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Research Article

ALOE VERA ETHANOL EXTRACT AS A THERAPY FOR ALVEOLAR MANDIBLE REGENERATION IN LIPOPOLYSACCHARIDE-EXPOSED RATS

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ABSTRACT

Objective: The study examined the effects of *Aloe vera* ethanol extract on alveolar mandible regeneration in rats (*Rattus norvegicus*).

Methods: Rats were divided into five groups as follows: Negative control group received no treatment, positive control group received *Escherichia coli* lipopolysaccharide (LPS) alone for 5 days, and Groups P1, P2, and P3 received LPS for 5 days followed by 40, 80, and 160 mg/kg BW *A. vera* ethanol extract, respectively, orally on days 6–12. Alveolar bone tissues were taken and histologically processed through hematoxylin-eosin staining. Osteoblast and osteoclast numbers in alveolar tissue were also examined.

Results: One-way ANOVA revealed strong relationships between the *A. vera* ethanol extract dosage and the numbers of osteoblasts ($r=0.921$, $p<0.05$) and osteoclasts ($r=-0.631$, $p<0.05$) in rats.

Conclusion: *A. vera* ethanol extract appears capable of stimulating alveolar bone regeneration following LPS exposure.

Keywords: *Aloe chinensis,* Baker ethanol extract, Lipopolysaccharide, Osteoblasts, Osteoclasts, Alveolar bone.

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INTRODUCTION

Periodontal disease is common and widespread and can affect children, adults, and the elderly. The 2003 World Oral Health Report identified periodontal disease as the fourth most expensive disease to treat. The Scheffler survey revealed that 75% of the American population has periodontal disease. The prevalence of periodontal disease in all age groups in Indonesia has reached 96.58% [1], whereas data from the Department of Health of Malang indicated that periodontal disease is the seventh most common disease in the state [2].

Periodontitis, a cause of tooth loss in adults, is a chronic infection of tooth-supporting tissues including the gingiva, periodontal ligament, bone, and cementum by bacteria. Substances produced by bacteria cause tissue inflammation and progressive alveolar bone damage, which are the main characteristics of periodontitis [3]. The major microbes that cause periodontitis are *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Actinobacillus actinomycetemcomitans*; however, other microbes including Gram-negative bacteria such as *Escherichia coli*, *Fusobacterium nucleatum*, and *Actinomyces israelii* can also trigger periodontitis [4].

Gram-negative bacterial species produce lipopolysaccharide (LPS), a structural component of the outer membrane that protects bacteria against host immune defenses [5]. LPS induces the production of local factors, namely pro-inflammatory cytokines such as interleukin-1α (IL-1α), IL-1β, tumor necrosis factor-α (TNF-α), and eicosanoids, including prostaglandin E2 (PGE2). Prostaglandins and pro-inflammatory cytokines promote destruction of periodontal tissue by stimulating osteoclast production and activity and decreasing the numbers and activity of osteoblasts [6]. Umezu *et al*. revealed that mice injected with *E. coli* LPS in the mucosal region of the maxillary first molar exhibit alveolar bone resorption caused by excessive osteoclast numbers and activity.

Alveolar bone regeneration is a suitable treatment goal for patients with periodontitis and severe bone destruction. Regenerative periodontal tissue healing occurs through the formation of new periodontal tissues, particularly the alveolar bone, functional periodontal ligament, and cementum. Increased osteoclastogenesis is one indicator of bone regeneration [7].

Several studies previously reported the efficacy of *Aloe vera* (*Aloe chinensis*) in healing bone defects. *A. vera* is considered to express biogenic stimulators and wound healing hormones because it promotes cell regeneration. *A. vera* contains acetylated mannose (acemannan), a large polysaccharide that promotes the formation of collagen type 1 fibers and acts as an immunostimulator enhancing T-helper immune responses against intracellular pathogens such as viruses, bacteria, and parasites. Another study conducted by Jittapiromsak *et al*. postulated that acemannan can stimulate bone morphogenetic protein-2 (BMP2) expression in pulpal fibroblasts and periodontal tissues, thus resulting in bone regeneration. Furthermore, Kresnohadi combined *A. vera* and xenograft cancellous grafts, thus observing increases in BMP2 expression and osteoblast counts as well as decreased receptor activator of nuclear factor kappa-B ligand (RANKL) expression, which is an indicator of decreased osteoclast production [8].

