Dental Research Journal

Original Article

Sponge amnion potential in post tooth extraction wound healing by interleukin-6 and bone morphogenetic protein-2 expression analysis: An animal study

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ABSTRACT

Background: Wound tooth extraction is a mechanical injury that traumatizes adjacent tissue. Sponge amnion contains growth factors that can promote postextraction wound healing. Amnion membranes can be transformed into sponge form rendering it easier to use. The aim of this study is to analyze interleukin-6 (IL-6) and bone morphogenetic protein-2 (BMP-2) expression in postextraction wound healing on the 1st and 7th day after sponge amnion application.

Materials and Methods: Twenty-eight Wistar rats were used in this experimental descriptive analytical study. Fourteen animals' first right anterior mandible tooth was extracted; then, the socket applied by sponge amnion and sutured (treatment group), while 14 others only sutured (as control group). The alveolar bone tissue of animal was observed 1st and 7th days after extraction and then was analyzed using immunohistostaining to identify the expression of IL-6 and BMP-2. Statistical analysis was performed using one-way ANOVA with the level of significance ($P < 0.05$).

Results: IL-6 expression in the treatment group was significantly lower than the control group on the 1st and 7th days ($P = 0.000$). BMP-2 expression in the treatment group was significantly higher than the control group on the 1st and 7th days ($P = 0.000$).

Conclusion: Sponge amnion can promote the healing process by increasing the expression of BMP-2 and decreasing IL-6 expression.

Key Words: Amnion membrane, bone morphogenetic protein-2, interleukin-6, wound healing

Received: September 2017
Accepted: April 2019

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INTRODUCTION

A wound can be defined as a disruption of tissue integrity resulting from mechanical or thermal trauma.[1] Tooth extraction leaves marks on the soft tissue as wound and on the hard tissue as socket. The resulting of socket will affect of the alveolar bone height so that prosthetic will be greatly affected. To preserve alveolar height, the postextraction healing process needs to be optimized.[2] Thus, some growth factors such as platelet-derived growth factors, epidermal growth factors, fibroblast growth factors, transforming growth factor-alpha (TGF-α), and TGF-β are required.[3]

The healing process is achieved by means of successive phases: homeostasis, inflammation, proliferation, and remodeling.[3] The inflammatory

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How to cite this article: Indrawati DW, Munadziroh E, Sulisetyawati TI, El Fadhlallah PM. Sponge amnion potential in post tooth extraction wound healing by interleukin-6 and bone morphogenetic protein-2 expression analysis: An animal study. Dent Res J 2019;16:283-8.

phase is characterized by migration and infiltration of inflammatory cells that release cytokines which play a role as mediator relaying signals between cells during the wound healing process. The molecular activity of IL-6 and tumor necrosis factor-α is activated on day 1, while TGF-β2 and TGF-β3 are expressed on day 7.[4] IL-6 as a signaling molecule pro-inflammatory cytokines was associated with impaired bone volume and function in bone healing.

The postextraction healing process involves bone regeneration through osteogenesis. One growth factor involved in osteogenesis is bone morphogenetic protein-2 (BMP-2) that initiates osteoblast differentiation through cellular pathways. Based on the findings of previous studies, the osteoinductive properties of BMP-2 will increase on the conclusion of the inflammatory phase.[2,5]

The amnion membrane constitutes biomaterial that can promote wound healing since it contains surfactant protein-A (SP-A) that interacts with macrophage and downregulates IL-6.[6] One form of biomaterial demonstrating the ability to promote wound healing in soft as well as hard tissue is the amnion membrane[7] whose unique characteristic lies in its ability to stimulate the ephitelization, control proliferation, and differentiation of fibroblasts.[8] The amnion membrane is a raw material, whereas the available fabricated biomaterial used to promote wound healing is guided tissue regeneration (GTR). Since the GTR used in dentistry is relatively expensive, amnion membrane is employed as a substitute.[9,10] In clinical dentistry, amnion membrane is used by periodontists during open-flap procedures, despite being difficult to manipulate due to its tendency to tear easily.[11] There had never been a study about the amniotic membrane made in sponge form. Therefore, the amnion membrane is made in sponge form to enhance the ease with which it can be used.

