Dental Journal
Majalah Kedokteran Gigi

Dental Journal
(Majalah Kedokteran Gigi)
2025 March; 58(1): 37–44

Original article

Effect of cocoa pod husk (*Theobroma cacao* L.) extract on alveolar socket post tooth extraction

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ABSTRACT

Background: *Cocoa pod husk (CPH; Theobroma cacao L.) contains tannins, alkaloids, saponins, and flavonoids triterpenoids that have antibacterial, anti-inflammatory, and antioxidant activities that can accelerate wound healing and alveolar bone regeneration.* ***Purpose:*** *This study aimed to analyze the effect of CPH extract gel on the number of fibroblasts, osteoblasts, and osteoclasts in Wistar rats' sockets after tooth extraction.* ***Methods:*** *The samples used were 36 male Wistar rats divided into three groups: the negative control group (placebo gel), the positive control group (Alveogyl; Septodont, UK), and the treatment group (100 mg/ml CPH extract gel), which in each application was given for three, seven, and fourteen days on the mandibular first molar post-extraction socket. The samples were sacrificed and tissue was processed on days 4, 8, and 15. The amount of fibroblasts, osteoblasts, and osteoclasts was identified and determined by the Image J software.* ***Results:*** *The Mann-Whitney U analysis showed significant differences ($p<0.05$) between groups. Fibroblasts and osteoblasts in the treatment group were significantly higher than the control groups. Osteoclasts in the treatment group were significantly lower than the control group.* ***Conclusion:*** *Ethanol extract gel of CPH significantly increased fibroblasts on days 3 and 5 and osteoblasts on days 7 and 14 and decreased osteoclasts on day 14 in post-extraction socket of Wistar rats.*

Keywords: *cocoa pod husk; fibroblast; osteoblast; osteoclast; post tooth extraction; Wistar rats*
Article history: *Received 8 January 2023; Revised 5 March 2024; Accepted 13 March 2024; Published 1 March 2025*

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INTRODUCTION

Tooth extraction requires treatment due to alveolar ridge resorption that occurs. Tooth extraction is known to cause complications, including infection of the wound and dry socket, one of which is triggered by traumatic tooth extraction.[1] In the wound healing process, the main cells involved are fibroblasts. When tissue becomes inflamed, fibroblasts quickly migrate to the wound area and proliferate to produce a collagen matrix and repair damaged tissue. Fibroblasts originating from fibrocytes around the wound tissue form collagen fibers that begin to appear on the third day, and collagen synthesis by fibroblasts reaches its peak on the fifth to seventh day.[2,3] The physiological processes of wound healing after tooth extraction include the proliferative phase, which includes epithelialization, angiogenesis, formation of granulation tissue, and deposition of collagen that occurs from the 4th to the 14th day after injury. The maturation and remodeling phase begins from day 8 after injury and lasts for about a year.[4] In some patients, tooth extraction requires rehabilitation treatment with dentures to regain esthetic and chewing functions, and for some time, there will be alveolar ridge resorption, which affects the quality of rehabilitation.[5]

Repairing tissue damage requires an understanding of the process of intramembranous bone regeneration from the alveoli after injuries such as tooth extraction, periodontal disease, or dental implants.[6] The process of bone resorption occurs due to increased formation and activity of osteoclast cells. Active osteoclasts will demineralize bone. After the process of resorption is complete, it will continue with the process of bone formation. Osteoclast cells will disappear and be replaced by osteoblast cell activity that

DOI: 10.20473/j.djmkg.v58.i1.p37–44

will synthesize as much bone matrix as it is resorbed.[7] Traumatic tooth extraction is thought to cause a prolonged inflammatory response.