Most current studies have found that molecular signals trigger the formation of complex tissues. Molecular biology studies identified BMP as a bone differentiation initiator. BMP regulates cartilage and bone differentiation as well as bone growth through progenitors that trigger osteoblast formation [9].

Thus, the effects of *A. chinensis* baker ethanol extract on LPS-induced alveolar bone destruction were examined in male rats (*Rattus norvegicus*) based on osteoblast and osteoclast counts.

METHODS

Research design

This *in vivo* study was randomized and controlled in nature.

Samples

The study samples included male rats maintained in the Pharmacology Laboratory of the Faculty of Medicine, Brawijaya University, Malang. The rats were 2 months old, healthy, active with normal behavior and weighed 200–250 g. Rats used in previous studies, those with a lack of appetite, those in poor condition as well as dead rats were excluded from the study.

Variables

The study variables were as follows:

a. Independent variable: *A. vera* ethanol extract;
b. Dependent variable: Number of osteoclasts;
c. Control: Simple criteria, method for administering LPS, and method for administering *A. vera* extract.

Six repetitions were performed. The rats were divided into five groups: A negative control group that received no treatment, a positive control group that received LPS alone, and three experimental groups treated with LPS followed by 200, 400, or 800 mg/200 kg BW of *A. vera* ethanol extract.

Setting and time of the study

The study was conducted at the Pharmacology and Anatomical Pathology Laboratory of the Faculty of Medicine, Brawijaya University, between December 2014 and February 2015.

PROCEDURE

Ethical clearance

The study was ethically approved by the Medical Research Ethics Commission of the Faculty of Medicine, Brawijaya University.

Sample preparation

Male rats were weighed using an analytical scale. The rats were allowed to adapt to the environment for 1 week and maintained in 40 cm × 30 cm × 30 cm^3 cages. Each cage included no more than two animals.

Dosage conversion

a. LPS dosage=5 µg/0.05 ml of PBS,
b. *A. vera.*

The conversion factor between a 70 kg man and 200 g mouse was 0.018. The recommended *A. vera* dosage for humans is 10–15 g/day. Thus, the *A. vera* dose for rats was calculated as follows:

=0.018×10 g
=0.18 g/200 g BW
=180 mg/200 g BW≈36 mg/kg BW.

The extract was prepared at three different dosages:

- 40 mg/kg BW for Group A
- 80 mg/kg BW for Group B
- 160 mg/kg BW for Group C.

Sample grouping

a. Group 1: Negative control group (K−) that was not administered LPS or *A. vera* ethanol extract;
b. Group 2: Positive control group (K+) administered LPS alone;
c. Group 3: (P1) Group administered LPS followed by 40 mg/kg BW *A. vera* ethanol extract;
d. Group 4: (P2) Group administered LPS followed by 80 mg/kg BW *A. vera* ethanol extract;
e. Group 5: (P3) Group administered LPS followed by 160 mg/kg BW *A. vera* ethanol extract.

Material preparation

Material used during the experiment included *E. coli* LPS (Sigma) to induce periodontitis and *A. vera* ethanol extract to stimulate alveolar bone repair.

LPS preparation

Totally, 10 mg of LPS was dissolved in 2 ml of PBS and stored in a sealed container at room temperature.

A. vera ethanol extract preparation

The *A. vera* ethanol extract was obtained through maceration. The process required approximately 200–400 g of *A. vera* powder as the main raw material, 200 ml of 96% ethanol for polysaccharide deposition, a deposition time of 10 h, and a precipitation temperature of 10°C. The preparations were then filtered into a porcelain bowl and allowed to stand uncovered at room temperature for 1 day. Following evaporation, the extract was stored in a container in a cool place and protected from sunlight [10].

EXPERIMENTS

Sedating the animals

Before treatment, the rats were sedated by administering an injection of ketamine (KTM 100; 40 mg/kg BW) into the right hind leg.

Applying the materials

LPS (5 µg/0.05 ml) was injected into the first incisive tooth gingival sulcus located on the mandibular right labia. PBS (0.02 ml) was injected using a 30-G insulin needle once daily for 5 days. The first incisor was selected because it is categorized as a front tooth and is, therefore, more visible than the other teeth.

