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Original Article

EFFECTIVENESS OF PROPOLIS EXTRACT GEL AS ADJUNCTIVE PERIODONTAL THERAPY: OVERVIEW OF FIBROBLASTS AND NEUTROPHILS (*IN VIVO* STUDY)

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ABSTRACT

Objective: Propolis has therapeutic activities contributed by its active secondary metabolites to fasten the healing of periodontitis. This study was to analyze the effectiveness of propolis extract gel as an adjunctive periodontal therapy.

Methods: The study used 50 periodontitis-induced Wistar rats, were divided into five treatment groups, and were treated by applying propolis gel with concentrations of 50%, 60%, and 70%, metronidazole gel, and placebo gel after mechanical therapy. The number of fibroblasts and neutrophils was obtained by removing the mandibular gingival tissue on the third and seventh days and were observed using a binocular microscope with 400x magnification using hematoxylin-eosin staining.

Results: The group administered with propolis extract gel showed a higher number of fibroblast cells and lower number of neutrophil cells. The 70% propolis gel appeared effective in inducing the fibroblasts and reducing the neutrophils ($p<0.05$).

Conclusion: Propolis extract gel can accelerate the healing of periodontitis and potentially be used as adjunctive therapy for periodontitis treatment.

Keywords: Propolis, Periodontitis, Fibroblast, Neutrophil

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INTRODUCTION

Periodontal diseases often occur in adults but can also found in children and adolescents and are also often found in the elderly [1]. Periodontitis develops over time with the accumulation of dental plaque that is not removed, which can result in bacterial dysbiosis which can progress over time to periodontitis and was characterized by periodontal pocket formation, gingival recession, tissue destruction, and loss of alveolar bone, which can ultimately lead to tooth loss. The severity of tissue damage is generally aligned with dental plaque levels, host defense factors, and other associated risk factors [2].

Periodontal disease is a global public health problem with the prevalence rate of up to 50% worldwide [3, 4]. The US Centers for Disease Control and Prevention and the American Academy of Periodontology estimated a prevalence of more than 50% in the United States and periodontitis is often encountered in older ages [5, 6]. The estimated prevalence of severe periodontitis in Indonesia is higher than the global average, with an age-standardized prevalence and incidence rate of 17% and 747 people per 100,000 population in 2010 (Kassebaum *et al.*, 2014). Based on 2018 National Basic Health Research (RISKESDAS) data, the prevalence of periodontitis in Indonesia in people aged ≥15 y is 67.8%.7 (Indonesian Ministry of Health, 2018). As a chronic form of noncommunicable disease, periodontitis shares risk factors with other noncommunicable diseases and has a bidirectional association with general health.8–10 The global prevalence of periodontal disease is expected to increase in the future due to the increasing number of elderly (elderly) [11, 12]. Tonetti *et al.* (2013) stated that periodontitis is a major public health problem due to its high prevalence and can cause tooth loss and disability, can have a negative impact on masticatory function and aesthetics, which is a source of social inequality and significantly disrupts the quality of life [12].

Moore *et al.* (1994) stated that microbiological studies carried out by cultivation showed that a lack of effort to maintain oral hygiene resulted in a shift in the dominant species to the subgingival area. Cytokines, chemokines and metalloproteinases are known to increase dramatically in periodontal tissue and gingival crevicular fluid in periodontitis [13, 14]. The subgingival environment in periodontitis is an area that can run into immune dysfunction that allows the proliferation of species that is normally controlled by host defenses.

Neutrophils are closely involved in the pathogenesis of periodontitis. Neutrophils represent the majority of leukocyte types (≥95%) that are recruited towards gingival crevicular and periodontal pockets, which are heavily populated by subgingival microbial communities [15]. Neutrophils peak on the third day and slowly return to baseline levels on day 7 [16-18]. Although neutrophils are necessary for immune homeostasis and contributing to healthy periodontal tissue, neutrophils can experience dysregulation in periodontitis conditions, which are hyperactive, excessive, or collapsed, which can cause tissue damage along with the release of toxic and inflammatory molecules, or protease that can degrade tissue [19]. Hajishengallis *et al.* (2020) stated that neutrophils can contribute to tissue damage in periodontitis, largely as a result of the defeat of neutrophils by microbes, resulting in dysregulation of the antimicrobial and inflammatory responses of neutrophils [20]. Maikawa *et al.* (2014) stated that the involvement of complement in both the dysbiosis and inflammatory processes that drive periodontitis occurs mostly through neutrophil-dependent effects, has encouraged many studies to develop interventions that can target the complement stage which has shown promising results in both periodontitis conditions. induced or naturally occurring in non-human primates [21, 22].

