Cytotoxicity Analysis Of Alginate Impression Materials Based Red Seaweed Extract On Cultured Gingival Fibroblast Cells

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ABSTRACT

Background: *Alginate is an impression material that is widely used in dentistry. Alginate can actually also be obtained from natural materials such as red seaweed. The impression procedure causes the impression material to come into contact with oral tissues including the gingiva. Ideally, the materials used must also meet requirements such as low toxicity or non-toxicity so that tissue damage does not occur. This study aims to analyze the cytotoxicity of alginate impression materials from red seaweed extract in gingival fibroblast cell.*
Method: *This experimental laboratory design using post-test only control group design. The research groups consisted of: sodium alginate extract group, red seaweed extract-based alginate impression material, positive control and negative control. Cytotoxicity was tested on gingival fibroblast cell cultures and the effect was analyzed using the MTT assay. Exposure to gingival fibroblast cell cultures was differentiated in three time durations: 5 minutes, 10 minutes and 15 minutes. Each time duration was repeated three times. MTT-formazan production is a method used to measure cell viability (living cells). The data obtained were statistically analyzed using two-way ANOVA test and Tukey HSD post hoc test.*
Result: *There was no significant difference in the average cell viability between the red seaweed extract-based alginate impression material group and the negative control group at an exposure duration of 5 minutes, which was more than 90%.*
Conclusion: *The red seaweed extract-based alginate impression material has no toxic effect on gingival fibroblast cells at 5 minutes exposure.*

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INTRODUCTION

Alginate impression material in dentistry is a material that is widely used to produce replicas of the shape of teeth and oral soft tissue.[1] The impression material is inserted into the mouth immediately after mixing and left in contact with the oral tissue for several minutes.[2] In this condition, the material may be toxic to cells or may cause tissue injury and damage to biological systems. The materials used should ideally meet requirements such as biocompatibility, degradability and low or no toxicity.

Alginate can be extracted from natural materials such as red seaweed (RS). The first stage of making alginate is to convert insoluble calcium and magnesium alginate into water-soluble sodium alginate by ion exchange under alkaline conditions. The results of several studies show that alginate has the ability to act as a stabilizer, emulsifier and has high viscosity. Apart from that, it is also capable of forming a gel, biocompatible, antioxidant, antibacterial, antiviral, anti-aging, anti-inflammatory, anti-cancer and biodegradable.[3,4,5,6] But the alginate extracted from RS still contains a large number of contaminants such as heavy metals which can cause toxic effects.[7]

The cytotoxicity of alginate impression material from RS extract is not yet known, so research is needed to analyze the cytotoxicity of alginate impression material from RLM extract in vitro, namely in gingival fibroblast cell cultures.

RESEARCH METHOD

The type of research is laboratory experimental with a post-test only control group design and has received approval from the Health Research Ethics Commission, Faculty of Dentistry, Jember University (No. 1998/UN25.8/KEPK/DL/2023). The research group consisted of: sodium alginate extract group, alginate impression material based on RLM extract, positive control and negative control. The number of repetitions is 3 times for each group. The cell culture used is gingival fibroblast cells which are a collection from the CDAST Molecular Medicine Laboratory, Jember University. Exposure to gingival fibroblast cell cultures was divided into three time durations, namely: 5 minutes, 10 minutes and 15 minutes.

Red seaweed (*Kappaphycus alvarezii*) extraction

The RS was washed with running water until the dirt was gone, then soaked in 0.1% KOH solution for 1 hour. The RS was washed again with running water to remove alkali residue. Next, the RS was dried in an oven at 60°C for 96 hours until the water content was less than 15%. The dried RS is ground using a blender until a fine dry powder is obtained.

