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The characteristics of swelling and biodegradation tests of bovine amniotic membrane-hydroxyapatite biocomposite

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ABSTRACT

Background: *A good biocomposite is a structure that can provide opportunities for cells to adhere, proliferate, and differentiate. It is affected by the characteristics of a material. As bone tissue regeneration occurs, biomaterials must have a high swelling ability and low biodegradability. The high swelling capability will have a larger surface area that can support maximal cell attachment and proliferation on the biocomposite surface, which accelerates the regeneration process of bone defects.* ***Purpose:*** *The study aimed to analyze the characteristics of swelling and biodegradation of bovine amniotic membrane-hydroxyapatite (BAM-HA) biocomposite with various ratios.* ***Methods:*** *The BAM-HA biocomposite with a ratio of 30:70, 35:65, and 40:60 (w/w) was synthesized using a freeze-dry method. The swelling test was done by measuring the initial weight and final weight after being soaked in phosphate-buffered saline for 24 hours and the biodegradation test was done by measuring the initial weight and final weight after being soaked in simulated body fluid for seven days.* ***Results:*** *The swelling percentage of BAM-HA biocomposite at each ratio of 30:70, 35:65, and 40:60 (w/w) was 303.90%, 477.94%, and 574.19%. The biodegradation percentage of BAM-HA biocomposite at each ratio of 30:70, 35:65, and 40:60 was 9.43%, 11.05%, and 12.02%.* ***Conclusion:*** *The BAM-HA biocomposite with a ratio of 40:60 (w/w) has the highest swelling percentage while the 30:70 (w/w) ratio has the lowest percentage of biodegradation.*

Keywords: *biocomposite; biodegradation; bovine amniotic membrane; hydroxyapatite; socket preservation; swelling*
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INTRODUCTION

The desired outcomes of biomaterials related to tissue engineering for clinical applications should include the attraction of natural resident stem cells to the site of injury and the suppression of inflammation, reduction of scar formation, enhancement of vascularization, and prevention of infection. The amniotic membrane is recommended for usage as an ideal biomaterial for wound healing due to its protein, cytokine, and growth factor concentration. The amniotic membrane can be used as a single or even double sheet to cover wounds on the body's exposed outer surfaces, such as the skin and cornea.[1]

The amniotic membrane has many different applications in medicine, but it is particularly useful for treating skin burns and preventing tissue adhesion during head, neck, abdomen, larynx, and genitourinary tract surgeries. The amniotic membrane can also serve as a barrier that protects internal organs.[2] For instance, wrapping the peritoneal cavity, tendon, spinal cord, and peripheral nerves in the amniotic membrane could prevent adhesion and minimize the formation of scars.[1]

Amniotic membrane is a biomaterial used widely in tissue regeneration because it has anti-bacterial and anti-inflammatory properties.[3] Amniotic membrane has growth factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), tissue inhibitor metalloproteinase (TIMP), transforming growth factor-β (TGF-β), and platelet-derived growth factor (PDGF).[4] One source of the amniotic

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membrane is bovine. The bovine amniotic membrane has some benefits over the human amniotic membrane, such as being easier to legalize, being considered more ethical, and not being impacted by religious rituals.[5]

The limitation of the amniotic membrane is its low mechanical properties and rapid degradation, which makes it easy to decompose and difficult to maintain the structural integrity required for bone regeneration.[6] The amniotic membrane is also easily torn by this feature when it is applied. The integrity of the structure, which is vital when mending extraction wounds with long-term treatment, will be impacted by how easily the amniotic membrane degrades. Modifying the amniotic membrane is needed to find a solution to this issue.[7] The amniotic membrane in combination with a bone graft will produce biocomposites that have the potential to support bone formation and provide better outcomes.[8]