Fibroblasts are cells that are centrally involved in the regeneration of periodontal tissue structures in response to healing and play important roles in synthesizing and organizing collagen fibers that connect the alveolar bone and gingiva to the cementum that lines the root surface of the tooth. The synthesis and remodeling of new collagen matrix is very important for the repair of functional periodontal tissue and this process is mediated by a number of

fibroblast populations located in the gingival connective tissue and periodontal ligament [23]. Fibroblasts enter the proliferation phase on the third day and regulate along with polarization towards anti-inflammatory and pro-angiogenic activities. Fibroblasts are the main contributor to the accumulation of extracellular matrix during the maturation phase starting on day [7, 24].

Local application of agents inserted directly into the pocket can provide greater effect than orally. Antimicrobials such as tetracycline, chlorhexidine, metronidazole, doxycycline 10%, and minocycline 2% have been used as local agents in the treatment of periodontal diseases [25, 26]. However, continuous using of antimicrobials can result in resistance to the targeted microbes. Chlorhexidine is considered as the gold standard agent in the field of periodontics because of its large antimicrobial spectrum, biocompatibility, and effectiveness [27]. However, long using of chlorhexidine is cytotoxic to fibroblasts which can interfere with wound healing performance [28].

Natural resources have become one of the most interesting research hotspots in recent years due to their low adverse reactions, lower costs, and accepted generally by society [29]. Propolis is a sticky natural material, also known as bee glue, which is produced by honey bees from sap, resin and mucin collected from various parts of plants, such as leaves, flower buds and tree bark, which the bees then mix with wax bees and several bee enzymes [30]. Propolis shows safety and efficacy and has potential benefits in dentistry and oral health care based on *in vitro*, *in vivo*, ex vivo studies, as well as clinical trials in humans [31]. The chemical compositions of propolis are diverse and depend on its geographical and botanical origin such as climatic factors, plant resources, the place of origin, and the time of collection carried out by bees [32]. Raw propolis cannot be used directly in analysis or treatment. Propolis must be extracted first to dissolve and release its most active composition. Propolis extract can contain concentrations of up to 70% [33].

Nakao *et al.* (2020) revealed that propolis can reduce the number of *Porphyromonas gingivalis* bacteria in gingival crevicular fluid [34]. The antimicrobial activity of propolis exists at two levels. The first level of antimicrobial activity is related to its direct action on microorganisms, and the other level is to stimulate the immune system, which results in the activation of the organism's natural defenses [35]. The contents of propolis, such as flavonoids and other important phenolic compounds, provide antibacterial activity. Flavonoids are one of the largest classes of small molecular secondary metabolites produced in various parts of plants. Flavonoids exhibit various pharmacological and beneficial health effects for humans, namely antioxidant activity, ability to scavenge free radicals, anti-inflammatory and anticancer. Therefore, flavonoids receive high attention from the pharmaceutical and health industries. This compound was found to be a potent antimicrobial agent against various pathogenic microorganisms *in vitro* [36]. Thus, this study was aimed at analyzing the effectiveness of propolis extract gel as an adjunctive periodontal therapy.

MATERIALS AND METHODS

The study was approved by the Animal Research Ethics Committees/AREC of Universitas Sumatera Utara (protocol number 0250/KEPH-FMIPA/2023). The *in vivo* study is experimental laboratory research with a posttest-only control group design. The study protocol was created according to Animal Research Reporting *In vivo* Experiments (ARRIVE) Guidelines. The research was done in the animal housing facility at the Faculty of Mathematics and Sciences at Universitas Sumatera Utara. A total of 50 male Wistar rats (Rattus norvegicus), weighing 200-250 g, aged 8-12 w with lower central incisors and good health were used. The animals were housed in groups of 3 under a 12 h light dark cycle at room temperature with free access to water and food.