In accordance with attempts to develop biomaterial technology, especially those conducted at Universitas Airlangga, the objective of the research reported here was to analyze the potential of the amnion membrane in sponge form to promote wound healing. Some studies have reported that amnion is an effective and very good tissue material to accelerate wound healing.[12,13] This amnion is used because the material available quite a lot in Bank Jaringan Rumah Sakit Umum Daerah (RSUD) Soetomo. Another function is the use of tissue waste as a form of creativity that is supported by science; therefore, a product that is very effective is obtained because this amnion has also been made in the form of a sponge, so it is very easy to use in clinical applications. The advantage of sponge amnion as a material in dentistry compared from previous studies in the form of amnion membrane/amniotic sheet is an easier application in scars because the shape of the sponge has an effect as hemostat. The weakness is the work is a little tricky because it has to change the amnion form from a membrane into a sponge which takes longer time and requires more extra precision because the drying system uses room temperature without using a dryer, therefore, the active components contained in the amnion not lost. The aim of this study is to analyze IL-6 and BMP-2 expression in postextraction wound healing on days 1 and 7 after sponge amnion application.

MATERIALS AND METHODS

Ethical clearance for this experimental descriptive analytical study was granted by the Health Research Ethics Committee of the Faculty of Dental Medicine, Universitas Airlangga No: 293/KKEPK. FKG/XII/2016. Sponge amnion is made from amnion membrane (made in Bank Jaringan RSUD Dr. Soetomo, Surabaya, Indonesia) which was washed with antibiotic solution, soaked in glycerine and dimethyl sulfoxide, and then stored by means of cryopreservation at −80°C. Before being processed, the stored amnion had been previously thawed, ground down to a specified quantity, and then mixed with glycerine at a ratio 1:1. The mixture was stored for two consecutive 24-h periods and subsequently dehydrated by lyophilization method and sterilized using gamma laser (Co-60, Nordion, Japan) at Batan Research. The ground particles 250 μm in size were mixed with glycerine as a binding agent.[14] The sponge amnion-making process was carried out at Bank Jaringan RSUD Dr. Soetomo, Surabaya, Indonesia.

In this laboratory-based experimental investigation incorporating a posttest only control group design and involving 28, 10–12-week-old male Wistar rats weighed 150–200 g. The rats being acclimatized over 7 days at room temperature under a constant 12-h light-dark cycle had free access to standardized pellet and water *ad libitum*. Before extraction of first right anterior mandible tooth, the rats were anesthetized

using a ketamine 20 mg/kg and then divided into four groups as K1, K7, P1, and P7.[15] Sponge amnion was placed in the sockets of Groups P1 and P7 (treatment groups), while Groups K1 and K7 (control groups) without sponge amnion. Those of all groups then were sutured using 3/0 black absorbable silk. The 1st day postextraction, the Groups K1 and P1 were sacrificed with their counterparts from Groups K7 and P7 undergoing the same procedure on the 7th day postextraction.

Alveolar bones were fixed in 10%, buffered formalin then being decalcified using 2% nitric acid, and processed for tissue embedding. Slide section 4 μm was made using a rotary microtome and stained by indirect immunohistochemical technique with IL-6 antibody (anti-IL-6 antibody, ab9770, Abcam, Cambridge, United Kingdom) and BMP-2 antibody (anti-BMP-2 antibody, ab14933, Abcam, Cambridge, United Kingdom) and counterstained using hematoxylin and eosin. Slides were examined concurrently by two pathologists using a light microscope (BX 41 series, Olympus Corporation, Tokyo, Japan) at ×400 magnification. The methods of quantification of the expression in immunohistochemistry slides using quantitative scoring system by counting the macrophages that expressed the IL-6 and osteoblast that expressed the BMP-2.[16] Data were then collected and shown as a mean value ± standard deviation. The differences between the groups were analyzed statistically by means of one-way ANOVA with significant of $P < 0.05$.

RESULTS

One-way ANOVA analysis showed there was a significant difference of IL-6 expression between the control and treatment groups (P = 0,000), while BMP-2 expression in the two groups also contrasted sharply (P = 0,000). The results of mean and standard deviation of IL-6 and BMP-2 expression can be seen in Table 1.

