ORIGINAL ARTICLE

The effect of application of bovine amniotic membrane on osteoblasts, osteocytes, and collagen in the post-extraction alveolar bone socket of Sprague Dawley rats

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ABSTRACT

Introduction: Tooth extraction can result in the loss of alveolar bone. The application of biomaterials such as Bovine Amniotic Membrane (BAM) that contain growth factors and collagen is expected to play a role in socket preservation. This study aimed to analyze the effect of BAM application on the growth of osteoblasts, osteocytes, and collagen in the histological slide of the alveolar bone socket of Sprague Dawley (SD) rats after tooth extraction. **Methods**: This research type was an experimental laboratory, with 16 male SD rats used in this group. The mandibles' central incisive teeth were extracted from the SD rats. Afterward, it was divided into two groups, specifically the control group (without treatment) and the treatment group (BAM application). After extraction, BAM with a size of 1.5 mm by 10 mm was applied to the alveolar bone socket in the treatment group. On the 14th and 28th days, the rats were euthanized and the alveolar bone sockets of mandibular tissue were taken and stained with Hematoxylin Eosin (HE) and Masson Trichrome (MT) to calculate the proliferation of osteoblasts, osteocyte cells, and collagen thickness. **Results**: According to the findings of all groups, the number of osteoblasts and collagen thickness were higher on the 14th day compared to the 28th, while osteocytes continued to increase from the 14th to 28th. The thickness of collagen, osteoblasts, and osteocytes in groups with BAM application was higher than without application. Statistical results showed that there were significant differences between groups ($p < 0.05$). **Conclusion**: The application of BAM can act as a material preservation socket. This biomaterial can accelerate recovery by increasing collagen thickness, osteoblasts, and osteocytes.

KEYWORDS

bovine amniotic membrane, collagen, osteoblast, osteocyte, socket preservation

INTRODUCTION

Alveolar bone resorption that occurs after tooth extraction results in damage to the periodontium and the loss of bone bundles, which will change the size and shape of the bones. Alveolar bone will decrease in dimensions due to resorption on the bone surface.[1] Several studies have shown that during the first three months of wound healing, alveolar bone resorption will occur in the range of 40-60% vertically and horizontally. An adequate alveolar bone is necessary for the success of denture restorations. Conversely, massive alveolar bone loss can complicate restorative procedures.[2] Therefore, the application of biomaterials for alveolar bone preservation is essential to reduce bone resorption and maintain adequate tissue. The application of this biomaterial can also stimulate healing or the formation of new bone in the alveolar bone socket. [3,4]

Bovine Amniotic Membrane (BAM) is rich in growth factors and collagen. BAM has the potential to stimulate tissue regeneration, thereby accelerating the re-epithelialization process and wound healing. BAM could accelerate the formation of collagen, which plays a role in the wound healing process.[5] BAM also has a role related to bone re-formation with osteoinductive properties. When bone regeneration occurs, the process occurs gradually and is characterized by cell migration, multiplication, proliferation, and differentiation. It involves several types of cells, including osteoblasts and osteocytes. Osteoblast expression in the process of osteogenesis can be stimulated by BAM.

Thus, osteoblast cells stimulated by BAM will mature and turn into osteocyte cells.[6,7] Related to the application of BAM in medical life, amniotic membrane can be used for burns, bone surgery, and ophthalmology in the form of hydrogel or eye drops because this material meets most of the criteria and works efficiently for the wound healing process.[8]

Wound healing after a tooth extraction is a process of replacement and repair of damaged tissue functions. This process involves the migration and proliferation of mesenchymal stem cells, collagen synthesis, and bone formation. The role of collagen in promoting the process of fibroplasia and helping the process of hemostasis is needed to restore the structure and function of tissue anatomy.[9] Furthermore, osteoblast cells are necessary for the process of bone formation. These cells directly contribute to osteogenesis.[10] In this process, osteoblasts in areas of bone destruction will express osteoids and produce new bone.[11] Osteoblasts produce a matrix that covers the older bone surface, resulting in the development of new layers of bone. The bone matrix then surrounds the osteoblast cells, which grow into osteocytes, mature bone cells.[12] Osteocytes play a role in bone remodeling by maintaining the integrity and vitality of new bone.[13]