Using a feeding tube, *A. vera* ethanol extract (1 ml) was administered for 7 days starting on day 6.

Alveolar mandible surgery

The surgery was conducted after 7 days of treatment with *A. vera*.

The rats were sedated using KTM (80 mg/kg BW), and their alveolar mandible bone was horizontally cut. The dead animals were then cleaned, sterilized using 70% alcohol, and buried into a 100 cm × 30 cm × 50 cm^3 hole in the backyard of the Pharmacology Laboratory. Each hole contained 24 dead rats. The mandibles were preserved using 10% formalin for 24 h and 10% EDTA for 14 days to decalcify the tissue. The solution was changed every 24 h, and the bones were washed under running water.

Paraffin block preparation

Tissues were dehydrated using acetone for 24 h and cleared using xylol twice for 1 h each. Next, infiltration was done using soft paraffin at 42°C–46°C twice for 1 h each followed by blocking with hard paraffin at 46°C–52°C for 1 h. Afterward, the tissue was cut vertically using a 4–6-µm microtome rotary and then heated at 60°C. The tissue was then soaked in xylol twice for 5 min each followed by a series of different concentrations of alcohol (95%, 85%, 70%, 50%, and 30%) twice for 3 min each.

For hematoxylin-eosin staining, tissues were incubated in Harris hematoxylin for 15 min followed by acid alcohol drops for 3–10 s and ammonium solution for 3–10 s. This was followed by counterstaining for 15–20 s and dehydrating using the ethanol series. Then, tissues were exposed to xylol for 5 min and mounted for observation using a digital microscope (five fields, ×400) to count the numbers of osteoblasts and osteoclasts with the assistance of an anatomical pathology lecturer and laboratory analyst.

Data analysis

Normality was assessed using the Shapiro–Wilk test because the number of samples exceeded 50. A two-tailed t-test was used to compare scores between two groups, with $p<0.05$ indicating statistically significant differences. Levene's test was used to compare normality and variance homogeneity between the positive control and experimental groups. When the data were normally distributed ($p>0.05$) and variance homogeneity was accepted ($p>0.05$), one-way ANOVA was conducted for hypothesis testing. *Post hoc* testing was then

used to identify significant differences based on ANOVA. Finally, the correlation-regression test was performed to assess the relationship between the *A. vera* dosage and osteoblast and osteoclast counts.

RESULTS

Figs. 1 and 2 show that osteoblast numbers were the lowest in the negative control group, whereas osteoclast counts were the highest in this group. Meanwhile, osteoblast counts increased with an increasing dosage of the *A. vera* ethanol extract; however, the opposite was seen in the case of osteoclasts. Figs. 3 and 4 show histological comparison of osteoblasts and osteoclast counts.

The Shapiro–Wilk test demonstrated that the data for osteoblasts and osteoclasts were normally distributed ($p<0.05$). The Levene's homogeneity test illustrated that the variance was homogenous in both osteoblast ($p=0.336$) and osteoclast data ($p=0.700$). Meanwhile, one-way ANOVA revealed significant differences in osteoblast ($p=0.000$) and osteoclast counts ($p=0.012$) based on the *A. vera* ethanol extract dosage.

The results of *post hoc* testing using the least significant difference test revealed significant differences between the control and experimental groups as well as among the experimental groups regarding the numbers of osteoblasts and osteoclasts.

The Pearson correlation test was employed to assess the correlation between the *A. vera* extract dosage and the numbers of osteoblasts and osteoclasts. A strong and positive correlation ($r=0.921$, $p=0.001$) was found between the *A. vera* extract dosages and osteoblast count, whereas an inverse correlation was observed between the dosage and osteoclast count ($r=-0.631$, $p=0.001$).

