Original article

The effect of an 8% cocoa bean extract gel on the healing of alveolar osteitis following tooth extraction in Wistar rats

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ABSTRACT

Background: Alveolar osteitis is a well-known complication that occurs following a tooth extraction when the clot within the socket breaks down too early, causing increased localised inflammation and extreme pain. Alveolar osteitis delays the wound healing process of the socket. The polyphenols in the cocoa bean (Theobroma cacao L.) can stimulate the wound healing process. Purpose: The aim of this study was to analyse the effect of an 8% cocoa bean extract gel on the healing of alveolar osteitis following a tooth extraction. Methods: This study is an in vivo experiment with a posttest-only control group design. Thirty-six male Wistar rats were divided into three groups: a negative control, positive control and an 8% cocoa bean extract gel. A tooth extraction was performed on the mandibular incisor, and alveolar osteitis was induced by the application of adrenaline using a paper point on the socket. On the 3rd, 7th and 14th days, the clinical wound size of the extraction socket was measured, and the rats were sacrificed to observe the number of macrophages, fibroblasts and osteoblasts microscopically. A two-way analysis of variance test and post hoc least significant difference test were used to analyse the data (p < 0.05). Results: The data analysis showed a significant difference in the clinical wound size of the extraction socket and the number of macrophages, fibroblasts and osteoblasts between the 8% cocoa bean extract gel and the control groups (p = 0.000). Conclusion: An 8% cocoa bean extract gel stimulates the healing of alveolar osteitis following tooth extraction in Wistar rats.

Keywords: alveolar osteitis; cocoa bean; socket healing; tooth extraction

INTRODUCTION

One of the most common procedures in oral and maxillofacial surgery is tooth extraction. A tooth extraction will cause an injury (or hole) called a socket. The procedure is considered successful if it is accompanied by an optimal wound healing process of the socket without any complications. Alveolar osteitis is one of the most common complications following a tooth extraction. The prevalence of alveolar osteitis ranges from 1 to 5% for a routine extraction, but it can be as high as 40% following an extraction of the mandibular third molar. The aetiology of alveolar osteitis is an early fibrinolysis in the extraction socket. The interaction between the excessive trauma during a tooth extraction and a bacterial invasion produce plasmin, which leads to a lysis of the blood clot. This condition delays the healing process due to the proliferation of the cells occurs from the circular gingival mucosa, which takes longer than a normal blood clot to form. The treatment of alveolar osteitis is performed by the placement of a local dressing, but the dressing can act...
as a foreign material that can prolong the wound healing process.\(^\text{2,3}\)

An alternative local dressing is required that is low in cost and has minimal side effects. Presently, the use of an herbal dressing as an alternative is highly recommended.\(^\text{4,5}\) Cocoa (\textit{Theobroma cacao} \textit{L.}) is a type of plant that is known to have many health benefits. In Indonesia, the cocoa bean is widely used as a food ingredient and in traditional medicine. Cocoa beans contain polyphenols that have antioxidant, anti-inflammatory and antibacterial activities that have the ability to stimulate the wound healing process. The polyphenols in cocoa beans promote the activity and proliferation of cells through affecting growth factor receptors, which accelerates the wound healing process.\(^\text{6}\)

According to a previous study by Kurniawati et al.,\(^\text{7}\) an 8% cocoa bean extract gel can reduce the number of neutrophil cells in the healing process of tooth extraction wounds in Wistar rats.

Based on this understanding, cocoa beans have much potential for use as a medicinal plant, but the effect of a cocoa bean extract on the healing process of alveolar osteitis is unknown. Thus, the purpose of this study is to evaluate the effect of an 8% cocoa bean extract gel on the healing process of alveolar osteitis through a clinical and histological examination.

**MATERIALS AND METHODS**

This study received ethical approval from the Animal Research Ethics Committees of the faculty of mathematics and natural sciences, Universitas Sumatera Utara (0390/KEPH-FMIPA/2021). This study is an in vivo experiment with a posttest-only control group design. Fresh cocoa beans were obtained from the Pamah area, Deli Tua district, North Sumatera, Indonesia. The cocoa beans were dried and ground into a powder and then macerated by soaking them in 70% ethanol for 24 hours. The cocoa bean extract was filtered and evaporated to obtain a concentrated cocoa bean extract. The gel base was made by dissolving 3 g of carboxyl methyl cellulose sodium (CMC-Na) powder into 100 ml of warm distilled water. To make the 8% cocoa bean extract gel, 8 g of cocoa bean extract were added homogeneously into the prepared gel base.

