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ORIGINAL ARTICLE

Cytotoxicity of Chitosan Derived from Shrimp for Bone Scaffold on Adipose Tissue-Derived Mesenchymal Stem Cells

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Abstract

Background: Chitosan as an organic constituent has widely been researched as biodegradable bone scaffold. However, some hesitation in some studies has intrigued to be observed. This study is aimed at observing the cytotoxicity of chitosan material with Adipose Tissue-Derived Mesenchymal Stem Cells (ASCs) obtained from human.
Methods: The material was served in 2 varieties among other raw and scaffold chitosans to prepare the bone scaffold candidate. Cytotoxicity was tested in vitro, using MTT*(3-(4.5-Dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromidefor)* assay standard protocol with ASCs as the cultured cell. The chitosan material was obtained from shrimps and processed into granules as raw chitosan. The raw chitosan was then processed into bone scaffold using frozen dried method. ASCs was gotten from the human tissue of a patient in a hospital with several criteria and certain indications. It was then cultured and put into the microplate. Afterwards, both scaffold and raw chitosan were added with Dulbecco's modified Eagle medium as the medium, and MTT solution as the reagent test. Both varieties of chitosan were later compared to the control cell which contained ASCs and the control medium which had blanks filled with cells.
Results: The result indicated that scaffold chitosan comes with no toxic effect, unlike raw chitosan. Although the raw chitosan displayed remarkably higher levels of cytotoxicity (P<0,01) than the control medium and control cell, the results also indicated that raw chitosan has a low-level cytotoxicity leading to the effect on ASCs and the cytotoxicity of chitosan depends on its properties.
Conclusion: This study indicated that raw chitosan gives more citotoxicity on ASCs compared to scaffold chitosan which has no citotoxicity against stem cells derived from human tissue.

Keywords: Chitosan; Cytotoxicity; MTT; Stem Cells

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INTRODUCTION

The study on chitosan as biomaterials for tissue engineering applications has intensified over the past 35 years. It is an important constituent of the exoskeleton in animals, especially in crustaceans, mollusks and insects. It is also the main fibrillar polymer in the cell wall of certain fungi (1). Chitosan is being applied in many areas of drug delivery and tissue engineering as a result of the broad range of compounds encompassed by this term (2). Since the ability of lisozyme and protease degrade chitosan, it is known as a biodegradable compound (3). Due to its biodegradability and untoxicity, its use in the pharmaceutical industry has been put into consideration. It reportedly possesses many beneficial properties such as anti-ulcer anti-acid properties, hypocholesterolemic action, wound-healing properties, antitumor, and hemostatic properties (4, 5).

In implant study, chitosan has been reported to be a biodegradable non-bearing loaded bone scaffold. One of the requirements for implant materials was not having toxic or pathological effects on the biological system. Chitosan is reportedly biocompatible with physiological medium (6). Two other reasons for the superiority of chitosan are its antibacterial effect (7) and antifungal effect (8). An in vivo study proved that chitosan has no toxic effect on cells. In contrast, it is reported to have disadvantageous effects on zebrafish embryo (9). Its physiological compatibility depends on preparation method and degree of deacetylation. Moreover, chemical modifications can lead to toxicity (6).

This hesitation of some reports results in further research about chitosan as biomaterial engineering. This phenomenon made the clearance study of the present research aimed at determining the toxicity phenomenon of chitosan on the cell.

METHODS

Chitosan material

Chitosan was derived from shrimps obtained from The Center for Application of Isotope and Radiation Technology, Indonesia

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National Atomic Energy Agency. Chitosan was divided into two forms, the Scaffold and Granule forms. Each chitosan was prepared for chitin deacetylation with 50% of sodium hydroxide at 90°C for 8 hours, and precipitated with 30% of hydrochloric acid (10). The raw chitosan was formed as granules followed by dissolution in acetic acid of 1% at 100ml per two grams, after which it was molded and freeze dried. The results were neutralised with 1% of NaOH and freeze dried. Afterwards, it was sliced into cuboid form using dimensions of 1mm x 2mm x 3mm. Every chitosan was sterilised by gamma irradiation with 25kGy.

ASCs

ASCs cells were obtained from the body fat tissue of a patient participant whose participation had been approved and legalised with ethical clearance with certificate number: 107/KEH/2018. The participant was a patient in The Education Hospital of Universitas Airlangga, Surabaya with selective indication and certain criteria such as caesarian section case due to fetal dystocia. Adipose tissue was collected, put into the transport medium and taken to Stem Cell Research Study Group Laboratory of Tropical Disease Institute for human ASCs collection, cultural expansion and cytotoxicity test. The Adipose tissue was cleaned with PBS solution, finely chopped and then flooded by collagenase enzyme. Afterwards, they were soaked and incubated at 37°C for 45 minutes to the pellets and embedded on 10cm plates until attachment. The plate was labelled with the patient's identity and then incubated at 37°C.

