ORIGINAL ARTICLE

Effects of *Stichopus hermanni* Gelatin and Local Hydroxyapatite on Osteoblast Number and the Viability of Fibroblasts

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ABSTRACT

Introduction: Alveolar bone resorption has significant influence on retention and stabilization of dentures. Hydroxyapatite is a major component of teeth and has a high bone density. Gelatin is commonly used as a bone graft substitute and has amino acid. *Stichopus hermanni* gelatin extract contains 74% gelatin. The aim of this research was to determine the effects of *S. hermanni* gelatin and local hydroxyapatite as bone graft substitute on osteoblasts and the viability of fibroblasts. **Methods:** The subjects were 3 months old male of Sprague Dawley rats were divided into 3 groups. The subjects were decapitated 3, 7, 14, 21, and 28 days after treatment. The bone defect was made on the femur condyle of Sprague Dawley rats. The defect area in the condyle femur was taken and made histology slides with Hematoxilin Eosine staining. A Trinoculer microscope was used to quantify the number of osteoblasts. The two-way ANOVA test was used to analyzed of the data. A toxicity test was performed using the MTT assay. The one way Anova was used to analyzed it. **Results:** The number of osteoblasts were significant difference between the *S. hermanni* gelatin local hydroxyapatite, *S. hermanni* gelatin, and gelatin groups after 3, 7, 10, 14, and 28 days of treatment ($p<0.05$). There were significant differences between the MTT absorbances measured in fibroblasts that were treated for 24 h with different concentrations of the *S. hermanni* gelatin local hydroxyapatite ($p<0.05$). **Conclusion:** The *S. hermanni* gelatin and local hydroxyapatite as bone graft substitute increased osteoblast number compared with *S. hermanni* gelatin or gelatin alone. Additionally, *S. hermanni* gelatin and local hydroxyapatite as bone graft substitute did not cause cytotoxicity on fibroblasts.

Keywords: Gelatin, Hydroxyapatite, Osteoblast, *Stichopus hermanni*

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INTRODUCTION

The purpose of using dentures is to replace missing teeth. Dentures can restore the function of mastication, speech, and appearance (1). To obtain an ideal denture, it is necessary to prepare the alveolar bone as it is the main support of the denture. The alveolar undergoes resorption 50% after tooth extraction, it will influence the denture usage (2). The resorption was subsequently slow, but continuous (3). The denture is not stable if it is not supported by the alveolar bone (4). Maintaining the alveolar bone is important to support the stabilization of the denture (5). The prevention of alveolar bone resorption is essential immediately after tooth extraction. The wound healing after tooth extraction is very important before denture insertion (6).

After tooth extraction, a common problem encountered is bone damaged. The resorption of the bone caused by tooth extraction to make the bone damaged (7,8). A graft can be used to promote bone formation, which allows the implant and denture to be installed (9). The increase in alveolar bone volume and rehabilitation of bone damage can be achieved using a graft (10).

Hydroxyapatite is a good biocompatibility material but it is porous and mechanically weak. New bone formation is promoted by hydroxyapatite through the mechanism of osteoconduction mechanism (11).

Bone consists of 65% inorganic matrix and 35% organic matrix (12). The inorganic matrix of bone contains inorganic salts, such as calcium fluoride, calcium carbonate, and calcium phosphate. The organic matrix of bone contains collagen fibers (12). Gelatin is used as a bone graft in tissue repair (13). Gelatin has the ability to attach to cells. The mechanical properties of gelatin are lower compared to bone (14). *Stichopus hermanni* gelatin extraction contains 74% gelatin (15,16).

Analysis of the proliferation and migration of fibroblasts in the wound area revealed that fibroblasts were very dominant and easily observed in the proliferative phase (17). The process of forming bone collagen involves the activity of osteoblasts and fibroblasts. The early

stages of osteoid formation requires osteoblasts, which synthesized collagen. Amino acids are essential for the osteoblasts to synthesize bone collagen. Further, calcium salts are needed for new bone calcification. Amino acids are obtained from the biodegradation of collagen fibers (17,18). Calf skin collagen, as a bone substitution material, increases fibroblast adhesion and promotes mitotic fibroblast activity (19). *Stichopus hermanni* extract contains 74% type 1 collagen fibers (15,20). The chicken scratch collagen as a bone substitute material did not cause toxicity in fibroblasts (21).

The purpose of this research was to determine the effects of *Stichopus hermanni* gelatin and local hydroxyapatite as bone graft substitute on osteoblasts and the viability of fibroblasts.

The authenticity of this research: A similar study was conducted previously that was tissue engineering scaffolds in stimulation of osteoblast responded to gelatin hydroxyapatite (22). However, the differences in this study were the use of *Stichopus hermanni* gelatin and local hydroxyapatite as bone graft substitute to determine their effects on osteoblasts and the viability of fibroblasts.

