

# Bovine sponge amnion stimulates socket healing: A histological analysis

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## ABSTRACT

Bovine sponge amnion (BSA) is made from bovine amniotic membrane, which contains several growth factors with the ability to suppress inflammation and stimulate the healing process. The healing process of the socket is clinically marked by the proliferation of fibroblasts, formation of new blood vessels, and closure of the wound site. Fibroblasts and new blood vessels are the common histological indicators of tissue regeneration. Twenty-four lower anterior teeth were extracted from the rats. The socket was then applied with BSA and sutured, while a control group was only sutured. The animals were sacrificed 3, 7, and 14 days after application, and the mandibular was isolated. Histological analysis of socket tissue used the staining of hematoxylin and eosin to analyze the new blood vessels and fibroblasts. An independent *t*-test was used to analyze the fibroblasts and new blood vessels in each group, with  $P < 0.05$  considered as significant. The number of fibroblasts is higher in the BSA group compared others groups at 3, 7, and 14 days ( $P < 0.05$ ). The new blood vessel count is higher compared to the control group at only 3 and 7 days ( $P < 0.05$ ). BSA aids the regeneration of the socket after tooth extraction by stimulating fibroblast proliferation and formation of new blood vessels.

**Key words:** Bovine sponge amnion, fibroblasts, new blood vessels, socket tooth extraction, tissue regeneration

## INTRODUCTION

Tooth extraction is a common procedure performed by a dentist. Tooth extraction means removing the tooth from the alveolar, which will cause wounding to both hard and soft tissues. After the extraction, the alveolar bone is of most concern to a dentist as it is an important aspect in building the replacement tooth needed to support mastication.

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As a consequence of tooth extraction, processes such as inflammation and a reduction of the dimension of the alveolar bone follow.<sup>[1]</sup>

The wound healing process after extraction is a normal biological process that occurs in the human body in response to injury. Wound healing is an interrelated process and must occur sequentially, at the right time and be uninterrupted.<sup>[2]</sup> The process consists of four stages, namely: Hemostasis, inflammation, proliferation, and remodeling. Various factors can influence wound healing, both accelerating and delaying it.<sup>[3]</sup>

Fibroblasts are a source of collagen synthesis in the wound-healing process, and this begins relatively early in

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the healing process, continuing for several weeks depending on the size of the wound. Collagen synthesis by fibroblasts reaches its peak on days 5 and 7 after wounding.<sup>[4]</sup> In the proliferation phase, endothelial cells in the venules will be stimulated by vascular endothelial growth factor (VEGF) to form new blood vessels<sup>[2]</sup> and fibroblast growth factor (FGF) to stimulate fibroblast proliferation.<sup>[5]</sup>

The acceleration of wound healing after tooth extraction is one of the means of reducing alveolar bone loss. One common procedure is socket preservation.<sup>[6]</sup> Currently, there is much biomaterial that can be used in socket preservation, one of which is bovine amniotic membrane (BAM).<sup>[3]</sup>

BAM has been widely used in dentistry as a gingival and periodontal therapy in guided tissue regeneration because it possesses anti-bacterial and anti-inflammatory properties and stimulates tissue regeneration.<sup>[7]</sup> Bovine sponge amnion (BSA) is a product of BAM. BAM contains several growth factors, such as VEGF, FGF, and epidermal growth factor (EGF), which have an important role in the physiological processes that lead to normal wound healing and tissue regeneration.<sup>[8-10]</sup> BAM can be prepared in the form of fresh membrane, dried membrane, or freeze-dried irradiated membrane.<sup>[11]</sup> BSA, as the other form of BAM, is also able to suppress inflammation and stimulate growth in the regeneration of alveolar bone after tooth extraction.<sup>[12]</sup>

Although BSA has the potential to stimulate tissue regeneration, there is no proof that it accelerates wound healing after tooth extraction by stimulating fibroblast and new blood vessel formation. Fibroblasts and new blood vessels are commonly used as the indicators of tissue regeneration by histological analysis. Based on these reasons, the analysis of fibroblasts and new blood vessels is needed to strengthen proof of BSA's involvement in the stimulation of tissue regeneration in the field of dentistry.

