitosan can actually be distinguished based on their nitrogen content. The polymer can be known as chitin if the nitrogen content is less than 7%, meanwhile, the polymer can be known as chitosan if the nitrogen content is more than 7%. Even though both polymers are found in nature, the terminology of chitosan nowadays is

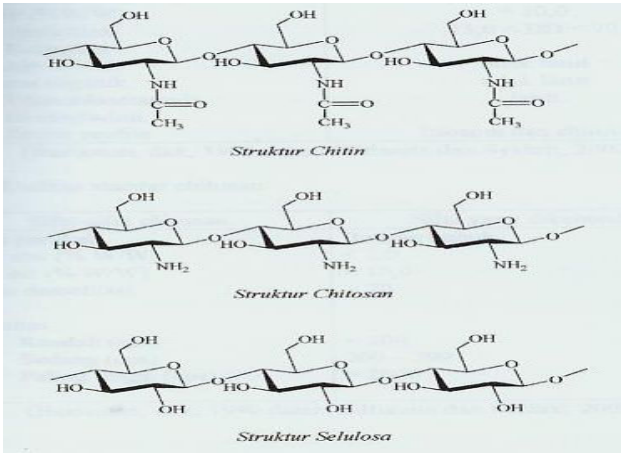

Figure 1. Chemical structure of chitin, chitosan, and cellulose.[9]

referred to chitin which acetyl structure can artificially be discharged.[8]

Chitin in tiger prawn shells, moreover, is produced as mucopolysacarida which relates to inorganic salt, especially calcium carbonate ($CaCO_3$), protein and fat including pigment. Therefore, deproteination process (protein separation), demineralization process (mineral separation), and depigmentation process (fat and color essence discharge) must be involved in order to obtain or produce chitin in tiger prawn shell.[10]

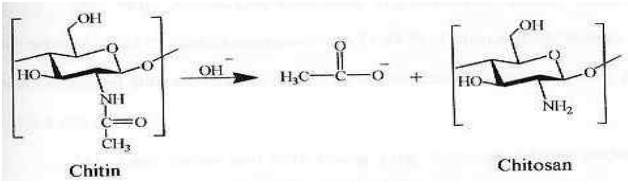

Figure 2. Distillation process of chitin into chitosan.[9]

Chitosan in some specification has actually been examined and produced by Chemistry Department of Science and Technology Faculty of Airlangga University. Thus, the chitosan is expected to be used later as biomaterial in dentistry.

In dentistry, however, if chitosan would be used orally, it must be biocompatible, which means that it could be accepted by human body, non toxic, non irritant, non carcinogenic, and also save without causing allergic reaction.[11]

Therefore, in order to reduce the bad effects of using chitosan, toxicity testing is needed. The toxicity testing can use animal testing in vivo or cell culture in vitro (cytotoxicity). The examination *in vivo* can give complete description about the response of the animal testing, but it can be difficult to comprehend the biologic response of the animal testing towards the tested material since the biologic response actually involves many stimulant reactions. Nevertheless, cell culture method is often used in examining biologic effects for several reasons such as: to reduce pressure from society towards the using of animal testing, to control the environment factors of cell culture (temperature, pH, and osmotic pressure), to obtain the result faster, and to expose the cell culture directly with the tested materials. Cell culture, moreover, is also very sensitive with toxic materials. Thus, not only can toxicity be measured quantitatively, but its response towards living cell can also be examined. Cell culture used in this experiment is BHK-21 cell from fibroblast of baby hamster's kidney. Fibroblast is often used by the researchers for toxicity testing of dentistry materials since it is the most important cell in the components of pulp, ligament periodontal and gingiva.[12]

This toxicity testing, furthermore, is done in some ways which are by measuring the number of cells and their development after being exposed with the tested materials, by examining cell status after the changing of membrane permeability, and by measuring toxic response based on enzymatic activities. Nevertheless, toxicity testing based on enzymatic cell activities is often done since this method can monitor specific function of cell metabolism, it needs only short duration (4 hours), gives quantitative results, has high sensitivity towards toxic materials, and has potentials for standardization of testing method.[12]

One of methods used for toxicity testing by monitoring enzyme activities is MTT assay. MTT is a yellow soluble molecule, which can be used to analyze cellular enzymatic activities. If the cell can reduce MTT, formazan produced will be blue-purple, insoluble, and precipitated in cell. The amount of formazan formed is proportional to enzymatic activities. This testing, furthermore, is measuring cellular dehydrogenizing activities, and changing the chemical material, MTT, through the number of cellular reductive materials into blue and insoluble formazan compound. MTT assay actually is based on the capability of living cells in reducing MTT salt. The principals of this assay are to break tetrazolium MTT ring (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the existence of dehydrogenase in active mitochondria, and then to produce insoluble blue-purple formazan product. The mechanism is that the yellow tetrazolium salt will be reduced in cell which has metabolic activities. Mitochondria of living cell has important role in producing dehydrogenase. If the dehidrogenase is not active because of sitotoxic effects, formazan will not be produced. Formazan production can be measured by diluting it and measuring the optic density of the solution produced. There are actually many protocols in using MTT assay, but the concentration of MTT used must be the same as to dilute 5mg/ml yellow MTT powder in PBS. The reaction of blue-purple color is used as the measurement of the number of living cells. The number of living cells can be measured as the product result of MTT by using spectrophotometer with 570–690 nm wave length.[13]