The results confirmed there to be a considerable contrast in IL-6 and BMP-2 expression between the control and treatment groups on the 1st and 7th day postextraction. There was a significant difference of IL-6 [Table 2] and BMP-2 [Table 3] expression on the 1st and 7th days. The results of examination with a light microscope at ×400 magnification can be seen in Figures 1 and 2. Positive results were showed by brown spots in Figures 1 and 2. From the results of this study, it appears that the treatment resulted in a decrease of IL-6 and an increase of BMP-2 on day 7.

Table 1: Mean and standard deviation of interleukin-6 and bone morphogenetic protein-2 expression

Group	IL-6	BMP-2
K1	10.57±1.51	5±1.15
P1	9.57±2.14	10.57±1.51
K7	9.86±1.86	2.71±1.38
P7	3.43±0.97	12.57±1.98

BMP-2: Bone morphogenetic protein-2; IL: Interleukin

Table 2: Result of least significant difference test of interleukin-6 expression

Group	K1	K7	P1	P7
K1		0.435	0.277	0.000*
K7			0.754	0.000*
P1				0.000*
P7				

*Significant difference

Table 3: Result of least significant difference test of bone morphogenetic protein-2 expression

Group	K1	K7	P1	P7
K1		0.01*	0.000*	0.000*
K7			0.000*	0.000*
P1				0.023*
P7				

*Significant difference

DISCUSSION

These results suggest that sponge amnion affects the acceleration of healing as evidenced by the decrease in the expression of IL-6 and increase BMP-2. The highest number of IL-6s was found in the K1 group compared to the others. IL-6 carries out functions in nonspecific and specific immunities. In nonspecific immunity, IL-6 stimulates hepatocytes to produce an acute phase protein with colony-stimulating factor that stimulates the progenitor in the bone marrow to produce neutrophils. In specific immunities, IL-6 stimulates the growth and differentiation of B-cells into plasma cells that produce antibodies.[17]

In the P1 group, IL-6 was not reduced in comparison to K1 because on day 1, it was still functioning, and in that condition, as an innate response, IL-6 will activate a growth factor to accelerate the healing process. On day 7, IL-6 began to decrease because its function was assumed by the growth factor TGF-β which is responsible for bone growth. On day 1, the

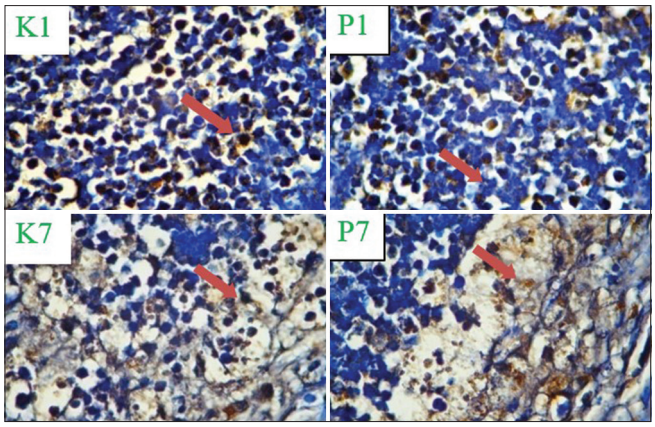

Figure 1: Interleukin-6 expression (arrow) on days 1 and 7 in Groups K1, K7, P1, and P7, ×400.

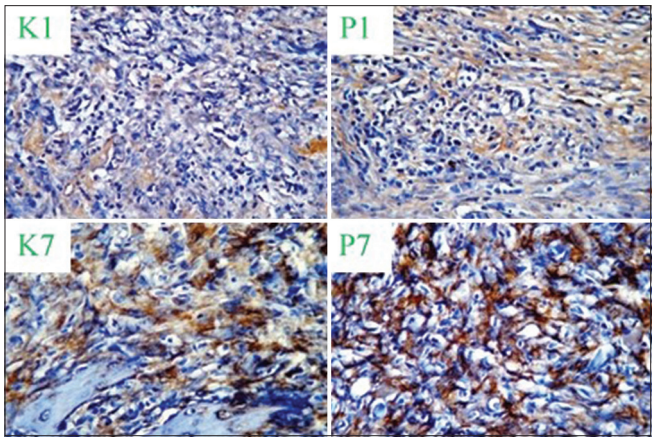

Figure 2: Bone morphogenetic protein-2 expression on days 1 and 7 in Groups K1, K7, P1, and P7, ×400.