## MATERIALS AND METHODS

### Animals

This research was performed in strict accordance with the protocols of the National Health Research Ethic and Guidelines (2017), Minister of Health, Republic of Indonesia. The protocol of the research was approved by Faculty of Dental Medicine, Airlangga University (Registered number 617/HRECC. FODM/VI/2019).

Twenty-four male Wistar rats, aged 2 months and weighing 140–160 g, were used as animal model. The experiments were conducted in the Laboratory of Animal, Faculty of Veterinary Studies, Airlangga University. The animal was placed in the different cages under a temperature control of 27°C in an artificial lighting rooms on the cycle of 12 h light/12 h dark, with a standard diet and free access for water.

### Bovine sponge amnion

BSA was made by and obtained from the Biomaterial Center, Bank Jaringan RSUD Dr. Soetomo, Surabaya, Indonesia. The BAM was screened for hepatitis B, hepatitis C, syphilis, and the human immunodeficiency virus (HIV). The BAM was washed with an antibiotic solution four times, and then stored in cryopreservation at –80°C for 24 h. After the process, the BAM was stored in a freeze dryer for 8 h, then mixed with glycerin at a ratio of 1:1 and stored for 24-h in two consecutive periods. Lyophilization method was used for the dehydrating process, and the sterilization was conducted with gamma laser (Co-60, Nordion, Japan) at Batan Research. The 250 µm ground particles were mixed with glycerin as a binder and formed into blocks measuring 1.5 mm × 5 mm to form BSA. Stored for two consecutive 24-h periods and dehydrated using the lyophilization method and sterilised using a gamma laser (Co-60, Nordion, Japan) at Batan Research. The ground particles, 250 µm in size, were mixed with glycerin as a binding agent and formed into blocks sized 1.5 mm × 5 mm to form BSA.

### Tooth extraction and bovine sponge amnion application

Before tooth extraction was performed, all animals were fed normally and were free to move around. The first anterior right mandible tooth of each Wistar rat was extracted under anesthesia using xylazine with 4 mg/kg dose (X1126, Sigma-Aldrich) and ketamine hydrochloride of 100 mg/kg (Ketalar, Warner Lambert, Ireland).

After extraction, the socket was given BSA and then sutured (Nylus nylon, nonabsorbable suture, Lotus surgical, India). The control group's sockets were only sutured, without application of BSA. At 3, 7, and 14 days, the socket was biopsied and subjected to histological analysis.

### Analysis of fibroblasts and new blood vessels

HE stain was performed for histological assessment, and the counting of new blood vessels and fibroblast was conducted under the ×400 magnification microscope. The counts were performed by a single operator in five fields of view.<sup>[13]</sup>

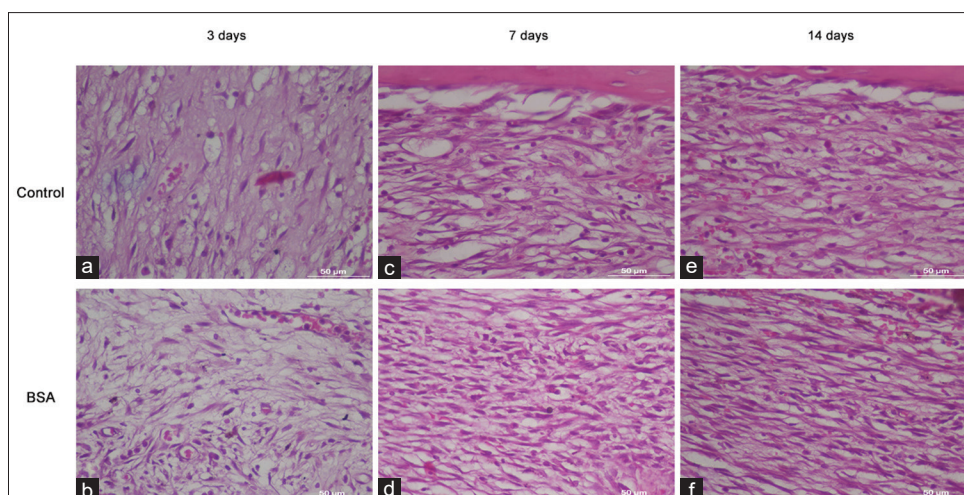
### Data analysis

The data were presented as mean and standard deviation (mean + SD). An independent *t*-test was done to assess the difference of fibroblast and new blood vessel numbers between each group, with a significance of  $P < 0.05$ .