The preparation of the experimental animals was carried out by acclimatising the rats for one week. Thirty-six healthy Wistar rats aged 2–3 months with weights of 200–250 g were divided into 3 groups. The negative control group was given 3% CMC-Na, the positive control group was given Aloclair® (Alliance Pharma, UK) gel and the experimental group was given the 8% cocoa bean extract gel. The gel base was made by dissolving 3 g of carboxyl methyl cellulose sodium (CMC-Na) powder into 100 ml of warm distilled water. To make the 8% cocoa bean extract gel, 8 g of cocoa bean extract were added homogeneously into the prepared gel base.

The mandibular incisor was extracted using a needle holder (OneMed Health Care, Indonesia). Following complete extraction, alveolar osteitis was induced by applying a 1:1000 adrenaline (Ethica Pharmaceutical, Indonesia) solution using a paper point (Gapadent, Vietnam) for one minute in the extraction socket, then the socket was left for three days. Following the induction of alveolar osteitis in the rats, 0.1 ml of the treatment was injected into the extraction socket using a 1 ml syringe (OneMed Health Care, Indonesia) twice a day (morning and evening) for 14 days.

On the 3rd, 7th and 14th days post treatment, the mesiodistal and buccolingual widths were measured using a digital caliper (Vernier, USA). The size of the clinical wound extraction socket was calculated by the formula used in the study by Mokhtari et al.,\(^\text{8}\) mesiodistal width \(x\) buccolingual width. Then, the rats were sacrificed via cervical dislocation and the mandibular socket tissue was excised using a blade and scalpel (OneMed Health Care, Indonesia). The fresh socket tissue was then put into a container and fixed in a 10% buffer formalin solution for tissue processing and hematoxylin-eosin staining (HE). The observation of the number of macrophages, fibroblasts and osteoblasts was carried out using a light microscope (Zeiss Primo Star, Germany) in five fields of view at 400 magnification. The data were analysed using the two-way analysis of variance (ANOVA) test and the post hoc least significant difference (LSD) test to compare the differences between all groups.

**RESULTS**

Based on the histological observations, there was a decrease in the number of macrophages accompanied by an increase in the number of fibroblasts and osteoblasts over time in all groups, as shown in Figures 1 and 2. There was an inflammatory response in the negative control group, which was characterised by the highest number of macrophages followed by the lowest number of fibroblasts (see Figure 1) and osteoblasts (see Figure 2). The positive control group and the 8% cocoa bean group showed the healing of alveolar osteitis, which was characterised by a lower number of macrophages with a higher number of fibroblasts and osteoblasts than the negative control group. The 8% cocoa bean group showed the best healing effect of alveolar osteitis, with a significant decrease in the number of macrophages and a significant increase in number of fibroblasts and osteoblasts.

The two-way ANOVA test results (Table 1) showed a significant difference in the mean number of macrophages, fibroblasts and osteoblasts between all groups (\(p = 0.000\)). The mean difference in the number of macrophages, fibroblasts and osteoblasts using the post-hoc LSD test are shown in Figure 3. For the number of macrophages, there was a significant difference between the 8% cocoa bean extract gel group and the negative control group on the 3rd and 7th days (\(p = 0.000\) and \(p = 0.001\), respectively), but there was no difference between the 8% cocoa bean extract gel group and the positive control group (\(p = 0.845\) and \(p = 0.859\), respectively). For the number of fibroblasts,
Table 1. Histological observation of the mean number of macrophages, fibroblasts and osteoblasts

<table>
<thead>
<tr>
<th>Histological observation</th>
<th>Observation day</th>
<th>Negative control (Mean ± SD)</th>
<th>Positive control (Mean ± SD)</th>
<th>Cocoa bean 8% (Mean ± SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage</td>
<td>3</td>
<td>25.36 ± 3.86</td>
<td>5.50 ± 1.36</td>
<td>5.22 ± 1.00</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>7.50 ± 3.85</td>
<td>2.07 ± 0.95</td>
<td>1.82 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>Fibroblast</td>
<td>3</td>
<td>80.85 ± 2.94</td>
<td>124.65 ± 3.14</td>
<td>128.72 ± 7.51</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>97.85 ± 3.85</td>
<td>163.27 ± 0.66</td>
<td>199.95 ± 10.46</td>
<td></td>
</tr>
<tr>
<td>Osteoblast</td>
<td>7</td>
<td>4.52 ± 1.11</td>
<td>18.75 ± 3.53</td>
<td>20.75 ± 1.26</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>12.30 ± 0.77</td>
<td>33.82 ± 1.80</td>
<td>43.87 ± 8.91</td>
<td></td>
</tr>
</tbody>
</table>

*Two-way ANOVA test; statistically significant with p < 0.05

Figure 1. Histological description of the macrophages (black arrow) and fibroblasts (red arrow) in the negative control group on day 3 and day 7 (A, B), the positive control group on day 3 and day 7 (C, D) and the 8% cocoa bean extract gel group on day 3 and day 7 (E, F) using HE staining at 400 magnification.