Animals were periodontitis-induced by ligation and bacterial infection. *Porphyromonas gingivalis* ATCC 33277 was cultured at Microbiology Laboratory at Universitas Sumatera Utara Hospital. Ligation with Silk 3/0 was done and placed simultaneously around incisor teeth in the subgingival position after adapted for one week. The animals were then infected with *Porphyromonas gingivalis* ATCC 33277 at the gingival sulcus. All experimental procedures were performed by an experienced clinician. Anesthesia was performed with ketamine before the induction and the treatment were applied to the animals. After 7 d, the periodontitis-induced Wistar rats were examined clinically and swabbed to check for *P. gingivalis* to confirm the periodontitis induction was successful. In this study, the propolis used in this study was sourced from the hives of *Geniotrigona thoracica*/kelulut bees from Kebun Efi, Kabanjahe. The hydroglyceric extract of stingless bee propolis was produced using a proprietary method that involves heating followed by filtration steps where the final product contains 100 % propolis extract. The propolis extract was then diluted and mixed accordingly to produce gel that contained hydroglyceric propolis extract. The gel products were produced in the Pharmacy Lab of Universitas Sumatera Utara. The gel contained propolis extract, aquadest, triethanolamine, and carbopol. Metronidazole gel was obtained from (Ti-es, Indonesia).

There were three treatment groups: the periodontitis-induced rats treated with 50% (I), 60% (II), and 70% (III) propolis extract gel. The positive control was the periodontitis-induced rats treated with metronidazole gel because of its usage as adjunctive periodontal treatment for its antibacterial and anti-inflammation properties. The negative control was the periodontitis-induced rats treated with placebo gel. Mechanical therapy was done by using a manual scaler. Then, depending on the group, the gel was applied once after the mechanical therapy was done for into the pocket of the gingiva by using disposable tip. Five rats from each group were anesthetized with ketamine on the baseline before the application of the gel, Mandibular segments were taken on the third day and the seventh day after treatment.

The histological parameter evaluated was the number of neutrophils and fibroblasts from the gingival tissue of periodontitis-induced rats at Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Sumatera Utara. An experienced clinician examined the histological parameter. Haematoxylin-Eosin staining was used to assessed the number of neutrophils and fibroblasts. Fibroblast cells and neutrophil cells in Wistar rats were counted using a binocular microscope with 400x magnification. The data obtained from the study were quantitative in the form of histological parameters before and after treatment on day 3 and 7. The Kruskal-Wallis test processed the data to see the difference between the groups on the third day and seventh day.

RESULTS

Evaluation of histopathological parameter showed that there were differences in the mean number of rat gingival tissue fibroblasts between groups; this can be seen in table 1.

Table 1 showed that there was a significant mean difference between propolis gel and the number of fibroblast cells on day 3, with the most effective concentration being 70% ($p<0.05$). Metronidazole gel had the lowest number of fibroblasts on day 3. There was no significant difference in the mean of propolis gel on the number of fibroblast cells on day 7 ($p>0.05$), but all groups treated with propolis had increasing fibroblasts number, with 70% propolis gel having the highest number of fibroblasts. Metronidazole gel had the lowest number of fibroblasts on day 7.

Table 1: The differences of the mean number of fibroblasts in the treatment groups

Day	Groups	The mean of fibroblasts±SD	*p*
Day 3	50% propolis gel	41.91±4.09	
	60% propolis gel	45.25±7.40	0.002*[a]
	70% propolis gel	42.87±7.74	
	Metronidazole gel	32.91±5.56	
	Placebo gel	44.95±5.39	
Day 7	50% propolis gel	43.41±3.35	

	60% propolis gel	45.95±8.37	0.864[b]
	70% propolis gel	49.50±10.87	
	Metronidazole gel	42.12±11.40	
	Placebo gel	42.55 ±7.08	

[a]One-Way Anova Test, [b]Kruskal-Wallis Test, *Significant (p<0.05), n=10

Table 2: The difference of the mean increase in the number of fibroblasts in the treatment groups

Groups	The mean increase of fibroblast cells±SD	*p*
50% propolis gel	1.50±0.74	
60% propolis gel	0.70±0.97	0.142
70% propolis gel	6.65±3.13	
Metronidazole gel	9.21±5.84	
Placebo gel	-2.40±1.69	

Kruskal-wallis Test. *significant (p<0.05), n=10

Table 2 showed that there was no significant difference in the mean increase in the number of fibroblast cells in all groups (p>0.05). Metronidazole gel group, as the positive control, has the highest mean increase in the number of fibroblasts and the placebo gel group, as the negative control, has decreased in the number of fibroblasts.