To overcome the occurrence of post-extraction complications, one of the drugs used as an additional therapy is Alveogyl (Septodont, UK). Alveogyl is a dressing material that contains butamben (anesthetic), iodoform (antibacterial), and eugenol (analgesic). Alveogyl is used as a topical treatment to treat symptoms of dry socket after tooth extraction.[8] Eugenol is reported to be cytotoxic and has a detrimental effect on cells, such as fibroblasts and osteoblasts. At high concentrations, eugenol can cause necrosis and interfere with the wound healing process.[9]

Cocoa (*Theobroma cacao* L.) is one of the most abundant plantation commodities in Indonesia. The high amount of cocoa bean production impacts the environment with increased cocoa pod waste. Cocoa pod husk (CPH) reaches 60% of the total cocoa pod production.[10] CPH is considered a waste product after processing cocoa beans and causes environmental problems, but it is rich in polyphenols and phytosterols, which can be extracted and used in various food and health products.[11]

CPHs contain alkaloids, flavonoids, tannins, saponins, and triterpenoids, which have antibacterial, anti-inflammatory, and antioxidant activities.[12] Flavonoids have antioxidant properties by binding to free radicals in tissues to inhibit cell membrane damage, thereby accelerating the cell proliferation phase. Flavonoids can reduce the inflammatory response by reducing neutrophils that produce matrix metalloproteinase, thereby triggering tissue regeneration by promoting collagen production and starting the new bone formation process.[13,14] The content of proanthocyanidin extract of CPH (*Theobroma cacao* L.) has been shown to be effective in increasing the thickness of the socket epithelium after tooth extraction in Wistar rats.[15] Polyphenols also have benefits for the wound healing process by expediting tissue regeneration due to accelerating the inflammatory process in the proliferative stage so that the healing process becomes faster.[16] The antioxidant activity of polyphenols is not only limited to inhibiting bone resorption but is also directed toward enhancing bone formation through enhancing osteoblast survival, function, and metabolism. In particular, a reduction in the rate of apoptosis through suppression of p53 signaling in mitochondria has been shown to be exerted by proanthocyanidins, due to their ROS-scavenging action.[17]

The direction of pharmaceutical-health technology development nowadays is focused on natural ingredients. It is relatively safer to use compared to drugs that contain chemicals that are considered harmful to the human body. Traditional medicine is generally considered safer since it has fewer side effects than chemical drugs.[18] Thus, research is needed on the role of CPH extract (*Theobroma cacao* L.), which is thought to be able to accelerate alveolar tissue regeneration after tooth extraction. The purpose of this study is to analyze the effect of ethanolic gel extract from CPHs on fibroblasts, osteoblasts, and osteoclasts numbers in the alveolar socket after tooth extraction in rats.

MATERIALS AND METHODS

This research is an experimental laboratory study with post-test-only control group designs. The procedure for the treatment of experimental rats met the eligibility requirements by the Health Research Ethics Commission of the Faculty of Dentistry, Universitas Jember with the Number 1372/UN25.8/KEPK/DL/2021. The subjects of this study were thirty-six of *Rattus norvegicus* strain Wistar, aged 12 to 14 weeks, male with a body weight of 200 to 250 grams. Before treatment, subjects received a minimum acclimatization period of 3–5 days, placed in cages in the same room and environment, Afterwards, the mandibular left first molar extracted using socket curettage, and they were divided into a negative control group (CMC-Na gel); a positive control group (Alveogyl; Septodont, UK);[8] and the treatment group (extract gel of CPH 100 mg/ml). Each group was divided into two subgroups based on the period of observation: three, seven, and fourteen days after tooth extraction.

Samples of forastero type CPHs (*Theobroma cacao* L.) were obtained from the plantations of the Jember Coffee and Cocoa Research Center in East Java, Indonesia. The CPHs were cleaned, cut into small pieces, and air-dried. After being baked and blended to a fine powder and weighed with an analytical balance (Ohaus, Parsippany, USA), 5 kg of CPH was found to produce ± 500 grams of cocoa pod powder. The extraction method used is the ultrasonic bath (Elma, Singen, Germany) method that was carried out at the Pharmacy Laboratory Universitas Jember. 50 grams of CPH powder plus 300 ml of 70% ethanol solvent was made in a ratio of 1:4 (w/v); 1:2 (w/v); 1:1.5 (w/v) was put into an glass container, and ultrasonicated for 3x3 minutes. Every three minutes, the mixture was stirred before being ultrasonicated again. The entire filtrate obtained was then put into a rotary evaporator (B-one, Jakarta, Indonesia). Afterward, it was transferred to a petri dish and put in an oven to be evaporated at 40°C until a constant weight extract was obtained. CPH extract was made in a gel preparation based on CMC-Na. A total of 48 ml of Aquadest was added with 2 grams of CMC-Na and stirred until homogeneous into a 4% CMC-Na placebo gel. A total of 22.5 grams of CMC-Na gel was added to 2.5 grams of CPH extract and stirred until homogeneous in a 100 mg/ml CPH ethanol extract gel.[19] CMC-Na is used because of its polymeric form, which is stable at pH 5–9, and takes a short time to acquire gel formation.[20]