This research provides new information for the development of treatments in the Dentistry Prosthodontics field. This study suggested that *Stichopus hermanni* gelatin and local hydroxyapatite may be used as alternative materials for the rehabilitation of alveolar bone without causing toxic side effects on fibroblasts.

MATERIALS AND METHODS

Determination of osteoblast number

Materials

Stichopus hermanni gelatin, local hydroxyapatite, xylaxin, formic acid, ketamine hydrochloride 10%, aluminum chloride, HCl 37%, and Mayer's Hematoxylin and Eosin.

Ethical statement

The research was performed after obtaining ethical clearance from the Unit Ethics and Advocation, Faculty of Dentistry, Universitas Gadjah Mada with registered number 001191/KKEP/FKG-UGM/EC/2017. The in vivo experiment was conducted at the LPPT UGM Laboratory and Veterinary Histopathology Laboratory UGM.

Research design

Sixty male Sprague Dawley rats that were 2.5-3 months old and weighed 250-300 g. All of the subjects was divided into 3 groups that received a different substituted material. Each group was divided into 5 subgroups after 3, 7, 14, 21, and 28 days.

Preparation of Stichopus hermanni gelatin

Stichopus hermanni gelatin extracted from *Stichopus hermanni* using chloroform and methanol in a ratio 1:1. According to the previous research (23)

Preparation of local hydroxyapatite

A gypsum powder (20 g) was mixed with 800 mL of 1 M diammonium hydrogen phosphate solution. It was then heated at 100°C for 20 min using a microwave digestion system.

Surgery

Ketalar (8 mg/100 g) was administered to the rats as anesthesia. The skin on the condyles of each femur of Sprague Dawley rats were opened with longitudinal cut. The fissure drill was used to generate a bone defect with a size of (3x3x2) mm^3 at LPPT UGM.

Bone substitution

After anesthesia was applied, the bone defects in group I rats were filled with a mixture of 65% local hydroxyapatite and 35% *Stichopus hermanni* gelatin. Group II bone defects were filled with *Stichopus hermanni* gelatin. Lastly, group III bone defects were filled with gelatin. Three rats in each group were sacrificed on days 3, 7, 14, 21, and 28.

Preparation of histology slides

Histology slides were prepared from the condyles of rat femurs that were substituted with *Stichopus hermanni* gelatin local hydroxyapatite, *Stichopus hermanni* gelatin, or gelatin. The histological sample was stained with Hematoxylin and Eosin staining. This performed at Veterinary Histopathology Laboratory, UGM.

Osteoblast counts

Using histological images, we determined the number of osteoblasts, which were identified by their cuboid shape and basophilic cytoplasm. Osteoblast counts were performed using a trinocculer microscope with a 400x zoom camera and obtained from 5 different fields. Each field has a diameter of 0.65 µm and an area of 1.99 $µm^2$. The statistically analyzed by a two-way ANOVA if they fulfilled the requirements of normality and homogeneity. This was followed by an LSD test.

Measurement of fibroblast viability

The in vitro fibroblast experiments were conducted at the Laboratory of LPPT UGM. Fibroblasts were the subject of this research.

Study design

The treatment group was divided into seven subgroups that received different concentrations of a *Stichopus hermanni* gelatin and local hydroxyapatite mixture. The concentrations were 0.015 mg/mL, 0.031 mg/mL, 0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL, and 1.000 mg/mL. The control group received growth medium only. There were 6 technical replicates for each concentration.

Materials

Stichopus hermanni gelatin and local hydroxyapatite, fibroblasts (obtained from LPPT UGM), phosphate buffer solution (Gibco, USA), M199 growth medium (Gibco, USA), trypsin (Sigma, USA), and MTT salt (Sigma, USA).

Research tools

Multi-well microplates (96 wells), inverted microscope, laminar air flow hood, CO_2 incubator (Jovan IG-150), cell counter, centrifuge, and micropipettes.

Course of Research

The viability cell test

Fibroblast cell suspensions of 20,000 cells/mL were prepared. Prepare fibroblast cell suspension 20,000 cells/ mL medium in a petri containing 96 wells micro-pitting. Each well was filled with 100 mL of the cell suspension. There was 6 replicated wells for each of the 7 different concentrations and the control. The concentrations were 0.015 mg/mL, 0.031 mg/mL, 0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL, and 1.000 mg/mL. The cultures were grown for 24 h in a 5% CO_2 incubator.