The objective of this experiment, finally, is to analyze the concentration of chitosan from tiger prawn shell waste which is produced by Chemistry Department, Faculty of Science and Technology, Airlangga University and does not

have toxic effects on BHK-21 cell culture testing. The result of this experiment is then expected to give information about the concentration of chitosan which does not give toxic effects on BHK-21 cell culture testing so that later it cannot only become references for next experiments on animal and patch test for humans, but can also add kinds of data specification about quality control of chitosan isolated from tiger prawn shell waste produced.

MATERIALS AND METHODS

This laboratory experimental research designs *Post test-only control group* experiment. The subject of this experiment is chitosan isolated from tiger prawn shell waste. The location of this experiment is at Pusat Veterinaria Farma (PUSVETMA–Centre of Pharmaceutical Veterinary) Surabaya and chemistry laboratory of Chemistry Department of Science and Technology Faculty of Airlangga University.

The materials used in this experiment were chitosan isolated from tiger prawn shell waste, composing of 100 g tiger prawn shells, HCl 1N, 3.5% NaOH, 50% NaOH, Acetone, 1% Acetate acid, sterile Aquadest, BHK-21 cell, culture media containing Eagle's minimum essential medium (MEM), RPMI-1640, 1% penstrep, 10% Foetal Bovine Serum (FBS), 100 unit/ml Fungizone, MTT reactor (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) Lot. 042K5313 (Sigma-USA), Phosphate Buffer Saline (PBS), and Dimethylsulfoxide AnalaR (DMSO) Lot 208KI6687088 (BDH-England). The tools used in this experiment, moreover, were 0.20 μm Filter Millipore Minisart Lot. 16534040736 (Sartorius), flask (Nunc), microplate 96 well Nunc Batch. 04644 (Nunclon-Denmark), pipette Pasteur, shaker Vari Shaker (Dynatech), Autoclave (Foundry), 5 cc Syringe injection (Terumo), 1 cc Syringe Tuberculin (Terumo), Eppendorf micropipette (Titertek, England), Incubator memmert W. Germany 37° C 5% CO_2, Laminar flow Oliphant Australia, and 630 nm ELISA reader Opsysmr Denmark.

In addition, there were some steps in preparing sample of tiger prawn shell (*Penaeus monnodon*). First, the shells of tiger prawn were washed cleanly and boiled in boiling water (± 100° C) for 15 minutes. Second, the shells were sun dried, then dissolved and filtered with 50 mesh size. Third, the phase of chitin extraction from the tiger prawn shells consisted of some processes, deproteination, demineralization, depigmentation, and distillation. In deproteination process the filtered powder of the tiger prawn shells was put into a beker glass, and then added with 3.5% NaOH solution with the ratio 1:10 (b/v). This process consisted of stirring process for 2 hours with temperature at 65° C. Afterwards, the solution was filtered by using filtering paper to obtain its residue. The residue is then washed by aquadest until the water pH became neutral, and dried in oven with temperature at 80° C until it was dried. The result of this process was known as crude chitin. In demineralization process: Crude chitin was put into beker glass, and then added with HCl 1N solution with the ratio 1: 15 (b/v). This process covered stirring process for 30 minutes at ambient temperature. Afterwards, the solution was filtered by using filtering paper to obtain its residue. The residue was then washed by aquadest until the water pH became neutral, and dried in oven with temperature at 80^o C until it was dried. The result of this process was known as chitin powder. In depigmentation process: the chitin powder was immersed in acetone for about 20 hours. The immersion result was then washed cleanly by aquadest and filtered with using filtering paper. After being dried in oven with temperature at 80° C, white chitin powder (cleaner) was obtained. In distillation process: the chitin powder was immersed in 50% NaOH solution with the ratio 1:10 (b/v) for 6 hours with temperature at 90° C. The result was then washed with aquadest and filtered with using filtering paper. After being dried in oven with temperature at 80° C, chitosan was obtained.[9]

The sample of this research was made of one gram chitosan powder diluted in 100 ml 1% acetate acid so that it became chitosan gel. Based on the standard procedure, 1% chitosan liquid was made of 1 gram chitosan gel mixed with 100 ml sterile aquadest (% b/v = 1 g/100 ml). Thus, chitosan solution with 0.25%; 0.5%; 0.75% and 1% concentration was made based on the procedure explained before. The sample was then sterilized in autoclave for 15 minutes. The sample was then ready to be tested with cell culture.