Figure 2. Histological description of the osteoblasts (black arrow) in the negative control group on day 7 and day 14 (A, B), the positive control group on day 7 and day 14 (C, D) and the 8% cocoa bean extract gel group on day 7 and day 14 (E, F) using HE staining at 400 magnification.
there was a significant difference between the 8% cocoa bean extract gel and the negative control group on the 3rd and 7th days (p = 0.000). On the 3rd day, there was no difference between the 8% cocoa bean extract gel and the positive control group (p = 0.155), but on the 7th day, there was a significant difference (p = 0.000). For the number of osteoblasts, there was a significant difference between the 8% cocoa bean extract gel group and the negative control group on the 7th and 14th days (p = 0.037 and p = 0.000, respectively). On the 7th day, there was no difference between the 8% cocoa bean extract gel group and the positive control group (p = 0.495), but on the 14th day there was a significant difference (p = 0.003).

The results of the clinical wound size of the extraction socket decreased over time in all groups, as shown in Table 2. The two-way ANOVA test showed a significant difference in the wound size of the extraction socket on the 3rd, 7th and 14th days between all groups (p = 0.000). The post hoc LSD test results (Figure 4) shows a significant difference between the 8% cocoa bean extract gel and the negative control group on the 3rd, 7th and 14th days (p = 0.000). On the 3rd and 7th days, there was no difference between the 8% cocoa bean extract gel group and the positive control group (p = 0.059 and p = 0.117, respectively), but on the 14th day there was a significant difference (p = 0.028).

Table 2. Clinical observation of the wound size of the extraction socket

<table>
<thead>
<tr>
<th>Clinical observation</th>
<th>Observation day</th>
<th>Negative control (Mean ± SD)</th>
<th>Positive control (Mean ± SD)</th>
<th>Cocoa bean 8% (Mean ± SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wound size of extraction socket</td>
<td>3</td>
<td>3.87 ± 0.03</td>
<td>2.20 ± 0.09</td>
<td>2.13 ± 0.03</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3.52 ± 0.08</td>
<td>1.79 ± 0.03</td>
<td>1.73 ± 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.85 ± 0.02</td>
<td>0.44 ± 0.09</td>
<td>0.36 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

*Two-way ANOVA test, statistically significant with p < 0.05

Figure 3. The mean number of macrophages, fibroblasts and osteoblasts in all experimental groups. *Post hoc LSD test; statistically significant at p < 0.05.

Figure 4. The mean clinical wound size of the extraction socket in all experimental groups. *Post hoc LSD test; statistically significant at p < 0.05.
DISCUSSION

The wound healing process of the extraction socket is an organised sequence of biological activities that restores the integrity of the mucosa and alveolar bone. Immediately following a tooth extraction, the socket will be filled by a blood clot through platelet aggregation and fibrin activation. The blood clot serves as a provisional matrix for healing cells to migrate to the wound area and becomes a reservoir for growth factors.1,9

Leukocytes, fibroblasts and new blood vessels will migrate to the blood clot to perform their function in the healing process. In the inflammatory phase, macrophages, phagocytose microbes and cellular debris produce high levels of free radicals, cytokines and growth factors that are important in stimulating cell migration and proliferation.1,2,10 In the proliferative phase, fibroblasts begin to migrate and degrade the fibrin clots. Fibroblasts play an important role in producing collagen to build an extracellular matrix to form connective tissue and support the growth of new alveolar bone in the socket.1,2,9

Osteoblasts are cells that play an important role in the process of new alveolar bone growth. Osteoblasts produce a bone matrix called an osteoid and organic components, such as osteocalcin, osteopontin and alkaline phosphatase. First, an amount of osteoid produced by the osteoblasts will form a woven bone. Then, osteoblastic and osteoclastic activity remodels the woven bone, leading to the growth of mature lamellar bone.1,2,9 In the healing of alveolar osteitis, an early destruction of the blood clot before the clot is replaced by granulation tissue inhibits the migration and proliferation of healing cells, subsequently inhibiting the healing process of the extraction socket.1,3

In this study, the 8% cocoa bean extract gel group had a greater reduction in the number of macrophages compared with the control groups from day 3 to day 7. This is because cocoa beans contain secondary metabolites, one of which is proanthocyanin.5 Proanthocyanin acts as an anti-inflammatory agent by inhibiting cyclooxygenase and lipooxygenase, which reduces the number of prostaglandin, thromboxane, prostacyclin, endoperoxide and leukotrienes produced, resulting in a reduction in the number of inflammatory cells that migrate to the injured area. Thus, the inflammatory reaction is shortened in duration and the wound healing process can move to the proliferative phase.5,11