Sodium alginate extraction

The first stage of alginate production is to convert insoluble calcium and magnesium alginate into water-soluble sodium alginate by ion exchange under alkaline conditions. 100 grams of RS dry powder was soaked in 1% HCl for 1 hour with a ratio of 1:30 (w/v). Next, wash with clean water until a neutral pH is obtained. Extraction was carried out using a 2% Na_2CO_3 solution with a ratio of 1:30 (w/v) while stirring and heating with a water bath shaker for 2 hours at a temperature of 60-70°C. The extraction results are filtered using a 150 mesh sieve to obtain a filtrate. Then the filtrate was bleached by adding 10% NaOCl to 4% of the filtrate volume for 30 minutes. 10% HCl was added to the filtrate until a pH of 2.8-3.2 was obtained and the alginic acid

precipitate obtained was then separated and washed until clean. The alginic acid precipitate is converted into sodium alginate by adding 10% Na_2CO_3 until a pH of 7 is obtained. The next stage is separating the sodium alginate by pouring the filtrate into isopropyl alcohol (1:2, v/v) little by little while stirring, then let stand for 30 minutes. The sodium alginate is dried using an oven at 60°C for 72 hours, then ground using a blender and finally filtered using a 60 mesh sieve, to obtain sodium alginate powder.

Identify red seaweed sodium alginate components

Identify the components of sodium alginate using an FTIR (Fourier Transform Infrared) spectrophotometer to obtain qualitative absorption peaks of the functional groups that make up sodium alginate. The spectrum of sodium alginate was measured, then the functional groups that make up sodium alginate were identified based on the spectrum results which showed the characteristics of the sodium alginate.

Making alginate impression materials based on red seaweed extract

The ingredients needed to make alginate impression materials based on RS extract are: potassium sulfate 10%, trisodium phosphate 2%, calcium sulfate 14%, diatomaceous earth (filler) 50%, HMPC (Hydroxy Propyl Methyl Cellulose) 6%, and sodium alginate 18% derived from RS. These ingredients are mixed and ground using a mortar and pestle, then ground with a blender and filtered until a homogeneous result is obtained.

Sample preparation

- Sodium alginate extract group: 4 grams of sodium alginate extract and 10 ml of sterile distilled water were manipulated using a rubber bowl and plastic spatula. Once homogeneous, the sample was put into a cylindrical mold with a diameter of 3 mm and a height of 1 mm until it was completely filled.
- RS extract-based alginate impression material group: 5 grams of RS extract-based alginate impression material powder and 2.5 ml of sterile distilled water were manipulated using a rubber bowl and plastic spatula. Once homogeneous, the sample was put into a cylindrical mold with a diameter of 3 mm and a height of 1 mm until it was completely filled.
- Positive control: 5 grams of Hygedent® alginate impression material powder and 11 ml of sterile distilled water manipulated using a rubber bowl and plastic spatula. Once homogeneous, the sample was put into a cylindrical mold with a diameter of 3 mm and a height of 1 mm until it was completely filled.

Before treatment, all samples were sterilized by exposure to ultraviolet for 1 hour.

Toxicity test

Fibroblast cells were taken from BHK-21 cell culture in the form of cell lines grown in Roux bottles. Once full the culture is harvested using trypsine versene solution. The harvest was grown in Eagles medium containing 5% fetal bovine serum albumin, incubated for 24 hours at 37°C. The cells were then transferred into Roux bottles at a density of $2x10^5$ cells/ml. Cells were cultured in each well (microplate 96) until confluent.

Observe the microplate containing fibroblast cells that have been incubated under a light microscope, whether there are enough fibroblast cells that have been planted in each well for treatment. Fibroblast cells that had been distributed into 96 well microplates were divided into 4 groups. The sodium alginate extract group is fibroblast cells exposed to sodium alginate extract, the RS extract-based alginate impression material group is

fibroblast cells exposed to RS extract-based alginate impression material, the positive control group is fibroblast cells exposed to Hygedent® impression material and the negative control group (control cells) which only contain fibroblast cells in the culture medium. The supernatant was taken after 5 minutes, 10 minutes and 15 minutes for analysis of its toxicity on fibroblast cells.