BHK-21 cell culture in cell-line form was cultivated inside a bottle. After confluent, the cell culture was harvested by using trypsine versene solution. The harvesting result was taken out little bit and then cultivated again into Rosewell Park Memorial Institute (RPMI-1640) media containing with 10% bovine serum albumin incubated for 24 hours with temperature at 37° C. Those cells, afterwards, were put into small bottles with 2×10^5 cell/ml density as the testing samples. This toxicity testing used 96 well plates of cell culture with flat base (Figure 3).

The testing was done based on standard protocol required for MTT *assay*. Each plate containing cells with 2×10^5 densities into 100 µl culture media. Before being tested, those samples had to be sterilized by *ultra violet* rays for 15 minutes. The samples were then put into well plates about 50 µl. Control cell and control media had to be prepared also. The control cell was that each well plate had to be contained only with cells and culture media. In this experiment, the testing was done in duplo. Those well plates were then incubated for 20 hours with temperature at 37° C. After that, each plate was contained with 5 mg/ml MTT which had been diluted in 25 μl PBS, and those well plates were then incubated for 4 hours with temperature at 37° C. The next step, samples were taken from each well plate which was added with 50 μl DMSO and piped up and down in order to dilute crystals formed. Those well plates were then incubated for 5 minutes with temperature at 37° C. Those well plates, afterwards, was monitored by ELISA reader with 630 nm wave length.[12] The result was

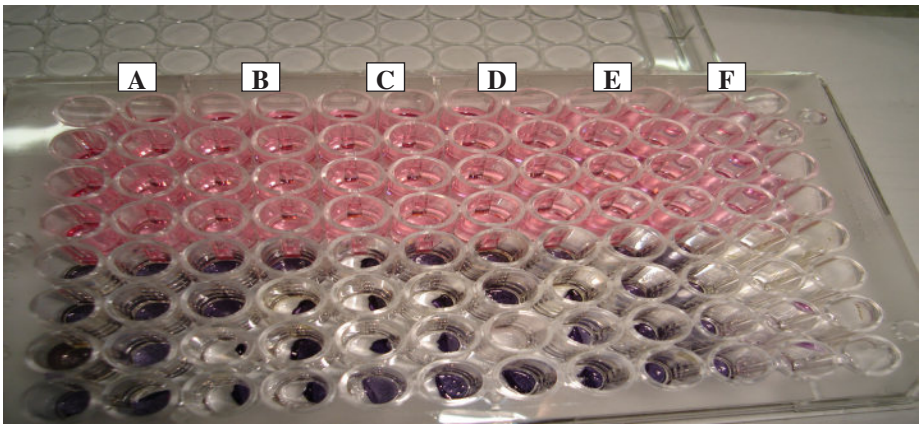

Figure 3. Toxicity Testing on 96 well plates.
Note: concentration of chitosan A: 1%, B: 0.75%, C: 0.5%, D: 0.25%, E: Control cell, F: Control media.

then shown in optical density (absorbents). The amount of absorbents in each well plate showed the number of viability cell in media culture. In order to analyze the percentage of living cell, the following formulation could be used:

$$\% \text{ living cell} = \frac{\text{treatment + media}}{\text{cell + media}} \times 100\%$$

The measuring result was then tabulated based on each groups, and tested by One-Way ANOVA with 5% level of significance. If there was significant difference, LSD test would be done.

RESULT

The research result of toxicity testing on chitosan from tiger prawn shell waste on cell culture, in which chitosan toxicity parameter in BHK-21 cell culture monitored based on the number of living cells/cell viability shown as optical density (absorbent), can be shown in table 1.

Based on table 1, the formazan optic density values are decreasing as the concentration is increasing. Kolmogorof-Smirnof Test shows that all groups have probability values more than 0.05 ($p > 0.05$). It means that all groups have normal distribution. In homogeneity test, however, the

Table 1. Average values, Standart Deviation, and Percentage of Chitosan (OD)

Concentration	Optic Density Values			PN	n
	$\overline{X}$	SD	%		
Control Cell	0.56650	0.17254	100.00	0.554	4
0.25%	0.53875	0.06698	93.16	0.447	4
0.50%	0.52625	0.06009	85.07	0.437	4
0.75%	0.42600	0.14615	78.48	0.609	4
1%	0.39725	0.11723	75.66	0.416	4

Note: x: Average of formazan optic density values; SD: Standart Deviation; %: Percentage average of formazan optic density; PN: Normal probability; n: number of samples.