## RESULTS

### Fibroblast number

The histopathology of tissue for fibroblast analysis is present in Figure 1. The fibroblast number in participants with BSA is higher compared to controls at 3, 7, and 14 days. The fibroblast number in participants with BSA after 3, 7, and 14 days ( $P = 0.000$ ) is higher compared to controls [Table 1].



**Figure 1:** Histopathological analysis of fibroblasts using hematoxylin and eosin staining, magnification  $\times 400$  (a,b) 3 days; (c,d) 7 days; (e-f) 14 days

**Table 1: Number of fibroblasts**

	Control	BSA	P
3 days	11.75 $\pm$ 0.50*	20.25 $\pm$ 0.96*	0.000
5 days	14.50 $\pm$ 0.58*	28.75 $\pm$ 0.50*	
7 days	19.75 $\pm$ 0.50*	31.75 $\pm$ 0.96*	

\*Significant difference in each group with independent t-test ( $P < 0.05$ ). BSA: Bovine sponge amnion

**Table 2: Number of new blood vessels**

	Control	BSA	P
3 days	7.50 $\pm$ 0.58*	9.50 $\pm$ 0.58*	0.003
5 days	10.25 $\pm$ 0.50*	13.00 $\pm$ 0.00*	0.000
7 days	9.00 $\pm$ 0.82*	10.00 $\pm$ 0.00*	0.05

\*Significant difference in each group with independent t-test ( $P < 0.05$ ). BSA: Bovine sponge amnion

### New blood vessel number

The histopathology of tissue for new blood vessel analysis is present in Figure 2. The new blood vessel number in participants with BSA is higher compared to controls at 3, 7, and 14 days. The fibroblast number in participants with BSA after 3, 7, and 14 days is higher compared to the control group ( $P = 0.003$ ;  $P = 0.000$ ;  $P = 0.05$ ; respectively) [Table 2].

## DISCUSSION

BAM has been used for a variety of medical purposes including the dressing of wounds and ulcers because it contains several growth factors, such as EGF, VEGF, FGF, tissue inhibitor metalloproteinase (TIMP), transforming growth factor  $\beta$  (TGF- $\beta$ ), and platelet-derived growth factor (PDGF) play an important role in the physiological processes that lead to tissue regeneration.<sup>[9,10]</sup> BAM also has several specific properties, including being bacteriostatic, anti-inflammatory, pain-reducing, protective of wounds, and a trigger for re-epithelialization. In addition, the presence of growth factors increases chemotactic activity,

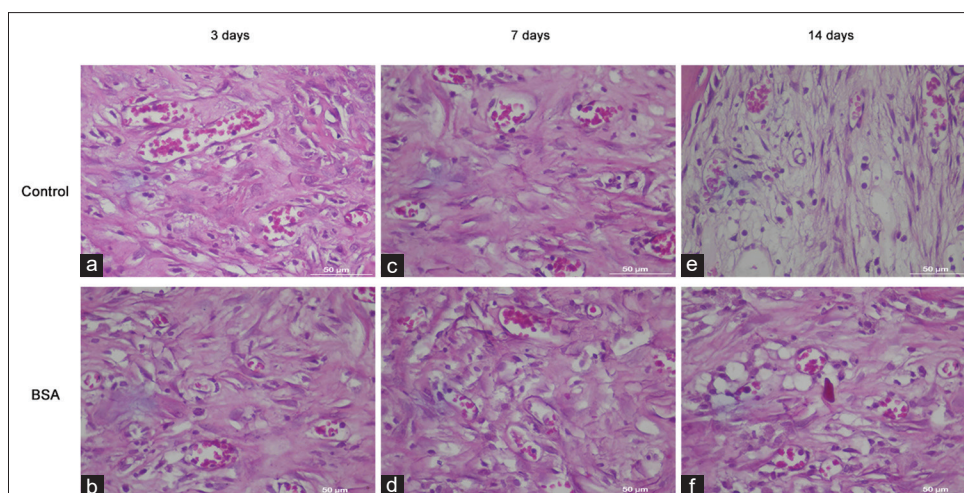
which stimulates various types of cells to migrate to the wound area. The growth factors produced by the amnion also stimulate cell proliferation and the synthesis of extracellular matrix (ECM) components.<sup>[14]</sup>

