ORIGINAL ARTICLE

Effect of *Citrus Sinensis* Peel Extract Gel on Periodontal Healing in Rat Model

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ABSTRACT

Introduction: Periodontitis is chronic inflammatory disease which could lead to the loss of teeth. *Citrus sinensis* peel extract gel is a potent host modulating therapy due to its rich flavonoid contents. The purpose of this study is to determine the efficacy of a 10% *C. sinensis* peel extract gel application following curettage. In periodontal disease healing in rat model. **Methods:** The lower incisor of 24 Sprague Dawley rat were ligated to induce periodontitis. The negative control group received 2% CMC-Na, treatment group received 10% *C. sinensis* peel extract gel, and the positive control group received Gengigel® gel following curettage. At days 3, 5, 7, and 14, two rats from each group were euthanized. Histological examination was conducted to assess angiogenesis, collagen density, fibroblast, and osteoclast count. Two-way ANOVA and the Post Hoc test were used to analyze the data. **Results:** Angiogenesis and fibroblast count were statistically significantly different in the treatment group ($p<0,05$) among the negative, treatment and positive control groups at day 3, 5, 7, and 14. Collagen density on treatment group was statistically different ($p<0,05$) between negative, control, and treatment group at day 7 and 14. At days 3 and 7, the osteoclast count in the treatment group was statistically different from that in the negative control group. **Conclusion:** Application of 10% *C.sinensis* peel extract gel following curettage increased angiogenesis, fibroblast count, collagen density, and decreased osteoclast count in periodontal tissue in rat models.

Keywords: Periodontitis, 10% *Citrus sinensis* peel extract gel, Angiogenesis, Fibroblast, Collagen density, Osteoclast

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INTRODUCTION

Periodontitis is a chronic inflammation that could lead to progressive damage to the supporting tissues of the teeth due to the penetration of microorganisms and microbial products. Periodontitis is characterized by gingival recession, periodontal pockets, decrease in alveolar crest height and loss of attachment of the teeth's supporting tissues. The pathological hallmarks of periodontitis include the breakdown of collagen fibers in the periodontal ligament (PDL), the destruction of fibroblasts in the PDL, alterations in the microvasculature, and the activation of osteoclasts that occurred along Initial lesion, early lesion, established lesion, and advanced lesion of periodontitis which involved the interaction between periodontopathogen and host immune cells (1).

Scaling and root planing has been considered as the gold standard for periodontitis treatment. However, moderate periodontal pocket required more complex periodontal surgery such as curettage. Curettage is a periodontal procedure that involves scraping the gingiva of the periodontal pocket in order to eliminate local factors, such as debris, pathogens and inflammatory tissues, as well as reduce pockets and facilitate new attachments (2). Nevertheless, curettage is frequently ineffective in deeper pockets. Therefore, the use of adjuvant therapy after surgery could increase periodontal repair. One of the promising natural herb which could be applied topically into periodontal pocket as an adjuvant therapy is 10% *Citrus sinensis* peel extract gel (3, 4, 5).

Citrus sinensis peel extract contains flavonoids, vitamin C, tannins, and saponins (6, 7). It also contains three types of flavonoids namely hesperidin, rutin, and quercetin (2, 8, 9). Hesperidin is a type of flavonoids mostly found in *Citrus sinensis* compared to other oranges, which is 28.5 – 73.8 mg/g FM (7). Hesperidin contains an angiogenic element that may promote the development of new blood vessels by inducing VEGF (10, 11). Additionally, hesperidin acts as an immunomodulator, stimulating the production of growth factors, such as TGF-β, fibronectin, fibroblast stimulating factor, and collagenase which stimulate the proliferation of fibroblasts (4, 12). Hesperidin could also inhibit NF-kB, thus lowering the number of osteoclasts. In addition, rutin could inhibit RANKL activation, while quercetin is able to inhibit NF-kB and AP-1. In fact, rutin and quercetin could also form type III collagen and produce IGF-1 as a mediator for fibroblast proliferation and collagen synthesis (2, 8, 9). Tannins and saponins increase the production of VEGF which then induces the proliferation of endothelial cells, thus increasing angiogenesis (13). Vitamin C could increase collagen deposition and the proliferation of endothelial cells during angiogenesis processes as well as accelerate the formation of the collagen triple helix; it is an important component in bone formation (14, 15, 16).

Gel is one of the formulations intended for topical treatment. Gel is a semi-solid substance composed of a suspension of dissolved substances. Effective delivery of therapeutic compounds is an advantage of utilizing a gel. The using of gel which contain active therapeutic compounds is beneficial in periodontal treatment since gel could deliver antibiotic or anti-inflammatory agent into periodontal pocket. Gel also could occupy the spaces inside periodontal pocket and stable enough from several hours to days. Gel is more effective than solution (17). In previous in vivo experiments, gel containing 10% *Citrus sinensis* peel extract was found to improve the epithelialization of gingival wound healing in rats compared to other gel. (14). The purpose of this study was to determine the effect of 10% *Citrus sinensis* peel extract gel applied following curettage on alveolar bone angiogenesis, periodontal ligament fibroblasts, and the density of collagen fibers and osteoclasts in alveolar bone in periodontitis healing in *Rattus norvegicus*.