After the cells had attached to the plate, the old medium was replaced with the new medium containing *Stichopus hermanni* gelatin and local hydroxyapatite. Cells were washed with PBS, and each well was filled with 100 mL of medium containing 7 different concentrations of *Stichopus hermanni* gelatin and local hydroxyapatite. The concentrations used were 0.015 mg/mL, 0.031 mg/mL, 0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL, and 1.000 mg/mL. The wells in the control group were filled with 100 mL of medium without *Stichopus hermanni* gelatin or local hydroxyapatite. Each concentration was repeated 6 times. The cultures were grown in an incubator at 37 C with 5% CO_2 for 24 h. Measurements were obtained from each well by adding MTT tetrazolium salt and determining the number of live fibroblasts. An ELISA plate reader was used to measure the absorbance at 550 nm to determine the cell viability of fibroblasts.

To calculate cell death, we used the formula of Hussein, 1993 (21). The average number of live cells in treatment wells was compared with that of the control group.

$$Mortality\ percentage\ (a) = \frac{ODK - ODP}{ODP} \times 100\%$$

ODK: Optical Density Control Absorbance
ODP: Optical Density Absorbance Treatment

The percentage of living cells = 100% - a

Indicator the living cells was used the formula which is 50% cells death, such as the lethal dose (LD_{50}) in vivo studies. The death cells by 50% compared with the average number of live cells in the control group a large number of living cells is considered 100% (22). Using an ELISA plate reader (wavelength of 550 nm), the average number of living cells in the *Stichopus hermanni* gelatin local hydroxyapatite treatment groups were compared with the control group, and the data were expressed as percentages.

RESULTS

In the *Stichopus hermanni* gelatin local hydroxyapatite group, osteoblasts were visible after the 3rd day (Table I). The number of osteoblasts kept increasing until day 14. It was observed from day 3 until day 14. The average highest number of osteoblasts was found on day 14. The same results occurred in the *Stichopus hermanni* gelatin and gelatin groups.

The two-way ANOVA test result indicated that there is a significant difference in the osteoblast number between group I, group II and group III ($p < 0.05$). There were a significant difference in the osteoblast number between groups on days ($p<0.05$). Furthermore, there was a significant difference in the osteoblast number on the interaction groups ($p < 0.05$). Analysis of LSD test results showed that there was a significant difference in the osteoblast number in all groups ($p<0.05$).

Table I: Mean and standard deviation of osteoblasts numbers in 5 visual fields (1.99/μm²)

Time Substitution	3 days	7 days	14 days	21 days	28 days
Stichopus hermanni gelatin-local hydroxyapatite	9.5400* ± 0.8076	12.9800* ± 0.5342	35.6500* ± 0.6215	32.3500* ± 0.2755	28.2400* ± 0.1207
Stichopus hermanni gelatin	8.1600* ± 0.7232	11.7200* ± 0.5145	33.1600* ± 0.1286	29.9400* ± 0.7586	24.5400* ± 0.7502
Gelatin	6.3900* ± 0.2724	10.1600* ± 1.5616	30.9650* ± 0.4132	26.4400* ± 0.5715	21.1400* ± 0.5191

*: Significant difference ($p<0.05$)

The observation of *Stichopus hermanni* gelatin and local hydroxyapatite-treated fibroblast cultures demonstrated that 24 h after treatment the growth of fibroblasts and cell attachment had occurred. In fact, the cells were very confluent. An ELISA plate reader was used to measure MTT absorbance as an indication of living cells. The average scores of cell viability after 24 h treatment are shown in Table II.

In the 0.250 mg/mL group, the average scores in the living cell were higher than the controls. The 0.0625 mg/mL group had the highest number on living cell compared with the lower dose treatment groups. These results demonstrate that gelatin does not reduce cell viability in fibroblasts and promotes the growth of fibroblasts.

The cell death percentage determined using the Hussein Formula showed that the percentage was less than 50%

Table II: Average and standard deviation of the viability of fibroblasts after 24 h

Control mg/mL	1.00 mg/mL	0.50 mg/mL	0.250 mg/mL	0.125 mg/mL	0.0625 mg/mL	0.031 mg/mL	0.015 mg/mL
1.548 ± 0.0030	1.294 ± 0.0090	1.368 ± 0.0054	1.529 ± 0.0092	1.595 ± 0.0090	1.699 ± 0.0043	1.637 ± 0.0020	1.586 ± 0.0026
(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)	(n=10)

at a dose of 1.000 mg/mL group and 0.500 mg/mL group, whereas at the lower doses, the percentage was less than 0% or was negative. The 24 h treatment with *Stichopus hermanni* gelatin and local hydroxyapatite did not cause cytotoxicity effects on fibroblasts and promoted the growth of more fibroblasts.