The results of this study are supported by a study conducted by Dugo et al.,11 which found that cocoa polyphenol extracts reduced the reactions of pro-inflammatory macrophages (M1 macrophages) and induced changes in the phenotype of macrophages to anti-inflammatory macrophages (M2 macrophages). Human macrophages can be divided into two groups based on their activation stages: M1 macrophages and M2 macrophages. M1 macrophages play a role in initiating the inflammatory process, while M2 macrophages are involved in the resolution of inflammation. At the end of the inflammatory phase, M2 macrophages will produce growth factors, such as transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF) and platelet-derived growth factor, which act as the initiators of the proliferative phase.11,12 Macrophages are the most dominant cells in the inflammatory phase, with the highest number on day 3 and a gradual decrease on day 7. The reduction in the number of macrophages indicates the end of the inflammatory phase and the beginning of the proliferative phase.10,12

The proliferative phase is characterised by fibroplasia.9,10 In this study, the 8% cocoa bean extract gel group had the highest number of fibroblast cells on the 3rd and 7th days compared with the control groups. Cocoa beans contain quercetin.9 Based on a study by Kant et al.,13 quercetin stimulates fibroblast proliferation by increasing TGF-β1 expression. Transforming growth factor beta 1 is a growth factor that stimulates the migration and proliferation of fibroblasts and supports the synthesis and deposition of collagen in the wound healing process.10,13

One of the factors that inhibit the healing process in alveolar osteitis is bacterial invasion. The bacteria involved in the pathogenesis of alveolar osteitis are generally anaerobic pathogenic bacteria.2,4 The theobromine found in cocoa beans is a potent antibacterial agent. Theobromine attaches to the bacterial cell wall, penetrates the biofilm generated by the bacteria and stimulates proteolytic enzymes, which damage the bacterial cell membranes. This causes the phagocytic activity of the bacteria that cause alveolar osteitis to be more easily carried out by inflammatory cells, speeding up the inflammatory phase so that the proliferative phase can begin, which is characterised by the increase in the number of fibroblasts.4 Fibroblasts begin to migrate on the 3rd day after the injury and continue to proliferate until their number peaks on the 7th day.2,10

Osteoblasts begin to migrate on the 7th day, and their number will continue to increase until the 14th day, after which the osteoblasts will differentiate into osteocytes. Osteocytes are mature osteoblasts trapped within the osteoid.9,14 In this study, the 8% cocoa bean extract group had the highest number of osteoblasts compared with the control groups on the 7th and 14th days. This is because cocoa beans contain anthocyanidins.5 In the study conducted by Xu et al.,15 anthocyanidins were found to increase the expression of VEGF, which is needed in the process of angiogenesis.

One of the crucial factors in the process of bone healing is vascularity. The granulation tissue that forms on the surface of the alveolar bone during the wound healing process following a tooth extraction requires good vascularity. Vascularisation is needed to provide a supply of nutrients and oxygen in the process of bone ossification.2,16 Cocoa beans also contain catechins.6 According to a study by Fajrjani et al.,17 catechins can stimulate osteoblast proliferation by increasing the expression of TGF-β1 and bone morphogenetic-2 (BMP-2). Transforming growth factor beta 1 is the most abundant growth factor in bone cells and stimulates osteogenic bone cell proliferation and
dissociation. In addition, TGF-β1 enhances osteoclast absorption and promotes new bone growth. Meanwhile, BMP-2 increases mesenchymal stem cell migration, proliferation and osteogenic differentiation.11,16,17

The positive control group in this study used Aloclair® gel, which contains 2% sodium hyaluronate. During the wound healing process, sodium hyaluronate binds well to the fibrin clot, promoting cell migration and proliferation to the wound area. In addition, sodium hyaluronate can also strengthen the bond between fibrin and collagen so that blood clots become more stable and less easily damaged.18

The wound healing process of the extraction socket can also be assessed clinically by the size of the socket wound. The faster the size of the wound decreases, the faster the wound healing process takes place.19 Based on this study, the cocoa bean extract gel group had the lowest mean clinical socket wound size compared with the control groups on the 3rd, 7th and 14th days. This was because the cocoa bean extract gel group had the highest number of fibroblasts. An increase in the number of fibroblast cells increases the wound closure process because fibroblasts differentiate into myofibroblasts, which play a role in wound contraction, thereby causing the wound to shrink in size.2,10

There were significant differences in the mean socket wound size and the number of macrophages, fibroblasts and osteoblasts between the cocoa bean group and the control groups. The cocoa bean extract gel produced a better effect on the healing process of alveolar osteitis compared with the control groups. This study concludes that the application of cocoa bean extract gel on the healing process of alveolar osteitis following a tooth extraction in Wistar rats, as evident from the decrease in the clinical wound size of the extraction socket, a decrease in the number of macrophages and an increase in the number of fibroblasts and osteoblasts.

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