The placebo CMC-Na gel, Alveogyl, and CPH extract gel were applied once a day for three, seven, and fourteen days. Experimental animals were sacrificed by injection of a lethal dose of anesthetic using a mixture of 100 mg/kg ketamine (Alfamine 10%; Alfasan Int., Woerden,

DOI: 10.20473/j.djmkg.v58.i1.p37–44

Netherlands) and 10 mg/kg xylazine (Alfazyne 2%; Alfasan Int., Woerden, Netherlands) i.m.[21] Tissue was taken on the left mandible and immediately fixed with 10% neutral formalin buffer for 24 hours, then decalcified with 10% formic acid (Sigma-Aldrich; Castle Hill, Australia) for 28 days. The tissue was processed by the paraffin embedding method and made sagittal incisions with a thickness of 5 µm using a microtome (Sakura Finetek, USA) and staining with Hematoxylin-eosin (HE09-40R, TissuePro, USA).

Fibroblasts, osteoblasts, and osteoclasts on the preparations were counted histologically using a binocular microscope (Olympus CX23, Guangzhou, China) with 400x magnification. Fibroblasts are cells that are oval with oval nuclei and dark purple with pink cytoplasm in the post-tooth extraction socket of male Wistar rats. Fibroblast were counted in the apical 1/3 of the three visual fields with a V pattern, namely on the left, middle, and right, whereas osteoblasts and osteoclasts were counted on the cervical third, middle third, and apical third of the alveolar bone margin on the mesial side of the tooth extraction socket of the lower mandibular left first molar in the rats. On hematoxylin eosin (HE) staining, osteoblast cells look cuboidal or columnar, are located at the edge of the bone, and have a round nucleus and a basophilic cytoplasm; while, the osteoclast cells appear large with multiple nuclei and are found in Howship's lacunae.[22] Calculating the average number of fibroblasts, osteoblasts, and osteoclasts was made with a binocular microscope (Olympus CX23, China) with 400x magnification on the cervical third, middle third, and apical third of the alveolar bone margin on the mesial side of the tooth extraction socket. Observation was confirmed with Image J software by three observers.

The data obtained was analyzed using SPSS software (IBM SPSS Statistics 26, New York, USA). Results are presented in terms of mean ± standard deviation (SD), normality test with Saphiro-Wilk, and homogeneity with Levene Test. The parametric data of fibroblasts was analyzed by one-way ANOVA and post hoc LSD, while nonparametric data of osteoblast-osteoclast was analyzed by Kruskal Wallis test and continued with Mann-Whitney U test to determine statistically significant differences between groups ($p < 0.05$).

RESULTS

Based on research on 36 samples of Wistar rats (*Rattus norvegicus*) divided into three groups, the following results were obtained. The graphical picture of the average number of fibroblasts in the control and treatment groups on day 3 and 7 are shown in Figure 1. The average number of fibroblasts in rat mandibular first molars showed that the negative control group on day 7 had the lowest value; meanwhile, CPH group on day 7 had the highest value. The one-way ANOVA test showed a significant difference in the number of fibroblast cells between groups ($p<0.05$). Figure 2A-F shows the histological appearance of the mandibular first molar in the apical third of the alveolar with HE staining in the negative control group (CMC-Na), positive control group (Alveogyl), and treatment group (CPH extract 100 mg/ml). The average number of osteoblasts in rat mandibular first molars increased significantly in the CPH group compared to positive control groups, both on day 7 and day 14 (Figure 3). There was a significant difference in osteoblast numbers, based on the results of the Mann-Whitney U test ($p<0.05$), in all groups except between the negative control group in day 7 and day 14 (Table 1).