The analysis of one-way ANOVA result showed there was a significant difference in cell viability after 24 h of treatment between the 0.015 mg/mL, 0.031 mg/mL, 0.062 mg/mL, 0.125 mg/mL, 0.250 mg/mL, 0.500 mg/mL, 1.000 mg/mL, and control groups ($p < 0.05$). The analysis result of the t-test demonstated that there were significant differences between group dose ($p< 0.05$).

DISCUSSION

The average number of osteoblasts was highest on day 14 in the groups. The analysis of two-way ANOVA and LSD analysis demonstrated that there was a significant difference between group I, group II and Group III, days, and interactions ($p<0.05$). The number of osteoblasts in the *Stichopus hermanni* local hydroxyapatite group was higher compared with other groups. It could promote osteoblast formation because *Stichopus hermanni* gelatin had type 1 collagen, it was higher than another group. Osteoid formation promoted by osteoblasts involves synthesized collagen. The organic mineral phase deposition of the new bone tissue was calcified. Fibroblast chemotaxis on the bone substitute surface promotes cell communication, proliferation, differentiation, and extracellular matrix formation (17, 18). The bone remodeling was based on the increased osteoblast number and activity of osteoblasts on day 7 and day 14. This result was likely caused by increased cell bonding, cell proliferation, and cell differentiation following inflammation in responsed againts to the wound, occured in first week (19). In the previous study demonstrated that similar result on the increased number of osteoblasts on day 7 and day 14 (20, 21). Osteoblasts produced collagen I type on day 14. Osteoblast number significantly increased on day 14 (22).

The results showed that osteoblast cell number was higher in the *Stichopus hermanni* gelatin local hydroxyapatite group. This result indicated that the high levels of type I collagen fibers produced in the *Stichopus hermanni* gelatin local hydroxyapatite mixture promotes more osteoblast formation. The *Stichopus hermanni* gelatin extracts contained higher collagen fiber as compared with other groups. Osteoblast required amino acid from the biodegradation process of collagen fibers to synthesize collagen for bone basic material (17, 22). In the calcification level, the calcium salt was needed for calsification material. It could be taken from hydroxyapatite to harden the new bone (23, 24). *Stichopus hermanni* gelatin extract contain treatment 74% gelatin and amino acids to synthesize collagen fibers. Gelatin had increased osteoblasts number in bone remodeling (23). The hydroxyapatite chicken scratch collagen could increase osteoblast and osteoclast number in bone remodeling (21). Gelatin had high biodegradability and biocompatibility, and it could promote cell proliferation, migration, and osteogenic differentiation (25, 26).

The toxicity test resulted using the MTT method in fibroblast cultures after 24 h of incubation with different doses of *Stichopus hermanni*-local hydroxyapatite showed that the materials increased the growth of fibroblasts. At 24 h following incubation, the percentage of cell death was under 50% in all groups.

The percentage of cell death in the 1.000 mg/mL, 0.500 mg/mL, and 0.250 mg/mL group was 15.52%, 11.86%, and 1.86%, respectively. In addition, the percentage of cell death was below 0% or negative in cells treated with 0.125 mg/mL and lower doses. Therefore, 24 treatment did not cause toxicity in fibroblast cell cultures. The results indicated that the toxicity limit of a substance in the cell culture was 100 µg/mL, which means a dose of 100 µg/ml did not cause death in 50% or more of cells (LD_{50}), and the substance was considered nontoxic (27). In the fibroblasts that were treated with the highest dose of 1 mg/mL, 15.52% showed no cell death of more than 50%, therefore the *Stichopus hermanni* gelatin local hydroxyapatite mixture did not cause toxic effects.
The analysis result of one-way ANOVA and t-test demonstrated that there was a significant difference in the MTT absorbance in the different concentration groups of *Stichopus hermanni* gelatin local hydroxyapatite. The results of this study indicated that fibroblasts could actively proliferative. The inside of living cells was where the reduction of the MTT salt occurs (28).

The calculations based on the percentage of cell death

formula showed that the percentage of cell death was less than 50% and even less than 0% or negative, therefore the 24 h treatment with collagen fibers had no toxic effects on cultured fibroblasts and promoted cell proliferation (27). In this research, the percentage of fibroblast cell death was low, and this was due to *Stichopus hermanni* gelatin and local hydroxyapatite materials did not cause cytotoxicity effects on fibroblasts and promoted the growth of more fibroblasts.

CONCLUSION

The *Stichopus hermanni* gelatin and local hydroxyapatite as bone graft substitute increased osteoblast number compared with either *Stichopus hermanni* gelatin or gelatin. The *Stichopus hermanni* gelatin and local hydroxyapatite as bone graft substitute did not cause cytotoxicity on fibroblasts.

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