The wound-healing process is a biological process that occurs in response to tissue damage. After tooth extraction, inflammation will occur as an initial response to this damage. Immediately after the tooth extraction occurs, the socket will be filled with blood which will then form a clot. The part of the periodontal ligament that is damaged contains mesenchymal cells and these blood vessels will come into contact with the blood clot. In the 1<sup>st</sup> week after extraction, the blood clot filling the socket will be replaced by granulation tissue, and this is then followed by the proliferation phase, indicated by the presence of fibroblasts and collagen deposition.<sup>[2]</sup>

BSA is formed from a mixture of BAM and gelatine through freeze drying. In this study, the BSA was formed into blocks with a size of 1.5 mm  $\times$  1 mm so that it was easier to apply in the socket. The application to the socket resulted in increased fibroblasts. TGF- $\beta$ , contained in BSA, stimulates fibroblast activity and stimulates FGF secretion. This causes increased fibroblast migration, so that this migration to the wound is faster. In addition, BSA contains extracellular metrics which increase chemotactic macrophage activity thus accelerating the release of PDGF and EGF, which can also accelerate fibroblast migration.<sup>[14]</sup>

Fibroblasts and endothelial cells are the dominant cells in the proliferation phase. Endothelial cells located in nontraumatized venules are stimulated by VEGF to begin forming new capillaries. The VEGF produce by keratinocytes at the wound margins, macrophages, fibroblasts, platelets and endothelial cells.<sup>[3,15]</sup> VEGF initiates angiogenesis events, especially endothelial cell migration and proliferation. In the proliferation phase, macrophages release VEGF which





**Figure 2:** Histopathological analysis of new blood vessel using hematoxylin and eosin staining. magnification  $\times 400$  (a,b) 3 days; (c,d) 7 days; (e-f) 14 days

stimulates the proliferation of endothelial cells to form new blood vessels.<sup>[16]</sup> In this study, it was seen that the number of new blood vessels is increased when BSA is applied. BSA containing VEGF causes the angiogenesis and vascularization process to take place faster.<sup>[8,16,17]</sup>

In the field of dentistry, BAM can be applied in the clinical setting as fresh membrane or freeze-dried membrane. For socket application, fresh membrane is not recommended because the membranes are fragile and need to be dealt with very carefully.<sup>[11]</sup> For accessibility, BAM for socket preservation is made into sponge form or BSA. The previous study showed, BSA applied in the tooth socket after extraction showed decreased interleukin 6 (IL-6).<sup>[12]</sup> Other research also confirmed that BSA was able to suppress IL-1 $\beta$ .<sup>[7]</sup> The over expression of IL-6 and IL-1 $\beta$  will delay healing.<sup>[18,19]</sup> Besides containing growth factors, BSA also contains surfactant protein A. This protein acts as an anti-inflammatory and stimulates growth factors to regenerate tissue. The exact mechanism of the anti-inflammatory properties of BSA and BAM is related to the reduction of inflammatory cells in the wound area, and this consequently reduces inflammatory mediators by serving as a barrier. Neutrophils and macrophages are inflammatory cells responsible for releasing matrix metalloproteinases and producing inflammation. This process is inhibited by TIMP, contained in BSA. All these processes stimulate the proliferation phase by increasing fibroblast and new blood vessel formation. The BSA itself provides a place for cell migration, such as fibroblasts from lamina propria, and the creation of new tissue,<sup>[20]</sup> as well as promoting recruitment of mesenchymal stem cells and retaining the native composition of its ECM and promoting angiogenesis.<sup>[21]</sup>

This study confirmed that BSA was not only able to suppress inflammation in the socket after tooth extraction<sup>[12]</sup> but also

able to stimulate the proliferation of cells involved in tissue regeneration. Future research is needed in the application of BSA to actual tooth extraction cases so that it can be used as a biomaterial for proper socket regeneration.

## CONCLUSION

BSA stimulates the regeneration of sockets after tooth extraction by stimulating fibroblast proliferation and formation of new blood vessels.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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