Osteoblast proliferation in osteogenesis can affect the alveolar bone socket wound healing process after tooth extraction. On the 14th day, the proliferative phase occurs, and bone remodeling is ongoing on the 28th day. On the 14th day after tooth extraction, collagen is formed, followed by increased growth factors to stimulate osteoblast cell activity. The osteoblast volume density also shows a significant increase on the 14th day. Meanwhile, a study showed that the number of osteoblasts in the bone remodeling process did not significantly change on the 28th day. On the 28th day, osteoblast cells begin to be replaced with mature osteoblast cells, and an increase in bone density is seen. Similarly, collagen also seems to decrease after the 14th day and will continue to decrease until the 28th day.[14,15]

Histological analysis can be used as an option for examining a tissue.[16] This method can be used to observe bone microarchitecture, showing the mineral components, bone cells, and physiological processes of bone formation and resorption.[10] The histological analysis will be carried out on alveolar bone socket preparations of SD rats by staining with HE for osteoblasts and osteocytes and MT for collagen.[17] Research shows that the anterior part of the mandible has the densest bone, so this area provides more bone cells for osteogenesis. Therefore, the application of BAM is expected to stimulate and induce bone formation and increase osteoblast activity.[18] Accordingly, this study aimed to analyze the effect of BAM application on the growth of osteoblasts, osteocytes, and collagen in the histological slide of the alveolar bone socket of Sprague Dawley rats (SD) after tooth extraction.

METHODS

The fabrication of biocomposite BAM started with BAM preparations. The process was carried out at the Installation Center for Biomaterials Bank Network at RSUD Dr. Soetomo. Fresh BAM comes from cows on farms in Malang, East Java. Fresh BAM was cleaned of blood clots and washed with a 0.05% saline solution four times for 10 minutes. After that, it was rewashed with distilled water until the saline solution was clean. The BAM was then blended with a 20- -mL saline solution until it was smooth and slurry. Following that, the BAM was placed in a petri dish container with a diameter of 10 cm. Then, it was put in the freezer at a temperature of -80°C for 24 hours. After that, freeze-drying took place for 24 hours at -100 °C. The resulting specimen was in the form of a sponge. The final stage was sterilization with 25 kGy gamma rays.[7]

This was experimental laboratory research that used 16 male SD rats. Each group consisted of 4 SD rats weighing 250–300 g, which were acclimatized for 7 days at the RSHP IPB Bogor. SD rats were given a portion of adequate food, water, ventilation, and lighting during the adjustment period. Tooth extraction was performed on the right mandibular central incisor and divided into two groups: the control group (without treatment) and the treatment group (with BAM application). Before the tooth extraction, experimental animals were previously anesthetized with ketamine (95 mg/kg) and xylazine (5 mg/kg). After the extraction, BAM was applied to the socket in the form of a sponge with a size of 1,5 mm x 10 mm, and sutures were performed on the socket wound post-extraction. On the 14th and 28th days, when the rats were euthanized, they were anesthetized using 95 mg/kg ketamine and 5 mg/kg xylazine. After the experimental animals died, the rats' blood was removed from the heart, the neck was relocated, the head was cut off, and the jaw was cut. The rats' blood was removed to ensure they were really dead. Jaw cutting was carried out longitudinally on the alveolar bone of the mandible in the anterior region.

The histological sample was cut at a size of 5 mm x 5 mm in each group. After that, the tissue was fixed in a 10% formalin buffer solution and decalcified with a20% citric acid solution for ± 3-4 days. Dehydration was then carried out with concentrated alcohol (70, 80, 90, and 100%), cleared with xylol, paraffin impregnation, embedding paraffin, and making paraffin blocks. After the paraffin block was cut with a microtome with a thickness of 5 µm, it was stained with HE and MT. After staining, slides were closed and labeled.