Citotoxicity assay

The cytotoxicity test was conducted by MTT assay standard protocol. ASCs were then seeded in a 96-well microplate with Dulbecco's modified eagle medium (DMEM) and incubated with 5% CO2 at 37°C for 24 hours. Subsequently, chitosan was put into a well which was then filled with DMEM and incubated with 5% CO_2 at 37°C for 20 hours. Twenty µl MTT solution (5mg/ml) was put in each well which was divided into 4 groups encoded based on side microplate. Furthermore, it was incubated at 37°C for 4 hours then 200µl of DMSO (Dimethyl sulfoxide) was added. The absorbance was read using ELISA (enzyme-linked immunosorbent assay) reader at 570nm wavelength (11). The data were finally interpreted with the following formula:

$$\% \text{ viability of cell} = \frac{optical\ density\ of\ the\ sample - optical\ density\ of\ the\ medium}{optical\ density\ of the\ control\ cell - optical\ density\ of the\ medium}$$

Statistical Analysis

The data were processed with root transformation and then analysed using parametric statistical Analysis of Variance and Tukey's test with significance level (P<0.05) by using SPSS 22 for Windows.

RESULTS

The results of this study were highly significant (P<0.01) as seen in the following Table 1. The results suggest that there is a highly significant difference among groups (P<0.001). The comparison between all groups and Control Medium was highly significant (P<0.001) for each. The comparison between Control Cell group and Scaffold Group was not significant (P=0.822). Raw chitosan seems to show a highly significant difference from Control Cell which is not added any materials and Control Cell group (P=0.010). The comparison between chitosan groups found to be (P=0.056).

DISCUSSION

Chitosan is known to be a non-toxic biodegradable biomaterial (2). Its substances such as biological matter brought about the thought that it could adapt inside the body, be biocompatible and it could also be unable to interact with the body system which means it could promote rejection such as inflammatory or hypersensitivity response. The remarkable properties of chitosan made it a relevant candidate for the preparation of biomaterial which has the ability to be substituted for missing or damaged tissues or organs and allow cell attachment and proliferation (6). Table 1 shows that scaffold chitosan was not toxic in ASCs. The differences between the control cell and scaffold chitosan group were similar (P>0.05). It can be inferred that the existence of scaffold chitosan does not harm the cell.

In contrast, the raw chitosan has a toxic effect on the cell, according to Table 1. It was appropriate that scaffold chitosan group was remarkably different from the raw chitosan group (P<0.000). In addition, the raw chitosan with both the control cell (P<0.000) and the control medium (P<0.004) were also as remarkably different as scaffold chitosan. This indicated that raw chitosan exerts a more intense toxicity effect on cells than scaffold chitosan. At variance with control medium, it was indicated that raw chitosan was not remarkably toxic to cells. It was also explained that the in vitro toxicity of chitosan varied. There are several factors capable of affecting the viability of a certain cell. Meanwhile, toxicity was

Table 1. The citotoxicity of chitosan in MTT assay

Group	X±SD
Control Medium	0.7611^{a}±0.001
Control Cell	1.0516^{c}±0.020
Scaffold Chitosan	1.0373^{c}±0.047
Raw Chitosan	0.9910^{b}±0.0115

The different superscript in the same column indicates a highly significant difference.
Notes:
Abbreviations: ASCs, Adipose Tissue-Derived Mesenchymal Stem Cells; MTT, 3-(4.5-Dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromidefor; PBS, phosphate buffer saline; DMSO, Dimethyl Sulfoxide; ELISA, Enzyme linked immunosorbent assay.

reported to be dependent on the degree of deacetylation and the molecular weight (2).

Toxicity was one of the several requirements of the biomaterial. The required properties of biomaterials among others are that they should be biodegradable, biocompatible, possess mechanical properties, scaffold architecture, manufacturing technology (12), functionality and sterilizability (13). Toxicity was one of the prerequisites of a biocompatible bone scaffold (14). The requirements of chitosan untoxicity have been studied in many different responses in vivo ranging from immunogenicity, allergenicity, reproductive toxicity, carcinogenicity and genotoxicity (15). Toxicity confirmation was needed, as done in this study, to hypothesize that the material for biomedical application was safe against the tissue. Therefore, it emphasized that the conformity of chitosan, as shown in Table 1, could be a beneficial bone scaffold.

The utilisation of biomedical material as bone scaffold has been widely studied. Not only biocompatibility stress shielding and foreign body reaction, but also bioactivity and osteoinduction have been gradually studied as materials in orthopedics (16). The ability of chitosan to retain the ASCs viability in vitro as well as scaffold chitosan group might be more eminent as biodegradable bone scaffold than the raw chitosan which gave low toxicity effects as seen in Table 1. ASCs have been used as a means of replacing faulty or depleted cells such as the ones that occur when there is non-union of complex bone fracture. ASCs were also applied therapeutically in a variety of disciplines such as orthopedic surgery, otolaryngology, neurosurgery and vascular surgery to increase healing, as ASCs have the multipotent ability to transform into various kinds of tissues (17). In addition, chitosan is capable of accelerating bone formation (18). It also has the property of being more osteoinductive than tissue culture plate control (19).

LIMITATION

The assay was only run in vitro without any variables related in the research.

CONCLUSION

The present study showed an in vitro MTT assay comparison between chitosan as a scaffold and raw chitosan. The result suggested that scaffold chitosan gives less toxic effect compared to raw chitosan which has less cytotoxicity.

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Conflict of interest: None to be declared.

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