Next, it was incubated for 24 hours in an incubator at 37°C. The 96 well microplate was removed from the incubator, 10 µl of MTT was added and dissolved in PBS 5 mg/ml, incubated again for ± 4 hours at 37°C. To the cell suspension, 50 µl of dimethylsulfoxide (DMSO) solution was added per well. The microplate was stirred mechanically for 5 minutes with a plate shaker until the formazan crystals dissolved. Live fibroblast cells will stain blue with formazan, while dead ones do not produce a blue color. Next, the absorbance of formazan was read spectrophotometrically with an ELISA reader at a wavelength of 620 nm and the percentage of cell viability was calculated based on the optical density (OD) or absorbance value obtained.

Statistic test

Data analysis used IBM SPSS Statistics 26. A two way ANOVA test was carried out to compare differences between groups followed by the Tukey HSD test with a p value <0.05 considered significant.

RESULT

The results of microscopic observation of gingival fibroblast cells using an inverted microscope with 10x magnification after being treated for 5 minutes can be seen in Figure 1.

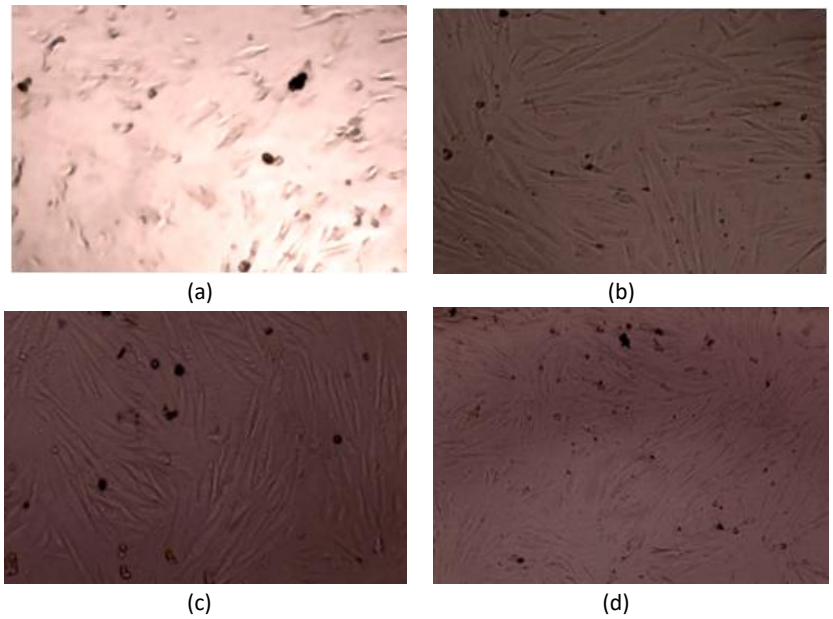

Figure 1. Microscopic images of fibroblast cells with 10x magnification at a time duration of 5 minutes: (a) sodium alginate extract group, (b) RS extract-based alginate impression material group, (c) positive control group, (d) negative control group.

Based on the reading of the absorbance or optical density (OD) value as well as calculating the percentage of cell viability in the toxicity test of the RS extract-based alginate impression material exposed to

gingival fibroblast cells for a duration of 5, 10 and 15 minutes, the following results were obtained (Table 1, Figure 2)

Table 1. Average OD and percentage of cell viability with exposure durations of 5, 10 and 15 minutes

Group	Exposure duration 5 minutes		Exposure duration 10 minutes		Exposure duration 15 minutes	
	Average OD	Cell viability (%) (x̄ ±SD)	Average OD	Cell viability (%) (x̄ ±SD)	Average OD	Cell viability (%) (x̄ ±SD)
Sodium alginate extract	0.033	40.179±1.999	0.053	41.276±3.674	0.029	37.587±1.253
Alginate impression material based on RS extract	0.289	118.146±49.036	0.178	80.160±14.283	0.148	74.477±4.302
Positive control	0.307	119.442±10.712	0.292	115.753±7.592	0.217	91.725±13.731
Negative control	0.247	100.000±3.998	0.247	100.000±3.998	0.247	100.000±3.998