One of the bone graft materials that is often used to support the bone healing process is hydroxyapatite.[9] Hydroxyapatite is a bioceramic material with a mineral composition similar to bones and teeth.[10,11] Hydroxyapatite has been used widely as a biomaterial for bone tissue replacement and repair because of its high osteoconductivity, non-toxicity, and good biocompatibility.[12] Hydroxyapatite can be obtained from bovine bone (bovine hydroxyapatite).[13] Bovine bone material consists of 93% hydroxyapatite and 7% β-*tricalcium phosphate* ($Ca_3(PO_4)_2$, β-TCP).[14] Bovine hydroxyapatite (BHA) has the same chemical composition as human bone, which affects bone metabolism.[15]

A combination of bovine amniotic membrane and hydroxyapatite will produce a new material in the form of a sponge-shaped biocomposite. The bovine amnion-hydroxyapatite membrane was expected to preserve the socket after tooth extraction. The characteristic properties of a biomaterial are important for successful tissue regeneration and must be able to adapt to the tissue that will be replaced.[16] A good biocomposite is a structure that can provide opportunities for cells to adhere, proliferate, and differentiate. It is affected by the characteristics of a material.[17]

The swelling test is a method used to determine the capacity of the material to absorb liquid. The swelling properties of the biomaterial have an impact on good cell proliferation. Higher swelling abilities increase the surface area of the biomaterial, thus facilitating cell attachment to the biomaterial.[18] With more cells attached, it is hoped that cell growth will also be faster. Swelling properties also play an important role in increasing the absorption of fluids from the body or media, as well as the transfer of nutrients and metabolic wastes.[19] The increase in the swelling ratio is caused by the presence of hydrophilic properties. Hydrophilic conditions are suitable for cell attachment and proliferation, therefore it accelerates the regeneration process.[20]

Biomaterials implanted in the body and in contact with biological systems can trigger a series of reactions between the biomaterials and host tissues. The ability of biodegradation is an important role in biomaterials' formation of new tissues because of its properties that can affect cell viability and proliferation. The biodegradation test is a parameter needed to see the time required for biomaterial to be degraded according to the formation of new tissue. The biodegradation test can indicate the biodegradability of a material.[21]

Biomaterials that have been implemented in the body must be able to maintain sufficient mechanical properties and structural integrity so that cell adaptation goes well and can store their extracellular matrix. Biomaterials that have biodegradable properties are expected to be able to create space for new bone tissue to grow.[22] Based on this, the study aimed to analyze the characteristics of bovine amniotic membrane-hydroxyapatite (BAM-HA) biocomposite through a swelling and biodegradation test.

MATERIALS AND METHODS

Ethical approval, which is managed as a condition for conducting research, has been obtained from the Research Ethics Commission of the Faculty of Medicine, Universitas Airlangga Surabaya (No. 400/HRECC.FODM/VII/2021). This research is an experimental laboratory with a post-test-only control group design. The data used primary data, which was directly collected by the researcher.

The manufacture of BAM-HA biocomposite was done at the Biomaterial Center Installation of the Tissue Bank Hospital by Dr. Soetomo Surabaya. The dry amniotic membrane was prepared at 3 grams (30:70 ratio), 3.5 grams (ratio 35:65), and 4 grams (ratio 40:60). The amniotic membrane was cut into pieces of about 2 cm and added with 40 ml of 0.9% NaCl. Then, the amniotic membrane with NaCl was soaked for 5 minutes until the liquid was absorbed. The amniotic membrane was blended for 10 minutes to produce a smooth amniotic slurry. The amniotic slurry was added with 7 grams of hydroxyapatite powder (30:70 ratio), 6.5 grams of hydroxyapatite powder (ratio 35:65), and 6 grams of hydroxyapatite powder (ratio 40:60), then stirred until homogeneous and put into a petri dish with a diameter of 10 cm. The petri dish was put into the freezer (Thermo, USA) at -80^{o}C for 24 hours, then freeze-dried (VirTis BenchTop^{TM} "K" Series) for 24 hours at -100^{o}C (Figure 1). Sponge-shaped biocomposite with a diameter of 10 cm was cut into pieces of 1.5x1.5 cm using a scalpel with a no. 15 blade and handle scalpel no. 3 (Figure 2).