In addition, osteoclasts were found to be higher in the CPH group compared to the positive control group

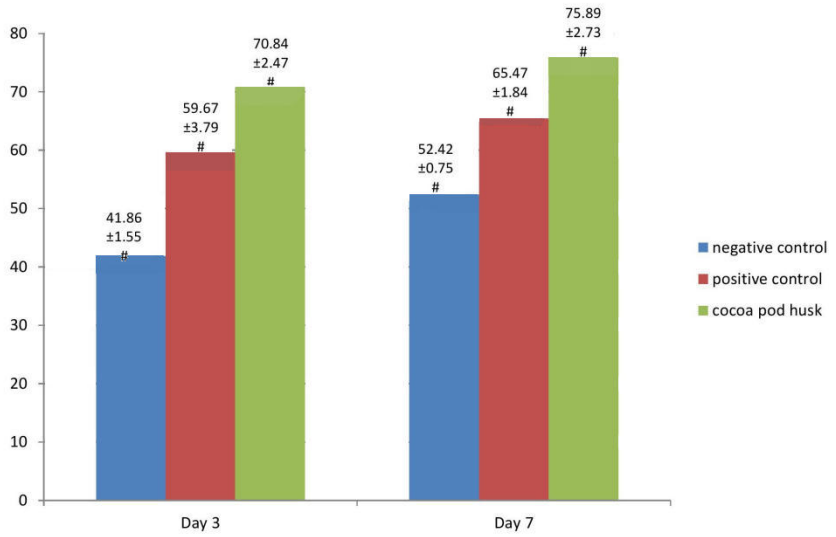

Figure 1. Quantitative analysis of fibroblast on day 3 and day 7 in CPH groups. Data was quantified by image analysis software as mean ± SD and analyzed by ANOVA and LSD post hoc test to determine differences between all groups #significance value =$p<0.05$.

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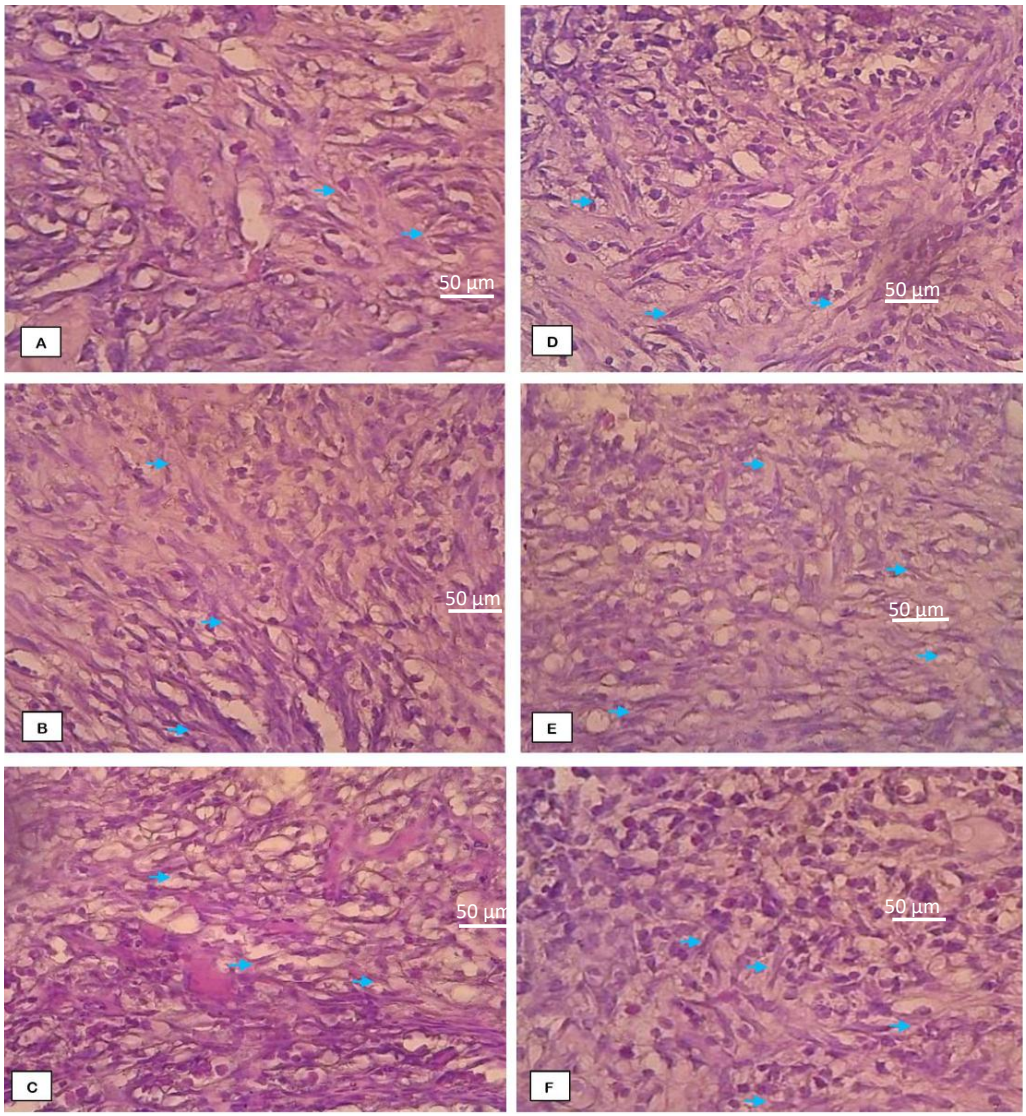