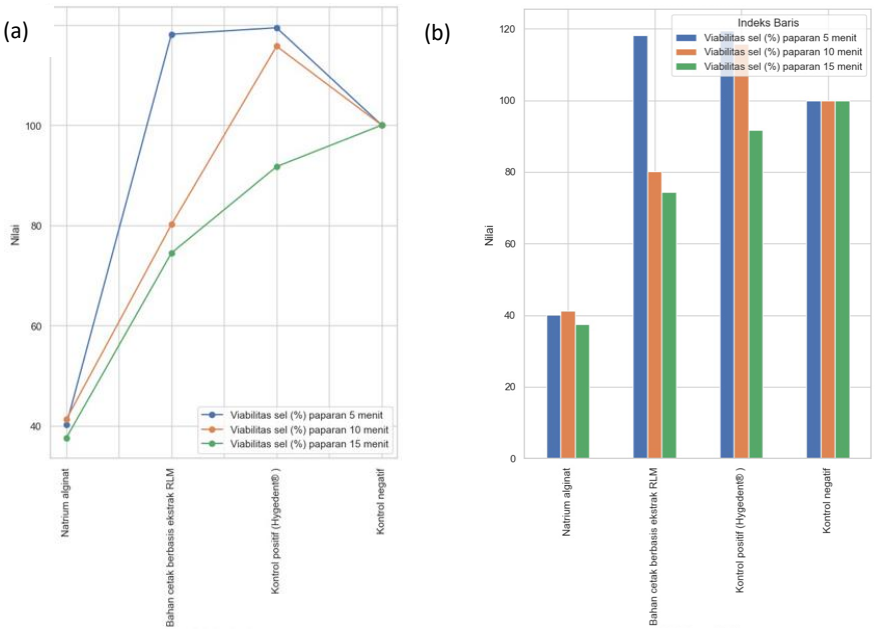

Figure 2. Line diagram (a) and bar diagram (b) average cell viability at exposure durations of 5 minutes, 10 minutes, and 15 minutes.

The classification of toxicity levels based on the percentage of cell viability is grouped as follows: non-toxic (more than 90% viable cells), slightly toxic (viable cells between 60% and 90%), moderately toxic (viable cells between 30% and 59%), and very toxic (less than 30% viable cells).[8]

The results of the two way ANOVA test showed that there was a significant difference in the average percentage of cell viability in each research group. Meanwhile, there was no significant difference in the average cell viability between research groups with exposure durations of 5 minutes, 10 minutes and 15 minutes. The Tukey HSD follow-up test showed the results: (1) there was no significant difference in the average cell viability in each research group with exposure durations of 5 minutes, 10 minutes and 15 minutes; (2) there is no

significant difference in average cell viability between groups with an exposure duration of 5 minutes; (3) there is a significant difference in the average cell viability between groups with an exposure duration of 10 minutes except between the RS extract alginate impression material group and the negative control and between the positive control and the negative control; (4) there was a significant difference in the average cell viability between groups with an exposure duration of 15 minutes except between the RS extract alginate impression material group and the positive control. and between positive control and negative control.