The biocomposite is in the form of a sponge with a sizing of 1.5x1.5 cm and various ratios weighed as the initial weight (Wi). The biocomposite was immersed in 10 ml of Phosphate Buffer Saline (PBS) solution at 37^{o}C for 24 hours. After that, the biocomposite was taken and then drained using Whatman filter paper for 3 seconds, and the final weight (Wf) was determined. The swelling ratio was calculated using the formula:

$$Swelling\ test\ (\%) = \frac{Wf - Wi}{Wi} x\ 100$$

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The biodegradation test was performed using a biocomposite is in the form of a sponge with a sizing of 1.5x1.5 cm and various ratios weighed as the Wi. The biocomposite was immersed in Simulated Body Fluid (SBF) solution at 37°C for seven days. After seven days, the biocomposite was taken and then dried using a freeze-drier for 48 hours at a temperature of -100°C. After that, the biocomposite was weighed again to determine the Wf. The biodegradation of biocomposites was calculated using the formula:

$$Biodegradation\ test\ (\%) = \frac{Wi-Wf}{Wf} x100$$

RESULTS

The average value of the swelling level of the BAM-HA biocomposite (Table 1) increased after immersion for 24 hours. The BAM-HA biocomposite with a ratio of 40:60 (w/w) obtained an average swelling rate of 574.19%, which was the highest rate compared to the ratios of 35:65 and 30:70 (w/w), which were 477.94% and 303.90%, respectively.

Statistical analysis of variance (one-way Welch ANOVA) is one of the parametric statistical tests to

Figure 1. The results of making BAM-HA ratios of 30:70 (A), 35:65 (B), and 40:60 (C).

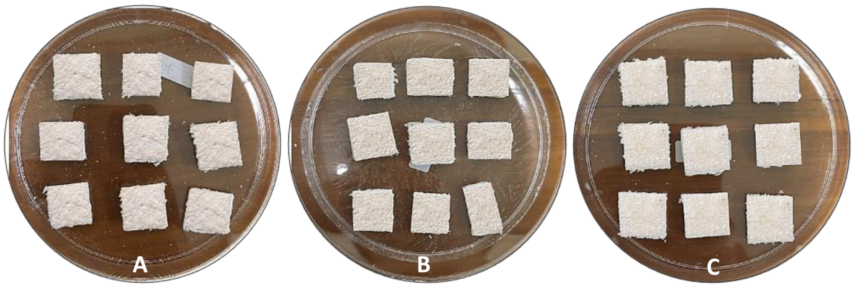

Figure 2. The results of the preparation of BAM-HA biocomposite ratios of 30:70 (A), 35:65 (B), and 40:60 (C).

Table 1. The average value of the swelling test

Biocomposite	Ratio	Swelling ratio (%)	
		Mean	SD
Bovine amniotic membrane-hydroxyapatite	30:70 (Control)	303.90	10.75
	35:65	477.94	10.90
	40:60	574.19	35.44

Table 2. The results of one-way Welch ANOVA statistical analysis on the swelling test

Robust tests of equality of means Swelling test	Statistic	df1	df2	Sig.
Welch	745.760	2	16.455	.000

Table 3. Post-hoc Tukey HSD statistical test results of swelling percentage

Ratio	30:70	35:65	40:60
30:70		0.000*	0.000*
35:65			0.000*
40:60			

*Significant (p < 0.05)

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determine differences between groups of data as a whole. The results of the one-way Welch ANOVA test showed a significance value of p = 0.000, which means that there was a significant difference between groups with a p-value of less than ($p < 0.05$) (Table 2). Therefore, further testing was carried out using the post-hoc Tukey Honestly Significant Difference (HSD) to find out which groups had significant differences. The post-hoc Tukey HSD test has been summarized and presented in Table 3.