IL-6 control group promoted transactivation of growth factors associated with the healing process.[18]

After day 1, IL-6 functioned as an adaptive response for days 3, 4, and 5. This situation indicated the start of the proliferation phase which produced growth resulting from active cell division with an increase in cell size. The stage following the proliferation of wound healing involved the integrity of physiological processes. According to data obtained from *in vivo* testing with microscopic observation in the group treated with amnion sponge on day 7 (K7), the expression of IL-6 decreased. This is because, on the 7th day of the healing process, IL-6 serves as a transactivation/expression of the gene directly to the bone.[19]

Sponge amnion contains SP-A,[6] one of the families of proteins possessing anti-inflammatory properties. SP-A binds to macrophages and affects the production of pro-inflammatory cytokines IL-6. SP-A macrophages can decrease the expression of Toll-like receptor-2 (TLR2) and TLR4 which will result in decreased activity of nuclear factor of kappa (NFκ) light polypeptide gene enhancer in B-cell inhibitor, alpha (IkBa). They will also provoke a decrease of kappa-light-chain-enhancer factor of activated B-cell NF-κB.[20] In addition, SP-A may also cause a reduction in reactive oxygen species which results in the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which also decreases, consequently affecting the activation of NF-κB. The decrease in NF-κB will affect the transcriptional process of cytokines and cause the production of IL-6 to decrease.[21]

In this study, BMP-2 increased after sponge amnion which, as a material, is an osteoconductive agent. Sponge amnion is believed to contain osteoinductive material that is able to induce BMP-2 during the osteogenesis process. BMP is a protein member of the TGF-β family that plays an important role in this process. Improved BMP-2 expression will help the process of differentiating precursor cells into condensed or osteogenic varieties.[22] Comparing with other study about amnion, amniotic membrane functioned as a reservoir for BMP-2, retaining more BMP-2 than poly(ε-caprolactone) mesh scaffolds through 21 days *in vitro*. As hypothesized, heterotopic mineralization was reduced with amnion surrounding collagen sponge compared to collagen sponge alone.[23]

The results showed there to have been an increase of BMP-2 on days 1 and 7 indicating a significant difference of BMP-2 expression between the control and treatment groups. This is because the amnion sponge has TGF-β content. TGF-β plays a role in the initiation of BMP synthesis signaling by osteoprogenitor cells resulting in the differentiation of osteoblast cells and osteoclast apoptosis that inhibit bone resorption.[24] TGF-β forms part of a superfamily, including BMP, that plays a role in the differentiation of undifferentiated mesenchymal cells into chondrocytes and osteoblasts and osteoprogenitor to osteoblasts.[4] In addition, the amniotic membrane also expresses BMP-2 and Type 2 collagen that has therapeutic potential as a treatment for damaged or diseased cartilage.[25] This suggests that the amniotic membrane increases BMP-2 expression in tooth extraction sites.

Sponge amnion has an influence on the growth factor which is one of the component amniotic membranes

and the content of SP-A which can accelerate the occurrence of the inflammatory phase. Amnion membrane will decrease TLR-2, TLR-4, and NADPH oxidase,[26] thus causing IL-6 to decrease and increase TGF-β; therefore, BMP-2 will increase.[27] Based on previous studies, increasing BMP-2 and decreasing IL-6 will quickly decrease the inflammatory phase, the remodeling process occurs faster, and it will speed up the wound healing process.[4,20-22] With the decrease in the inflammatory reaction, there will be an increase in the proliferation process and collagen synthesis will also increase and this will accelerate the wound healing process.

CONCLUSION

There was a difference in the number of IL-6 and BMP-2 expressed on days 1 and 7 resulting from sponge amnion posttooth extraction in Wistar rats. Sponge amnion can promote the healing process by increasing the expression of BMP-2 and decreasing expression of IL-6. This is because one of the functions of sponge amnion is that of a growth factor playing an important role in the processes of cell proliferation and differentiation. As BMP-2 increases during these processes, there will be an increase in osteoblast production which will enhance the healing process.

Financial support and sponsorship
Nil.

Conflicts of interest
The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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