The slides were analyzed under a ZEISS AXIO SCOPE A.1 series microscope equipped with an AxioCam digital camera and using ZEN 3.4 software with 40 times magnification. Each histological preparation was observed in five areas. The number of cells was counted in five visual fields using Image J software. The inclusion criteria for these slides were that the specimen and staining slides were successfully carried out, the results of the microscopic picture of the MT and HE slides were clearly visible, and collagen, osteoblasts, and osteocytes could be identified properly. Osteoblast cells are cuboidal or columnar, while osteocyte cells appear to be trapped in lacunae within their own

matrix with HE staining.[19] In addition, collagen could be seen in blue and inelastic fibers with MT staining. Collagen slides were analyzed based on scoring criteria: 0 (no appearance of collagen fibers visible); 1 (collagen fibers look very thin or very little); 2 (collagen fibers look thin spread); 3 (collagen fibers look thick spread); 4 (collagen fibers look thick clumped).[20]

RESULTS

The data from this study were obtained from the application of BAM to the alveolar bone sockets of SD rats after extraction of the right mandibular central incisors. In this study, histological analysis was carried out on osteoblasts and osteocytes by staining with HE and measuring collagen thickness using MT. Observation and analysis were carried out after the rats were euthanized on days 14 and 28 by counting the number of osteoblasts, osteocytes, and collagen thickness in histological preparation. The results of the histological image can be seen in Figures 1 and 2.

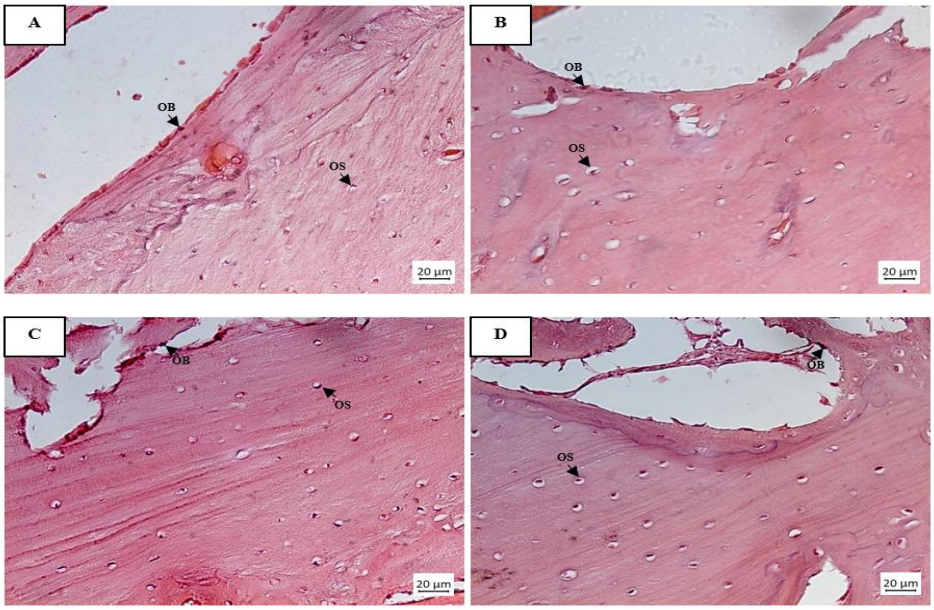

Figure 1. Histology of Osteoblasts and Osteocytes in the alveolar bone sockets of SD rats. (A) Group without treatment on day 14 (B) Group without treatment on day 28 (C) Group BAM on day 14th (D) Group BAM on day 28. HE staining, 400x magnification.

Figure 1 is a histological image of the 14th- day osteoblast cells in the control and BAM groups. Osteoblast cells are characterized along the alveolar bone sockets shown in Figures 1A and 1C (arrows OB). The histological image of the control or without treatment groups had a lower number of osteoblast cells (Fig. 1A) compared to BAM (Fig. 1C). The calculation of the mean value and standard deviation of the number of osteoblast cells is shown in Table 1. Statistical data using one way ANOVA post-hoc LSD showed a significant difference ($p < 0.05$) between the control group and the BAM groups (Table 1).

Table 1. Observation results of osteoblast cells in alveolar bone socket preparations of SD rats days 14 and 28.