Table 3: The differences of the mean number of neutrophils in the treatment groups

Day	Groups	The mean of neutrophil±SD	*P*
Day 3	50% propolis gel	1.05±0.85	
	60% propolis gel	1.00±0.72	0.040*
	70% propolis gel	3.50±3.58	
	Metronidazole gel	1.12±1.57	
	Placebo gel	0.00±0.00	
Day 7	50% propolis gel	0.45±0.32	
	60% propolis gel	0.60±0.84	0.907
	70% propolis gel	1.43±2.87	
	Metronidazole gel	0.87±1.46	
	Placebo gel	3.80±5.68	

Kruskal-wallis Test, *Significant (p<0.05), n=10

Table 3 showed that 70% propolis gel had the highest number of neutrophils and the placebo gel had the lowest number of neutrophils on day 3. 70% propolis gel had the highest number of neutrophils and metronidazole gel had the lowest number of neutrophils on day 7. There was a significant difference in the mean of propolis gel on the number of neutrophil cells on day 3 (p<0.05) with the most effective concentration was 70% (p<0.05). There was no significant difference in the mean of propolis gel on the number of fibroblast cells on day 7 (p>0.05), but there was still decreases in neutrophil cells in all propolis gel groups.

Table 4: The difference of the mean decrease in the number of neutrophils in the treatment groups

Groups	The mean decrease of neutrophils±SD	*p*
50% propolis gel	0.60±0.53	
60% propolis gel	0.40±0.12	0.139
70% propolis gel	2.07±0.71	
Metronidazole gel	0.25±0.11	
Placebo gel	-3.80±5.68	

Kruskal-Wallis. Test *significant (p<0.05), n=10

Table 4 showed that there were decreases in the mean number of neutrophils in rat gingival tissue between groups except in the placebo gel group. There was no significant difference in the mean decrease of neutrophil cells (p>0.05), but propolis gel groups experienced decreases in neutrophil count. Table 4 showed that the 70% propolis gel group experienced the largest decrease in neutrophil count (2.07±0.71) and the placebo gel (-3.80±5.68) experienced an increase in neutrophil count on day 7.

DISCUSSION

The healing of can be seen from clinical parameters such as pocket depth, attachment loss, and alveolar bone loss that occurs [9]. Histological parameters are one of the parameters that can be used to evaluate the success of the therapy being tested to prove its effectiveness in accelerating healing and reducing inflammation [34]. This study used propolis extract gel, metronidazole gel (positive control), and placebo gel (negative control) as periodontal support therapy in Wistar rats (*in vivo*). The selection of Wistar rats was based on the periodontal tissue structure of rats, which was similar to that observed in the tissue structure of rats.

Hematoxylin-eosin staining has been tested over time as the standard staining for histological tissue examination. This simple coloring combination is able to highlight the fine structure of cells and tissues [37]. Presumptive or correct histological diagnosis is the starting point for relevant molecular investigations accompanied by the results of other clinical and laboratory tests that can provide important information for patient care management.

Table 1 shows that there was an increase in the number of fibroblast cells on day 7 with the highest number found in the 70% propolis extract gel group. The data obtained was supported by the statistical test, which showed that there was a difference in the mean number of fibroblast cells on day 3 (p<0.005). Table 2 shows that all treatment groups experienced an increase in the number of

fibroblast cells except for the placebo gel group. The data in table 2 shows that there was no difference in the mean increase in the number of fibroblast cells in the propolis gel group compared to the positive control group. Propolis gel has been proven to have anti-inflammatory properties and the results of this study are in line with previous research, which stated that propolis gel has anti-inflammatory capabilities and plays a role in the wound healing process. Gingival fibroblasts are an interesting sources of induced stem cells and are considered as one of the components that play a major role in regenerative dentistry [38, 39]. Gingival fibroblasts can also differentiate into vascular endothelial-like cells and vascular smooth muscle-like cells [40, 41]. Fibroblasts are part of connective tissue that are closely involved in the wound healing response in the inflammatory process by contributing to the formation of new tissue and the subsequent process of tissue remodeling [23].