Figure 2. Histological appearance of the rat's alveolar bone after extraction of mandibular first molar in the apical third with HE staining in a microscope with 400x magnification. A) negative control group day 3; B) positive control group day 3; C) CPH treatment groupday 3; D) negative control group day 7; E) positive control group day 7; F) CPH treatment group day 7 (blue arrows: fibroblast, scale bar: 50 µm).

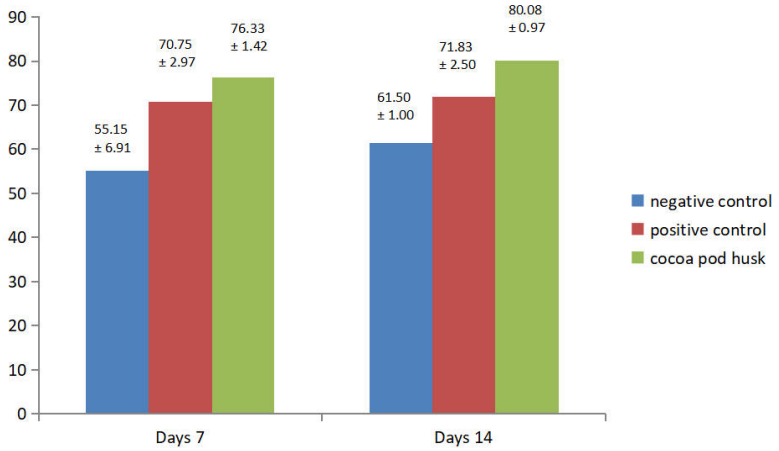

Figure 3. Quantitative analysis of osteoblast on day 7 and day 14 in control group and experimental groups. Data was presented as mean ± SD, analyzed by Kruskal Wallis and Mann-Whitney U test.

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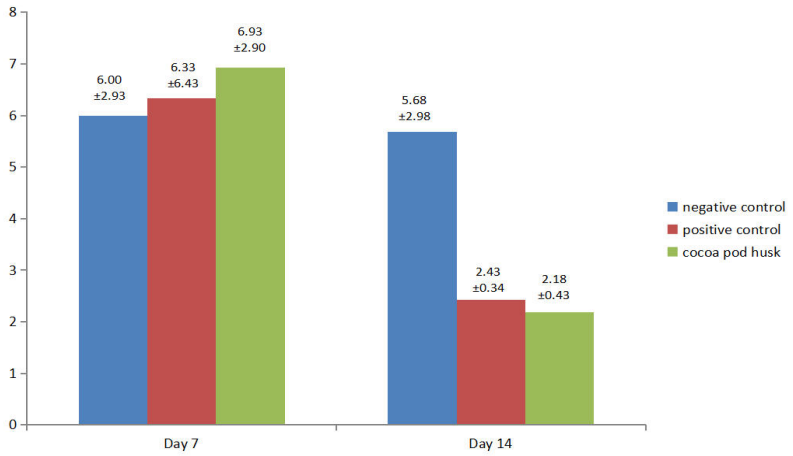

Figure 4. Quantitative analysis of osteoclast on day 7 and day 14 in the socket of control and experimental groups. Data was expressed as mean ± SD, analyzed by Kruskal Wallis ($p<0.05$), and followed by the Mann-Whitney U test.