Parameter	Mean±SD	
	Control Group	BAM Group
Osteoblasts day 14	8,87 ± 0,75*	17,53 ± 0,30*
Osteoblasts day 28	6,93 ± 0,50*	8,93 ± 2,36*

*Significantly different ($p < 0,05$)

The description of 14th-day osteocyte cell proliferation in the control and BAM groups can also be seen in Figures 1A and 1C. Osteocytes, characterized by cells located within the lacunae of the bone matrix, are shown in Figure 1A (OS arrows). Similar to osteoblast cells, the histology of the control group also showed a lower number of osteocyte cells compared to BAM. The mean values and standard deviation of osteocyte cells in the control and BAM groups, respectively, are shown in Table 2. Statistical results using one-way ANOVA post-hoc LSD also found significant differences ($p < 0.05$) in the control and the BAM groups (Table 2).

Table 2. Observation results of osteocyte cells in alveolar bone socket preparations of SD rats on days 14 and 28

Parameter	Mean±SD	
	Control Group	BAM Group
Osteocytes day 14th	8,43 ± 0,91*	13,17 ± 0,40*
Osteocytes day 28th	13,17 ± 0,40*	19,80 ± 2,07*

*Significantly different (p < 0,05)

On day 28, images of osteoblast cell proliferation in the control and BAM groups are shown in Figures 1B and 1D (arrows OB). Based on these figures, the control group (Figure 1B) showed a lower number of osteoblast cells compared to the BAM group (Figure 1D). Table 1 shows the mean value and standard deviation of osteoblast cells in the control and BAM groups, respectively. Statistical results using one-way ANOVA post-hoc LSD also found significant differences (p < 0.05) in the control group and the BAM group (Table 1).

On day 28, the proliferation of osteocyte cells in the control and BAM groups is shown in Figures 1B and 1D (arrows OS). Based on these figures, the control group still showed a lower number of osteocyte cells compared to the BAM group. The mean values and standard deviation of osteocyte cells are shown in Table 2. Statistical results using one-way ANOVA post-hoc LSD also found significant differences (p < 0.05) in the control group and the BAM group (Table 2).

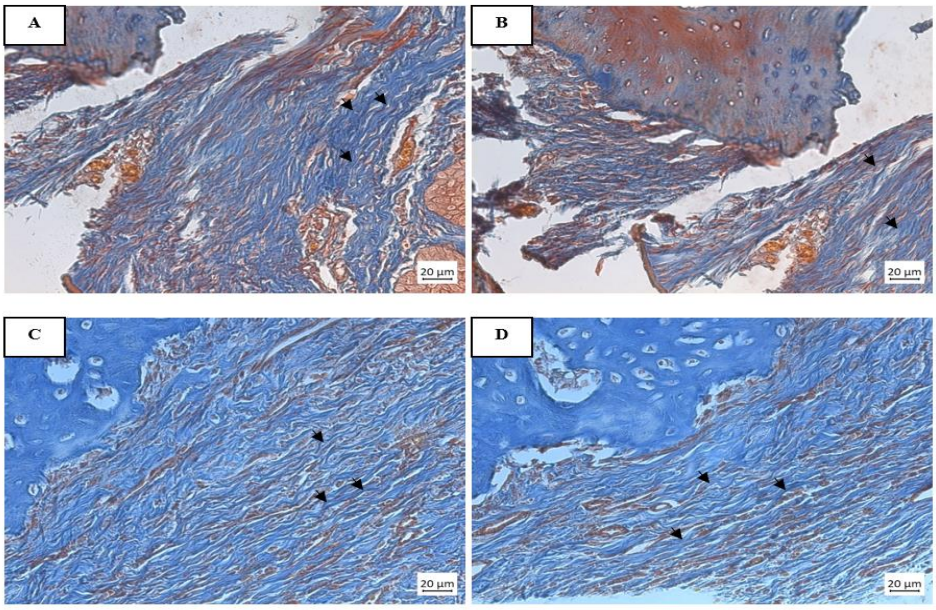

Figure 2. Histology of Collagen in the alveolar bone sockets of SD rats. (A) Group without treatment on day 14 (B) Group without treatment on day 28; (C) Group BAM on day 14; (D) Group BAM on day 28. MT staining, 400x magnification.