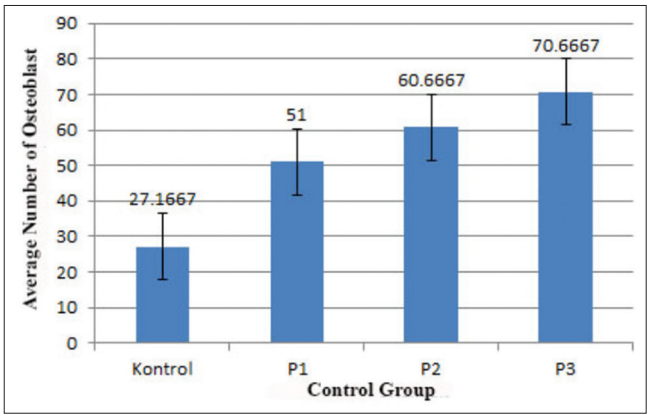

Fig. 1: Average number of osteoblasts in the groups. Description: K/P0: Lipopolysaccharide (LPS) treatment alone, P1: LPS treatment followed by 40 mg of *Aloe chinensis* baker ethanol extract, P2: LPS treatment followed by 80 mg of *A.chinensis* baker ethanol extract, P3: LPS treatment followed by 160 mg of *A. chinensis* baker ethanol extract

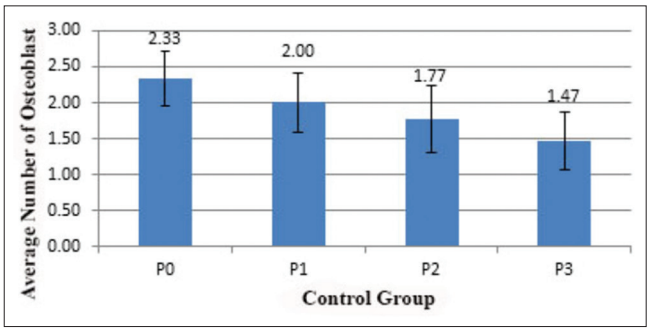

Fig. 2: Average number of osteoclasts in the groups. Description: K/P0: Lipopolysaccharide (LPS) treatment alone. P1: LPS treatment followed by 40 mg of *Aloe chinensis* baker ethanol extract, P2 LPS treatment followed by 80 mg of *A. chinensis* baker ethanol extract, P3 LPS treatment followed by 160 mg of *A. chinensis* baker ethanol extract

DISCUSSION

The study results revealed significant differences in osteoblast and osteoclast numbers between rats exposed to LPS alone and those exposed to LPS followed by *A. vera* ethanol extract. LPS stimulates osteoclast production, thereby resulting in tissue resorption. LPS functions as an endotoxin by binding to CD14 receptors in macrophages and monocytes. This binding induces the production of arachidonic acid (AA), which stimulates the secretion of cytokines such as IL-1α, IL-1β, IL-6, TNF-α, and PGE2 [11]. Prostaglandins and pro-inflammatory cytokines, which play important roles in bone pathology, are associated with bone destruction caused by localized chronic inflammation by increasing osteoclast formation, differentiation, and activation directly as well as by inhibiting osteoblast function [12]. Previous studies conducted by Indahyani *et al.* also proved that exposure to *E. coli* LPS leads to periodontitis.