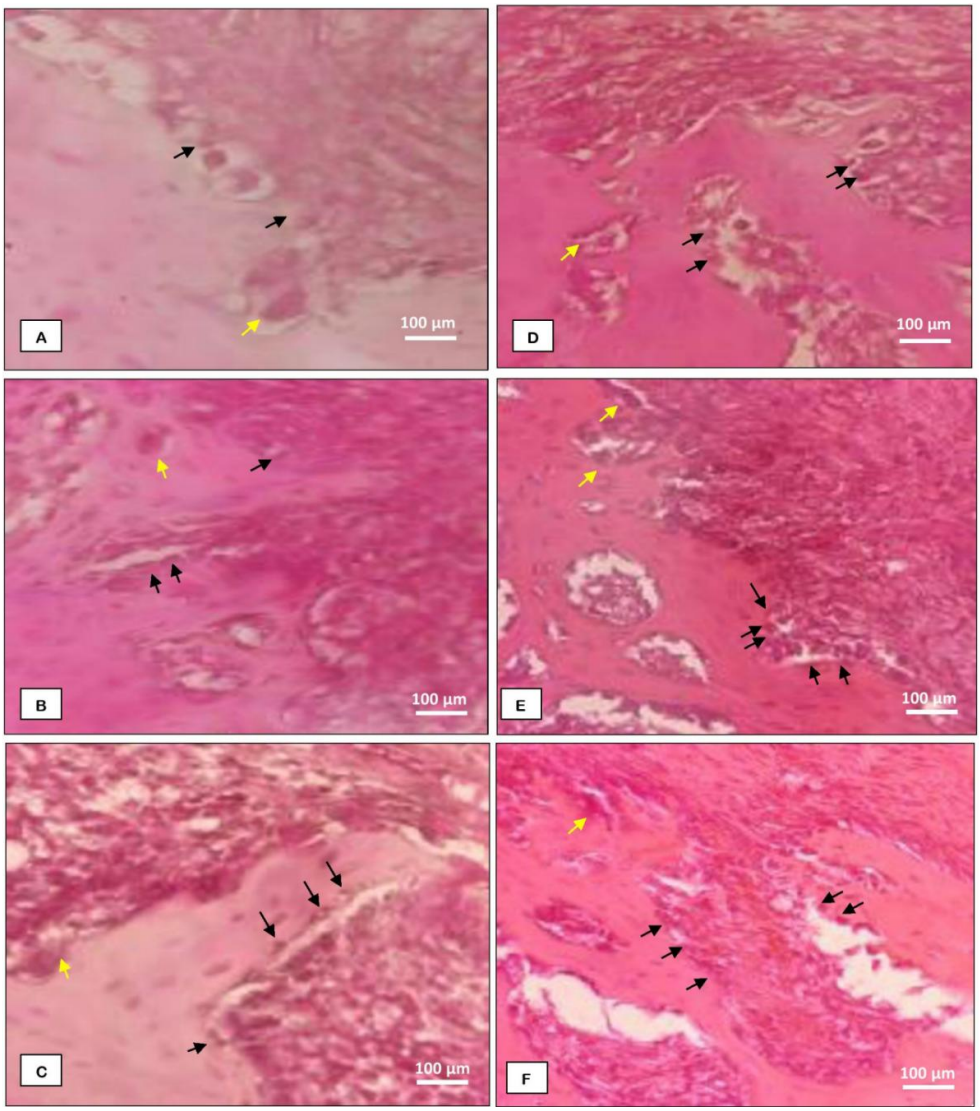

Figure 5. Histological appearance of the rat's alveolar bone after extraction of the mandibular first molar in the apical third with HE staining in a microscope with 400x magnification. A) negative control group day 7; B) positive control group day 7; C) CPH treatment group day 7; D) negative control group day 14; E) positive control group day 14; F) CPH treatment group day 14. (black arrows: osteoblast, yellow arrows: osteoclast; scale bar: 100 µm).