DISCUSSION

Biomaterials in the field of dentistry must meet the requirements, namely good biocompatibility, non-toxic, non-irritant, and not detrimental to the biological environment. Evaluation of dental materials can be carried out using cytotoxicity tests, to measure the toxic effect of the material on cells or to find out whether the material that meets the requirements to be accepted into the network.[9,10]

The cytotoxic effect of a material is measured by measuring cell viability in culture. This research uses fibroblast cell cultures taken from gingival tissue. Cultured gingival fibroblast cells are characterized by a high level of differentiation. In addition, gingival fibroblasts also maintain specific features and can represent a good simulation of in vivo conditions.[11,12]

The MTT (dimethylthiazol-diphenyltetrazolium bromide) test is one of the methods used in cytotoxic tests.[13] This method is a colorimetric method, where the MTT reagent is a tetrazolium salt which can be broken down into formazan crystals by the succinate tetrazolium reductase system found in the cell respiration pathway in mitochondria. which is active in living cells. These formazan crystals give a purple color whose absorbance can be read using an ELISA reader. The stronger the intensity of the purple color formed, the higher the absorbance value. The higher the absorbance value, the greater the cell viability.[14] Cell viability is the possibility of cells being able to live after being exposed to a material.

The impression procedure takes a few minutes and the contact time with the patient's mouth is very short.[1] The exposure duration of 5 minutes is assumed to be the length of time the impression material is in contact with the oral cavity tissue each time an impression is made. Printing can sometimes be done more than once to get accurate print results, so this research was carried out with three exposure times, namely 5 minutes, 10 minutes and 15 minutes.

The results of this study showed that gingival fibroblast cells exposed to RS extract alginate impression material for 5 minutes had an average cell viability of more than 90% and the results of data analysis had no significant differences with the negative control which was the cell control group. Based on these results, it can be said that the RS extract alginate impression material is categorized as a non-toxic material because no cytotoxic effects were detected in gingival fibroblast cells that were exposed to the RS extract alginate impression material for 5 minutes.

RS contains chemical compounds that have biological activity (bioactive substances). The results of screening for bioactive compounds in RS (*K. alvarezii*), which were detected were alkaloids, tannins/polyphenols, saponins and flavonoids.[15,1617] These bioactive ingredients are ingredients that can function as antioxidants. Antioxidants reduce the formation of free radicals and can help protect cells from damage due to exposure to free radicals. The body's defense system in the form of an antioxidant enzyme

control system works to regulate the reactions that form free radicals as needed and neutralize excess free radicals that can damage tissue in the body.[18,19,20,21]

In conditions where free radicals are formed, levels of hydrogen peroxide and superoxide increase. Antioxidants have an impact on reducing the formation of free radicals because they function as scavengers of hydroxyl radicals. Antioxidants are compounds that can counteract the negative effects of oxidants by donating one electron to the oxidant compound so that the activity of the oxidant compound can be inhibited. Antioxidants can also inhibit oxidation reactions by binding free radicals and reactive molecules so that cell damage can be prevented.[22]

Apart from that, RS also contains carotenoids which are a group of natural pigments and antioxidants that can reduce free radicals. Several carotenoids have been studied extensively, one of which is β-carotene. The mechanism of β-carotene as an anti-free radical is to protect cell membranes and maintain the integrity of cell membranes through free radicals, so that lipid peroxidation in cell membranes can be prevented.[23]

Meanwhile, at exposure durations of 10 minutes and 15 minutes, there was a significant difference between the RS extract alginate impression material and the negative control. RS extract alginate impression material exposed to gingival fibroblast cells for 10 minutes and 15 minutes is considered slightly toxic because the average cell viability is in the range of 60%-90%. The toxic effect is likely due to the alginate extracted from RS containing high M monomers. This mononer is more immunogenic and 10 times more effective in increasing cytokine synthesis compared to monomer G. Sodium alginate itself contains a hydroxyl ion (OH^-) component which is a reactive free radical and is able to react with cell membranes so that the cell structure undergoes irreversible changes. Another cause of sodium alginate's toxicity is the possibility that it still contains large amounts of active ingredients such as heavy metals, endotoxins, proteins and polyphenols which can increase the host's immune response and thus reduce its biocompatibility.[4]

CONCLUSION

The red seaweed extract-based alginate impression material has no toxic effect on gingival fibroblast cells at 5 minutes exposure.

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CONFLICT OF INTEREST

All authors declare that we have no conflicts of interest.

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