Table 2. The result of One-Way ANOVA test of formazan optic density values on 0.25%, 0.5%, 0.75%, 1% chitosan and control cell

Variation Source	Quadrate Total	Db	Quadrate Average	F	P
Among Treatments	0.089	4	0.022	1.524	0.246
Under Treatment	0.219	15	0.015		
Total	0.308	19			

Note: db: Free Degree; F: F count; P: Probability

value obtained is about 0.233 ($p > 0.05$) which means that all groups are homogeny. Thus, one way Anava test is needed to be done with the result as in table 2.

The result of One-Way ANOVA test shows that there is no significant difference of optic density values of each chitosan concentrations with probability value = 0.246 ($p > 0.05$). In other words, chitosan with concentration from 0.25% to 1% can not influence formazan optic density values.

DISCUSSION

In recent years, biomaterial has been known to have many special characteristics that can be used for many clinic applications. Chitosan is one of biomaterials which is continuously improved for many clinical applications. Chitosan with chemical formulation of 2-amino-2-deoxy-D-glucan, produced by distillation process of chitin, is chitin derivative polysaccharide which can be used widely for biomedical applications.[15]

The use of chitosan is generally including biochemistry, enzymology, microbiology, drugs/pharmacy, food and nutrition, agriculture, waste management, paper industry, textile, membrane /film, cosmetics, etc. In the last decade, chitosan, moreover, can be used for many clinical applications.[8] Chitin and Chitosan can give effects on fibroblast cell proliferation of human skin and keratinocyte in vitro. Chitosan chloride CL 313A has stimulant effects on fibroblast cell proliferation depending on high distillation degree. The combination of chitosan and arginine can be used as biomaterial for anticoagulant.[10]

In this research BHK-21 cell culture is used for toxicity testing of chitosan which is isolated from tiger prawn shell waste. The chitosan with the concentration of 0.25%; 0.5%; 0.75% and 1% is used based on the previous research that Minimum Inhibitory Concentration (MIC) of chitosan in *S. mutans* is about 1%. Based on this research, the estimated density values of formazan optic is decreasing as the concentration is increasing from 0.25% to 1%. The percentage of living cell of all groups is up to 50% if using parameter CD_{50}. Statistic measurement using One-Way ANOVA with level of significance 5% shows that the increasing of chitosan concentration from 0.25% to 1% can not influence optic density values of formazan. It means that there is no toxicity on BHK-21 cell culture since chitosan does not have function cluster and structure which can make chitosan becomes toxic. A material usually can produce toxic especially because of its toxic cluster and structure. The process of chitosan production, moreover, has passed deproteination phase which is a phase of separating or breaking bond between protein and chitin so that chitosan will not become toxic.

Since chitosan is not toxic, the combination of chitosan and other materials can be used as drug delivery system and oral vaccine carrier. Chitosan, furthermore, cannot only be used as scaffold for tissue engineering, but can also interact with living tissue in implantation procedures.[3]

Some researches show that chitosan can be used as substitute materials for bone. The combination of phosphor-chitosan and calcium phosphate cements, for example, can produce compression power and modulus Young which is enough for bone cement material. The combination of chitosan-citrate acid and calcium phosphate cements, moreover, can become bone cement materials with good concentration.[16]

The research on the difference of chitosan concentration on cell culture with MTT assay is done in order to analyze the percentage of living cell with different concentration of chitosan. MTT assay is done based on the ability of living cell in reducing MTT salt. The principal of this assay is to break tetrazolium MTT ring, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), which has yellow color because of dehydrogenesis in active mitochondria, and to produce insoluble blue-purple formazan product. The mechanism of the yellow tetrazolium salt will be reduced in cell which has metabolic activities. In this mechanism, mitochondria of living cell have an important role in producing dehydrogenises. If the dehydrogenises are not active because of cytotoxic effects, the formazan is not produced. The formazan production can be measured by dissolving it and measuring optic density of the solution produced. The lower the percentage of optic density is, the fewer the number of active metabolic cells that can reduce MTT. The number of living cell detected by spectrophotometer or ELISA reader is the result of MTT product. The purpler the color is, the higher the absorbent values are and the more the number of living cells.[14]

Spectrophotometer is used in this toxicity testing since it can make the testing not only faster and easier than plat measuring method, but also more sensitive than visual reader method. Measuring with spectrophotometer is the best method which can give the best result since chitosan is highly viscous so that the visual monitoring can hardly be done on generating result of BHK-21 cell.

Based on the laboratory experimental research on toxicity testing of chitosan from tiger prawn shell waste on BHK-21 cell culture with MTT *assay*, it can be concluded that chitosan with concentration 0.25% to 1 % in tiger prawn shell waste does not have toxic effect on BHK-21 cell culture since chitosan does not have any structure which can cause toxic reaction.

Based on this research result, finally, the next phases of biocompability test (secondary test and tertiary test) are needed to be done.

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