There was a significant difference in the average level of swelling between the BAM-HA biocomposite ratio of 30:70 with a ratio of 35:65 (w/w) (p = 0.000), BAM-HA biocomposite ratio of 30:70 with a ratio of 40:60 (w/w) (p = 0.000), and the BAM-HA biocomposite ratio of 35:65 with a ratio of 40:60 (w/w) (p = 0.000) ($p < 0.05$).

The average value of the biodegradation rate of BAM-HA biocomposite in various ratios is shown in Table 4. The BAM-HA biocomposite 40:60 (w/w) ratio obtained the highest average value of the biodegradation rate at 12.02%, followed by 11.05% at 35:65 (w/w), and 9.54% at 30:70 (w/w).

The results of the one-way Welch ANOVA test showed a significance value of p = 0.000, which means that there was a significant difference between groups with a p-value of less than 0.05 (Table 5). Therefore, the post-hoc Tukey HSD analysis test was continued to find out which groups had significant differences. The post-hoc Tukey HSD test has been summarized and presented in Table 6.

Data analysis was performed using the post-hoc Tukey HSD test. It showed that there was a significant difference in the mean level of biodegradation between the BAM-HA biocomposite group with a ratio of 30:70 and a comparison ratio of 35:65 (w/w) (p = 0.000) and BAM-HA biocomposite with a ratio of 30:70 and a comparison ratio of 40:60 (w/w) (p = 0.000).

DISCUSSION

The selection of biomaterials for making bone tissue regeneration material is a significant matter in the socket preservation procedure. The requirements for biomaterials in bone tissue regeneration, include having a porous structure that promotes cell proliferation and distribution so that it can support the formation of new bone tissue. Additionally, it can provide a temporary structure that will degrade over time with the formation of new tissue.[23,24]

Socket preservation biomaterial candidates used in this study were obtained by synthesizing bovine amniotic membrane biomaterials and BHA with a combined ratio of 30:70, 35:65, and 40:60 (w/w) using the freeze-drying method. The freeze-drying process can form a porous structure, which can affect the outer surface of the biocomposite to become hydrophilic.[25,26]

The characteristic properties of a biomaterial are important for the success of tissue regeneration and must be able to adapt to the tissue to be replaced.[16] Swelling is one of the important properties in the application of biomaterials in bone tissue regeneration. Swelling in biomaterials can play a role in cell infiltration into biomaterials.[27] The results of the swelling test showed that the average swelling ratio was increasing from a ratio of 30:70 (w/w) of 303.90±10.75%, 35:65 (w/w) of 477.94±10.90%, 40:60 (w/w) of 574.19±35.44%, and after the one-way ANOVA test, showed that there was a significant difference between groups with a p-value of less than 0.05.

This study showed that the BAM-HA biocomposite with the larger bovine amniotic membrane ratio of 40:60 (w/w) obtained the highest percentage of swelling results compared to the 35:65 and 30:70 (w/w) ratios. That is because the bovine amniotic membrane biomaterial in this study contains a polymer in the form of collagen.[28]

Table 4. The average value of the swelling test

Biocomposite	Ratio	Biodegradation ratio (%)	
		Mean	SD
Bovine amniotic membrane-hydroxyapatite	30:70 (Control)	9.54	1.43
	35:65	11.05	1.42
	40:60	12.02	0.48

Table 5. The results of one-way Welch ANOVA statistical analysis on the biodegradation test

Robust Tests of Equality of Means				
Biodegradation test	Statistic	df1	df2	Sig.
Welch	14.048	2	14.307	.000

Table 6. Post-hoc Tukey HSD statistical test results of biodegradation percentage

Ratio	30:70	35:65	40:60
30:70		0.024*	0.000*
35:65			0.180
40:60			

*Significant ($p < 0.05$)