The phase of new tissue formation begins around day 2 to day 10 after injury, which involves proliferation and migration of epithelial cells, connective tissue and endothelial tissue [42]. Table 2 showed that there was no significant difference in the mean increase in the number of fibroblast cells in all groups ($p>0.05$), with the positive control has the highest number of fibroblasts. All propolis group increased the number of fibroblasts, and the negative control has a decreasing number of fibroblasts. Fibroblasts, which play an important role in the formation of new tissue, which is a biological structure, can have a variable duration of healing and remodeling process and depend on many factors such as the location and size of the injury, tissue vascularity, the large number of cells that have the potential to repopulate, and the functional role of the tissue itself, where wounds whose edges are close (primary intention) can heal relatively quickly, while wounds whose edges are not close (secondary intention) show slower healing. One important aspect of the healing response in periodontal tissue is the regeneration of collagen fibers that connect the cementum that protects the tooth root surface with the alveolar bone, where fibroblasts in the lamina propria of the gingival connective tissue secrete and regulate different collagen networks [23]. Local fibroblasts can transform into myofibroblasts, which are stimulated by cytokines and growth factors [43].

Table 3 showed that the highest mean number of neutrophil cells was found in the 70% propolis extract gel group and the lowest mean was found in the placebo gel group on day 3. The data obtained was supported by the statistical test showing that there was a significant difference in the mean number of neutrophil cells on day 3 ($p<0.05$). Table 4 showed that the highest decrease in the average number of neutrophil cells was found in the 70% propolis extract gel group and an increase in the average number of neutrophil cells was found in the placebo gel group, though test showed that there was no significant difference in the mean decrease in the number of neutrophil cells in the treatment groups ($p>0.05$).

Propolis component that has anti-inflammatory properties, such as caffeic acid, can inhibit the synthesis of eicosanoids from arachidonic acid and suppress the activity of COX-1 and COX-2 enzymes, thereby inhibiting the release of inflammatory mediators such as PGE-2 (prostaglandin), leukotrienes and thromboxane, which results in increased expression IL-10 as propolis dose increases. Flavonoid compounds and CAPE can also inhibit leukotrienes from arachidonic acid through the lipoxygenase enzyme [44,45]. Propolis, as an anti-inflammatory agent, is able to inhibit prostaglandin synthesis, resulting in increased phagocytic activity and speeding up the healing process of epithelial tissue [46].

One of the main components of propolis, caffeic acid phenethyl ester (CAPE), which is a biologically active compound with anti-inflammatory properties, is able to inhibit Nuclear Transcription Factor-Kappa B (NF-kB) and release IL-1β, which stimulates interleukin-2 (IL-2) which triggers T-cell proliferation, which affects the cyclooxygenase and lipoxygenase pathways [47, 48]. Lipoxygenase is the main enzyme in neutrophils, which produces leukotrienes, while cyclooxygenase produces prostaglandins, which are mediators of inflammatory reactions. Defenses that can influence the reduction in leukotriene production will influence the activity of neutrophil phagocytes which will suppress the inflammatory process [49]. Cyclooxygenases activity, especially cyclooxygenase-2, is increased in periodontitis in response to periodontal pathogens [50–52]. Neutrophils in periodontal disease patients result in an increase in lipoxin A4, especially in patients experiencing localized aggressive periodontitis.52 Chronic periodontal lesions are characterized by hyperactivation of neutrophils with activated lipoxin pathways. Insufficient amounts of lipoxin result in resolution of inflammation not being achieved [53].