DOI: 10.20473/j.djmkg.v58.i1.p37–44

(Figure 4). The results of the Mann-Whitney U test for the number of osteoclasts showed that there was a significant difference ($p<0.05$) The results of the Mann-Whitney U test for the number of osteoclasts showed that there was a significant difference ($p<0.05$) among the C-7, C+14 and CPH14 group; CPH7, C+14, and CPH14 group, and among the C-14, C+14, and CPH14 group. Meanwhile, significant differences also existed between C-7 and C+7 group, CPH7 and C-14 group; C+7 and CPH7 group, C-14, C+14, and CPH14 group; between CPH7 and C-14 group. There was no significant difference between the C+14 group and the CPH14 group (Table 2). Histological appearance of osteoblast and osteoclast of the mandibular first molar in the apical third of alveolar with HE staining is showed in Figure 5. The examination of osteoblast and osteoclasts were carried out under a binocular microscope at 400x magnification.

The ratio of osteoclasts to osteoblasts can show the role of osteoclasts in the bone resorption process and osteoblasts in bone formation. The results showed that the ratio of the number of osteoclasts and osteoblasts from day 7 to day 14 in each group increased (Table 3). Based on the regression and Friedman test showing $p = 0.000$, there was a significant difference in terms of cell type and treatment on days 7 and 14.

DISCUSSION

Flavonoid of CPH extract has an antioxidant effect that can accelerate the inflammatory phase by capturing free radicals and preventing oxidation reactions by increasing the activity of the enzymes superoxide dismutase and glutathione transferase. Procyanidins stimulate macrophages to produce growth factors and cytokines, such as Interleukin-1 (IL-1), Interleukin-4 (IL-4), Interleukin-8 (IL-8), transforming growth factor-β (TGF-β), and epidermal growth factor (EGF). TGF-β and EGF function for the induction, proliferation, and migration of fibroblasts in the production of extracellular matrix. IL-1, IL-4, and IL-8 induce the process of chemotaxis of fibroblasts and keratinocytes, collagen and proteoglycan synthesis, and margination and maturation of keratinocytes as well as activate fibroblast proliferation and macrophages to initiate the chemotaxis process.[23]

The study's result was in accordance with the research conducted by Luthfi et al.,[24] where there was an increase in the number of fibroblasts reaching a peak, but in this study, it was not observed on day 7 but day 5. An increase in fibroblasts was followed by an increase in FGF-2 expression until day 7; the same results were obtained in a study by Oentaryo et al.[25] The rise in fibroblasts is also due

Table 1. Mann-Whitney U test for the number of osteoblasts in each sample group

	C-7	C+7	CPH7	C-14	C+14	CPH14
C-7		0.021*	0.021*	0.248	0.021*	0.021*
C+7			0.021*	0.021*	0.663	0.021*
CPH7				0.021*	0.021*	0.021*
C-14					0.021*	0.021*
C+14						0.021*
CPH14						

Note: C-7: Negative control group administering CMC-Na gel on day 7; C+7: Positive control group administering Alveogyl on day 7; CPH7: Treatment group administering CPH extract gel on day 7; C-14: Negative control group administering CMC-Na gel on day 14; C+14: Positive control group administering Alveogyl on day 14; CPH14: Treatment group administering CPH extract gel on day 14. (*): indicated significant difference ($p < 0.05$)

Table 2. Mann-Whitney U test for the number of osteoclasts in each sample group

	C-7	C+7	CPH7	C-14	C+14	CPH14
C-7		0.772	0.554	0.766	0.019*	0.020*
C+7			0.564	0.661	0.557	0.243
CPH7				0.384	0.020*	0.021*
C-14					0.019*	0.020*
C+14						0.369
CPH14						

Note: C-7: Negative control group administering CMC-Na gel on day 7; C+7: Positive control group administering Alveogyl on day 7; CPH7: Treatment group administering CPH extract gel on day 7; C-14: Negative control group administering CMC-Na gel on day 14; C+14: Positive control group administering Alveogyl on day 14; CPH14: Treatment group administering CPH extract gel on day 14. (*): indicated significant difference ($p < 0.05$)

Table 3. The ratio of osteoclast to osteoblast in rat tooth extraction sockets after administration of CPH extract

Day	Negative control	Positive control	CPH extract	p
Day 7	1:9.2	1:11.2	1:11	0.000
Day 14	1:10.8	1:29.6	1:36.7	