Figures 2A and 2C are histological images of collagen on the 14th day in the control and BAM groups. The histological image of the control group had the lowest collagen thickness (Fig. 2A) compared to BAM (Fig. 2C). Meanwhile, on the 28th day after tooth extraction in the alveolar bone socket, collagen synthesis in the control group and BAM is shown in Figures 2B and 2D. Based on these figures, the control group showed lower collagen thickness compared to BAM. The calculation of the mean value and standard deviation of collagen thickness in the control group and BAM, respectively, is presented in Table 3.

Table 3. Observation results of collagen in alveolar bone socket preparations of SD rats on days 14 and 28.

Parameter	Mean±SD	
	Control Group	BAM Group
Collagen day 14	2,07 ± 0,23*	3,13 ± 0,23*
Collagen day 28th	1,93 ± 0,12*	2,60 ± 0,20*

*Significantly different (p < 0,05)

Based on the results of this study, the proliferation of osteoblast cells was higher than osteocyte cells on the 14th day. On the 28th day, osteoblast cells decreased, while osteocyte cells increased. Statistical tests on both osteoblast and osteocyte cell counts still showed a significant difference in the control group and the BAM group, which can be seen in Tables 1 and 2. In a one-

way ANOVA analysis, it was found that there was interaction between groups of osteoblast cells and osteocytes.

In addition, statistical results using one-way ANOVA post-hoc LSD also found a significant difference ($p < 0.05$) in collagen thickness in the control group and the BAM group (Table 3). Based on the results of this study, both the control and BAM groups showed higher collagen synthesis on day 14 than on day 28. The collagen density statistical test showed a significant difference ($p < 0.05$) in the control group and the BAM group which can be seen in Table 3. In the One-Way ANOVA analysis, significant differences were also seen in both the treatment day group and the material application.

DISCUSSION

The biological process of wound healing after tooth extraction occurs through several phases, including hemostasis, inflammation, proliferation, and remodeling. After the bleeding in the socket wound stops, it will be replaced by granulation tissue. Granulation tissue is gradually replaced with a temporary connective tissue matrix rich in collagen fibers, and the proliferative phase of the wound healing process begins.[21] In addition, collagen is the main protein present in the bone matrix. COL1 is the most abundant collagen protein type, widely used as a bone-preserving component to induce tissue regeneration in damaged bone.[22] At the end of the inflammatory phase, macrophages release growth factors that cause osteoblast cells to activate.[21,23] Osteoblast cells are markers of alveolar bone healing by expressing bone-forming proteins.[24] These osteoblast cells appear during the proliferative phase and continue into the bone remodeling phase.[23,25] Over time, osteoblast cells will undergo a maturation process that can undergo apoptosis or differentiate osteocyte cells. Calculation of the number of osteoblast cells and osteocytes was carried out because these two cells are markers of osteogenesis. Osteoblasts and osteocytes must work in balance properly to remodeling process during osteogenesis, also collagen thickness was calculated because it is a parameters of the bone healing process.[26,27]

In this study, the histological appearance of collagen which is shown in Figure 2 appeared to reach its peak on the 14th day in each group. The increase in collagen thickness is explained by the calculation results in Table 3. This is in line with previous research, which said that the thickness of collagen would continue to increase from the 3rd day after tooth extraction to the 14th day with a denser collagen image.[28,14] However, collagen thickness decreased on the 28th day in each group. This decrease in collagen thickness occurs because collagen fibers begin to be replaced with bone matrix.[14] The results in Table 3 are in line with previous studies, which said that there was a rapid increase in the level of collagen thickness until the 21st day, and the thickness would continue to decrease until the 90th day.[14,29] The value in the control group was not too high because this group did not get any medication to support the healing process. However, it remained a normal inflammatory phase, resulting in low collagen stimulation.[28]