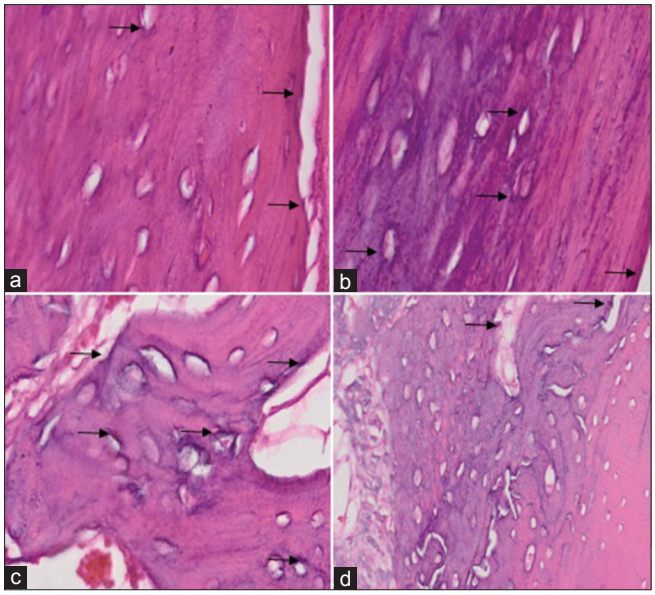

Fig. 3: (a-d) Histological comparison of osteoblast counts

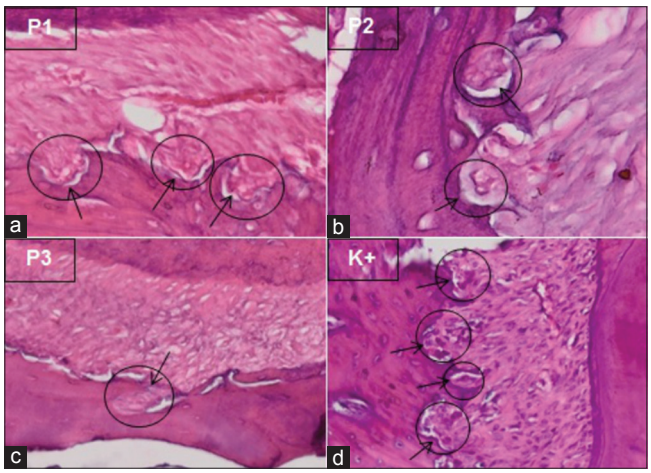

Fig. 4: Histological comparison of osteoclast counts. Description: (a) P1: Lipopolysaccharide (LPS) induction +40 mg of *A. vera* extract, (b) P2: LPS induction +80 mg of *A. vera* extract, (c) P3: LPS induction +160 mg of *A. vera* extract, (d) K+: LPS induction alone

The finding that the osteoblast numbers increase with increasing *A. vera* extract dosage corroborates with the hypothesis that acemannan can stimulate BMP2 expression and type 1 collagen fiber formation in periodontal tissue, thus facilitating bone regeneration by increasing osteoblast counts and decreasing osteoclast numbers in the alveolar bone. Acemannan is the largest polysaccharide capable of reducing inflammation through prostaglandin synthesis [13]. *A. vera* extract also inhibits AA-induced prostaglandin and thromboxane production through antagonism by eicosapentaenoic acid (EPA) [14,15]. In the body, EPA mostly interacts with AA metabolites. EPA is a polyunsaturated fatty acid that acts as a precursor for prostaglandin-3 (which inhibits platelet aggregation), thromboxane-3, and leukotriene-5. This substitution may result in the decreased release of pro-inflammatory signals, thus leading to decreased cytokine production.

A decrease in inflammatory mediator levels may also decrease osteoclastogenesis. Osteoblasts and stromal cells produce osteoprotegerin (OPG), which competes with RANKL for binding to RANK, thus suppressing osteoclast formation. When the OPG concentration exceeds that of RANKL, OPG binds to RANK and inhibits binding by RANKL. A study conducted by Kresnohadi demonstrated that acemannan can increase BMP2 expression and thereby lead to increased osteoblast formation and decreased expression of IL-1β and RANKL, which are markets of osteoclastogenesis and alveolar bone resorption [16].

The finding of the correlations between the *A. vera* extract dosage and osteoblast and osteoclast counts corroborates with data reported by Manoglas, as cited by Lindawati that BMP-2 stimulates osteoblastogenesis with increasing *A. vera* dosage [17]. In addition, *A. vera* also contains Vitamin A, which has significant roles in cell differentiation and strengthening of collagen and Vitamin C bound stimulating type-1 collagen, accumulation of osteoblasts, and matrix mineralization in osteoblasts. Vitamin C also maintains bone mass by stimulating osteoblast formation to promote the development of new bone and suppressing bone resorption by inhibiting osteoblast formation [18-20].

Linear regression analysis demonstrated that the R^2 was 0.398, thus illustrating that most effects of *A. vera* extract on osteoclasts were attributable to factors not analyzed in this study. These include the animal's conditions and the extract storage time, and other chemicals present in *A. vera*.

The hypothesis that *A. vera* extract can stimulate alveolar bone regeneration was successfully proved, as indicated by the increased number of osteoblasts and decreased a number of osteoclasts in LPS-treated rats.

CONCLUSION

A. vera ethanol extract appeared to promote alveolar bone regeneration in LPS-treated male rats based on the findings of increased osteoblastogenesis and decreased osteoclastogenesis. The *A. vera* extract dosage positively correlated with osteoblast counts and negatively correlated with osteoclast counts.

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