Sahib *et al.* (2020) showed that treated wounds had increasing epithelial tissue coverage accompanied by a decrease in the number of neutrophils and macrophages and an increase in fibroblasts compared to the group not given propolis [54]. Marquele-Oliveira *et al.* (2019) showed that propolis induces inflammation during the healing process followed by a substantial reduction in inflammation. The study stated that propolis-enriched cellulose membranes induced higher levels of inflammation with neutrophil infiltration in wounds compared to the control group on the second day. However, the propolis group experienced much less inflammation on the seventh day and had faster wound healing rates [55].

Propolis, with its inflammation-modulating properties, can play a role in regulating the immune system [56]. Hasmy *et al.* (2017) stated that propolis can improve the healing process of gingivitis by reducing the number of neutrophil cells, increasing the number of fibroblasts, and accelerating the formation of new blood vessels (neoangiogenesis) *in vivo* [57]. Sukmawati *et al.* (2021) stated that patients who were treated with propolis irrigation after gingival curettage had better periodontitis recovery parameters as measured by plaque index, pocket depth, and decreased interleukin-1β (IL-1β) compared to patients treated with conventional tetracycline treatment [46].

Gjertsen *et al.* (2011) stated that 50% propolis was significantly more effective in maintaining periodontal ligament cell viability compared to milk, saline, and Hank's Balanced Salt Solution (HBSS) and reduced the level of fibroblast apoptosis compared to HBSS by increasing mitochondrial enzymatic activity [58,59]. The mechanism underlying the potential cell viability of propolis is associated with the active compounds contained in propolis, such as flavonoids and caffeic acid phenethyl ester (CAPE), which have cytoprotective and DNA-protective effects in inflammatory pathological conditions [60–62]. The anti-inflammatory properties of flavonoids are in accordance with the results obtained from research by Alipour *et al.* (2021) which states that flavonoids derived from plants exhibit anti-inflammatory properties [63]. The toxicity of propolis against fibroblast cells is low and is able to inhibit apoptosis and stimulate proliferation of fibroblast cells and has been evaluated for its biocompatibility in subcutaneous tissue as well as reducing inflammation significantly through the role of IL-10, as an inflammatory cytokine TGF-β, as a regulator of cell proliferation and differentiation; VEGF, a protein that helps the angiogenesis process; and IL-8, a pro-inflammatory cytokine recruited by neutrophils for the repair of the damaged tissue, carried out in mouse models [44, 64-66].

Good regulation of inflammation can result in faster healing progress. Eyarefe *et al.* (2019) stated that treatment with propolis induces higher levels of inflammation in wounds. Propolis induces higher inflammatory infiltrates such as monocytes, macrophages, eosinophils, mast cells and platelets. However, the inflammatory response regressed on day 8 and induced significantly better wound healing compared to the untreated control group which experienced a worse and persistent healing response on day 16 [67]. The results of this research are in accordance with the research results obtained, namely that there was a decrease in the number of neutrophil cells after the application of propolis gel. Propolis appears to trigger an intense inflammatory response early in the healing process. However, inflammation then appears to decrease markedly after a sharp increase in inflammation in the initial phase, resulting in significantly faster healing of damaged tissue.

Chronic periodontitis treatment may require additional therapy to achieve optimal results. Propolis is a natural material that has advantages compared to synthetic products, such as low toxicity, minimum side effects, and does not cause bacterial resistances.

Propolis has anti-inflammatory and antibacterial properties. In this study, the use of propolis gel with a concentration of 70% had the best effectiveness as an additional therapy that can accelerate periodontal tissue healing and does not cause destructive effects. Propolis can be a promising alternative ingredient as an additional therapy for periodontitis patients.

CONCLUSION

Propolis extract gel can accelerate the healing of periodontitis and potentially be used as adjunctive therapy for periodontitis treatment. Propolis gel with a concentration of 70% had the best effectiveness to promote faster healing of periodontitis.

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AUTHORS CONTRIBUTIONS

JM, AD, PW, WA, SI, OAH: Researcher, Critically Revised the Manuscript. JM: Writing, Analysis, Interpretation. JM, WA, SI, OAH: Conception and Data Design. JM, AD, PW: Analysis, Writing and Performed the Experiments.

CONFLICT OF INTERESTS

Declare that there is no conflict of interest regarding the publications of this paper.

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