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These findings are appropriate with several findings from previous research that demonstrates collagen is one of the polymers with a high-water absorption capacity. A high amount of collagen will increase the water absorption properties of the biomaterial. Collagen is a polymer with many hydrophilic groups, which can form hydrogen bonds with the surrounding solution. The low contact angle of biomaterials with hydrophilic characteristics can result in low surface tension. If the surface tension of the liquid is low, the surface of the biocomposite material will contact it more easily. As a result, the biocomposite can absorb liquids more readily.[29]

Several other studies have shown that the increase in the percentage of swelling is directly proportional to the increase in the amount of gelatin, which is widely known as a biomaterial that has hydrophilic properties, while the increase in the amount of hydroxyapatite is inversely proportional to the percentage of swelling. The higher hydroxyapatite ratio in the biocomposite caused a decrease in the percentage of swelling and its swelling ability.[19,30] By attaching calcium and phosphate to the hydrophilic groups of COOH or NH_2, hydroxyapatite forms a cross-link between polymer chains and reduces the biomaterial's hydrophilicity.[31]

Another property that plays a role in the success of bone tissue regeneration is biodegradability. Biodegradable biomaterials were expected to provide a space for the growth of new bone tissue.[22] According to the data, the BAM-HA biocomposite ratio of 40:60 (w/w) had the highest average value of biodegradation at 12.020±0.483%, followed by 11.045±1.415% at a ratio of 35:65 (w/w). Meanwhile, the BAM-HA biocomposite with a ratio of 30:70 (w/w) had the lowest percentage of biodegradation, which was 9.541±1.428%. The results of the one-way Welch ANOVA test showed a value of significance of p = 0.000, which means that there is a significant difference between groups with a p-value of less than 0.05. The lowest percentage of a biodegradation rate was obtained at a 30:70 (w/w) ratio, with the highest composition hydroxyapatite ratio. That is because hydroxyapatite can reduce the pore size of the biocomposite.[30] As a result, the lowest percentage level was attained at the 30:70 (w/w) ratio, which had a higher hydroxyapatite content than the 40:60 and 35:65 (w/w) ratios. Meanwhile, the average percentage of degradation rate was high for the 40:60 (w/w) ratio because it contained less hydroxyapatite and more bovine amniotic membrane than the ratios of 35:65 and 30:70 (w/w).

The inclusion of the hydroxyapatite ratio can result in a reduction in porosity size. Therefore, it may have an impact on a biocomposite's ability to access liquids. When the biomaterial immerses in the SBF solution, the solution forms a link with the biomaterial's surface. By capillary action, the fluid will progressively seep into the biomaterial. During this phase, the pore wall will serve as a new connection between the liquid and the biomaterial. A decrease in porosity size will result in a smaller surface area. As a result, liquid accessibility reduces, which results in a slower rate of biodegradation.[32] The level of biomaterial degradation is influenced by chemical properties, polymer composition, and environmental conditions.[18]

This study seeks to identify the appropriate combination ratio between the bovine amniotic membrane and hydroxyapatite and develop a bone biocomposite that produces swelling and biodegradation properties suitable for bone tissue regeneration. As bone tissue regeneration occurs, biomaterials must have a high swelling ability and low biodegradability. The high swelling capability will have a larger surface area that can support maximal cell attachment and proliferation on the biocomposite surface, which accelerates the regeneration process of bone defects.[27] Biocomposite can be used as bone graft material, preferably having a low level of biodegradation for bone regeneration to occur.[26]

The results show that the percentage of swelling increased with the concentrations of bovine amniotic membrane increasing and the concentrations of hydroxyapatite decreasing. Additionally, the percentage of biodegradation decreased with the concentrations of hydroxyapatite increasing and the concentrations of bovine amniotic membrane decreasing. The BAM-HA biocomposite with a ratio of 40:60 (w/w) has high swelling and biodegradation ability, while the ratio of 30:70 (w/w) has low swelling and biodegradation ability.

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