DOI: 10.20473/j.djmkg.v58.i1.p37–44

to the saponin compounds that act as immunomodulators by increasing the production and migration of macrophages to the wound area, thereby increasing the secretion of IL-1 cytokines and the proliferation of fibroblasts in the wound area. In addition, saponins play a role in activating the function of TGF-β. TGF-β will stimulate the migration and proliferation of fibroblasts.[26] Tannins in cocoa pod extract are thought to have a cellular mechanism for cleaning free radicals and reactive oxygen, as well as increasing the formation of capillary blood vessels and fibroblast migration and proliferation by inducing TGF-β.[27,28]

The number of osteoblasts in the CPH extract group showed higher results when compared to the positive control group. Alveogyl has an active ingredient in the form of iodoform, which is known to inhibit microbial growth, especially aerobic and facultative microorganisms.[29] The eugenol content in Alveogyl is known to have an anti-inflammatory effect by lowering IL-1, IL-6, and tumor necrotizing factor-α, which can reduce inflammatory cells.[30] Meanwhile, CPH extract contains tannins, alkaloids, saponins, and triterpenoids, which are known to have antibacterial, antioxidant, and anti-inflammatory activities. The CPH gel acts as a barrier to prevent pathogens from entering the wound while killing the microorganisms that do enter.[31] This can prevent infection in the wound, thereby helping the healing process.

Osteoblasts are known to originate from MSCs. MSCs in the osteoblast differentiation stage require the expression of specific genes, such as runt-related transcription factor 2 (RUNX2), that can increase osteoblast-related genes, such as alkaline phosphatase (ALP). At the proliferative stage, influenced by the activity of the ALP enzyme, osteoblast progenitor cells will turn into pre-osteoblasts. Then, at the maturation stage, pre-osteoblasts will become mature osteoblasts as indicated by increased expression of Osterix and secretion of bone matrix proteins, such as osteocalcin, bone sialoprotein I/II, and type I collagen.[31,32] Osteogenic characteristics stimulate the formation of osteoblasts. Expression of growth factors, including Bone Morphogenetic Protein-2 (BMP2), first regulates RUNX2, making cells more susceptible to BMP2, which then activates RUNX2 and other downstream mediators, inducing osteoblastogenesis.[33] Wounds from tooth extraction require fast healing so that complications do not occur afterward. Osteoclasts are an indicator of whether a tooth extraction wound is healing well or not.[34]

The ratio of osteoclasts and osteoblasts can show the dominance of the number of osteoclasts as cells that have a role in the bone resorption process to the number of osteoblasts as cells that have a role in bone formation. The results showed that the ratio of the number of osteoclasts and osteoblasts on day 7 to day 14 in each group increased (Table 3). The highest increase occurred in the CPH treatment group. This means that the bone resorption process is lower than the bone formation process in the molar tooth extraction socket. In the statistical analysis, the number of osteoclasts between all groups on day 14 did not show a significant difference. The high number of osteoclasts on day 7 after tooth extraction has an important function for the resorption of defects in the alveolar bone because the reduced number of osteoclasts in the early phase of remodeling can actually cause retention of damage (dead bones), so that the process of new bone formation by osteoblasts cannot occur.[35] Meanwhile, osteoclasts in the CPH group on day 14 show lower results when compared to the negative control group. This decrease is because cocoa pods contain flavonoids that can inhibit p38 phosphorylation. Extracellular signal-regulated kinase ½ (ERK) and c-Jun N-terminal kinase (JNK) belong to the mitogen-activated protein kinase (MAPK) family, which regulates the pathway of osteoclast formation and a further decrease in the expression of tartrate-resistant acid phosphatase (TRAP), an osteoclast marker associated with osteoclast differentiation.[36] In addition, triterpenoids in CPH are able to inhibit the process of osteoclast genesis by inhibiting the expression of TRAP,which has a function in osteoclast activity that causes the formation and function of osteoclasts to decrease, and their number will also decrease in the socket.[37] This study's limitation was that it only used a single dose of the extract, so the effective dose could not be observed.

In conclusion, extract of CPH (*Theobroma cacao* L.) has the potential for osteogenesis by significantly increasing fibroblasts and osteoblasts while also decreasing osteoclasts on the alveolar socket in white rats (*Rattus norvegicus*) post tooth extraction. Further research on CPH extract is necessary to study the molecular mechanism of osteogenesis biomarkers.

ACKNOWLEDGEMENT

Authors gratefully thank to the Institute of Research and Community Service, Universitas Jember, Indonesia.

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DOI: 10.20473/j.djmkg.v58.i1.p37–44