On the 14th day, osteoblast cells showed a higher average number in each group compared to the 28th day. This shows that on the Figures 1A and 1C, on the 14th day the bone healing process was dominated by osteoblast cells activity.[9] In Table 1, the number of osteoblast cells on day 14, as described in this study, is consistent with previous studies which stated that alveolar bone healing increased from day 7 to day 14 after tooth extraction. The proliferative activity of osteoblast cells is an indicator of new bone formation, which reached its peak on the 14th day and started to decreased until the 28th day, when the osteoblasts had matured.[15,30]

On the 28th day, the osteoblast cells transformed into osteocytes. This result is shown in Figure 1B and 1D. Therefore, this study showed that on the 28th day osteocytes increased and osteoblasts decreased. This is in inline with previous studies which stated that osteoblast cells decreased from day 14 to day 28, while osteocyte cells continued to ,so that higher numbers were obtained on day 28.[15,30] The results based on Table 2 described that on the 28th day the wound healing process in the alveolar bone socket after tooth extraction was dominated by osteocyte cells activity. This is indicated by the fact that the number of osteocyte cells has increased compared to osteoblast cells.[15] Zubaidah et al[31], stated that osteocytes also showed that osteocytes started to grow on the 14th day after the evaluation, and they kept growing for another 28 days following the application of hydroxyapatite bovine tooth graft and PEG as the carrier. Thus, osteoblast and osteocytes as components of bone regeneration are also inducing bone healing.

Based on this study, the thickness of collagen, osteoblast cells, and osteocytes was less in the control group than the BAM group. This is because no material was applied to the alveolar bone socket after the tooth extraction. Although the control group did not receive material for the alveolar bone socket, the collagen's thickness and the proliferative activity of osteoblasts and osteocytes still occurred through the normal wound healing process.[32] The normal wound healing process will increase the thickness of collagen when entering the proliferative phase and release growth factors to stimulate the proliferative activity of osteoblast cells, which in turn will induce osteocyte cells in wound healing.[33,31]

In this study, the BAM application group had higher collagen thickness, number of osteoblasts, and osteocytes compared to the control group. This is because BAM, in the form of a sponge, contains collagen, which can absorb blood, so that bleeding wounds stop more quickly and speed up the haemostasis and coagulation phases.[34] BAM also contains anti-inflammatory, so it can accelerate healing time, and the inflammatory phase becomes shorter.[23] In addition, BAM has several growth factors, such as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), and platelet-derived growth factor

(PDGF). The application of BAM to the alveolar bone socket will stimulate these growth factors to induce osteoblast cell proliferation initiated by fibroblast activity. TGF-β and FGF stimulate fibroblast activity.[35] All of these growth factors are highly essential during all phases of wound healing. However, since most of them are growth factors, if TGF-β is overexpressed it can lead to fibrosis and hypertrophic scar formation.[8]

Furthermore, PDGF and EGF will cause increased migration of fibroblasts and accelerate migration to the extraction wound. The migration of fibroblasts can encourage angiogenesis, namely the formation of new blood vessels.[23] The formation of new blood vessels is a sign of entering the proliferative phase that allows mesenchymal stem cells to differentiate into osteoblasts to produce bone.[11] In the proliferative phase, osteoblast progenitor cells show alkaline phosphatase (ALP) activity and are considered pre-osteoblasts. The transition from pre-osteoblast to osteoblast is characterized by increased osterix (Osx) expression and bone matrix protein secretion. In addition, osteoblast cells undergo morphological changes to become large and cuboidal in shape.[26] Therefore, release of growth factor largely will mediate the acceleration of the wound healing process. BAM, in particular, also has a role related to bone reformation with osteoinductive properties. Osteoblast expression in the process of osteogenesis can be stimulated by BAM.[5,6]

CONCLUSION

The application of BAM to the alveolar bone socket of SD rats can act as material for alveolar bone socket preservation. After extraction, it produced better results than the control group (without treatment). This is also shown by increasing collagen thickness, osteoblasts, and osteocytes. The number of osteoblast cells decreased on the 28th day and was replaced by osteocyte cells. Collagen thickness increased from day 14th to 28th. Therefore, the healing of soft and hard tissues could be increased by the administration of BAM biomaterials.

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