MATERIALS AND METHODS

This study was ethically approved by The Research Ethics Commission, Faculty of Dentistry, Universitas Gadjah Mada with reference number 00252/KKEP/FKG-UGM/EC/2019. Three materials were used in this study: 10% *Citrus sinensis* peel extract gel for the treatment group (group II), 2% CMC-Na for the negative control group (group I) , and 0.25% hyaluronic acid (Gengigel® Gel) for positive control group (group III). *Citrus sinensis* peel extract was produced using maceration with 70% ethanol as the solvent. *Citrus sinensis* peel that has been dried and ground into a powder is combined with 70% ethanol, agitated for 30 minutes with an electric stirrer, and then let to stand for 24 hours. The results of stirring are filtered three times. The filtration process produces dregs and filtrate. The filtrate was evaporated at 70^{o}C using a vacuum rotary evaporator to produce a thick extract. After heating the fluid extract to 50 oC, a dry *Citrus sinensis* peel extract was obtained. In addition, 1 g of *Citrus sinensis* peel extract was blended with 9 g of 2% Na CMC to produce 10 g of *Citrus sinensis* peel extract gel.

The subjects of the study consisted of 24 male *Rattus norvegicus* of Sprague dawley strain aged 2-3 months of which the body weight was 120-150 grams. Three groups of rats were randomly chosen and divided; each of which consisted of eight animals. Prior to inducing periodontitis, these *Rattus norvegicus* were given an intraperitoneal administration of 50 mg/kgBW ketamine HCL and 5 mg/kgBW xylazine to provide a sedative effect (18). Periodontitis induction was done by binding and suturing 3.0 silk ligature as an irritant in "8" around the lower central incisors as in Fig.1. The ligature was circled four times, then the knot was fixed to the interdental gingiva. Ligation was maintained for 7 days (19). The characteristic of periodontitis was evaluated by the presence of accumulated debris and plaque, the formation of periodontal pockets with a depth of less than 5 mm, bleeding on probing, and tooth mobility. The periodontal pocket was evaluated using University of North Caroline 15 (UNC-15) Periodontal probe which has markings every 1 mm. The tooth mobility degree 2 (2 mm mobility) was used as parameter of periodontitis.

Curettage on the gingival wall of the periodontal pocket was performed by placing a curette on the lower side of the junctional epithelium where the sharp side of the curette faced the wall of the periodontal pocket, followed by scraping with vertical stroke until completely removed (20). The three materials were topically applied 1 time after curettage, using an injection syringe of which the needle had been removed, into the periodontal pocket. The amount of gel to be applied into periodontal pocket is about 0.5 cc (4, 19). Euthanasia was performed on days three, five, seven, and fourteen using a cervical dislocation method. Histological preparations were then taken from the periodontal tissues of the mandibular central incisor of *Rattus norvegicus* by Hematoxylin-Eosin (HE) and Mallory Trichrome stain. Fibroblast and angiogenesis evaluation were assessed on day three, five, seven, and fourteen. While collagen density and osteoclast were assessed in day three, seven, and fourteen. The consideration of the timing of assessment day for histological analysis was based on the pattern

of activity of cells and related tissues from previous studies. The pattern of changes in fibroblasts and angiogenesis followed the pattern of day three, five, seven, and fourteen. While the pattern and peak of osteoclast activity and collagen density occurred on day three, seven, and fourteen (4, 12, 13).

Angiogenesis, periodontal ligament fibroblasts, and osteoclasts were observed using a light microscope equipped with a 520x magnification OptiLab Viewer with 5 fields of vision, while the density of the collagen fibers with 6 fields of vision (21). Every section of the histological tissues, apart from the density of the collagen fibers, was observed by 2 observers who had previously been calibrated and the results were averaged. The density of the collagen fibers was determined using Image J software, with the results expressed as a percentage.

The Shapiro-Wilk test was used to determine the normality of the obtained data, and Levene's Test was used to determine the homogeneity. If the data were normally distributed and homogeneous, a Two-way ANOVA was conducted, subsequently by a Post Hoc test using the Least Significant Difference (LSD) method. All tests were conducted with a 95% significance level ($p<0.05$).

RESULTS

The study's findings indicated that topical application of 10% *Citrus sinensis* peel extract gel could increase alveolar bone angiogenesis, the number of periodontal ligament fibroblasts, and the density of alveolar bone collagen fibers, while decreasing the number of osteoclasts. The selection of rats with periodontitis induced by induced ligation of the mandibular anterior teeth was the most novel aspect of this study. The topical application of 10% C. sinensis gel to healthy gingival wounds in rats has been the subject of previous research. The novel aspect of this study is the research on animal models with periodontitis.

Angiogenesis

It can be concluded from Table I that each group's blood vessel count increased from day 3 to day 7, but decreased from day 7 to day 14. The Anova test with two variables revealed a significant effect ($p<0.05$), followed by a Post Hoc test using the Least Significant Difference (LSD) method. On days 3, 5, 7, and 14, the Post Hoc LSD test revealed a significant difference ($p<0.05$) between the CMC-Na group, the *C.sinensis* group, and the hyaluronic acid group group. However, no significant difference ($p>0.05$) was observed between the *C.sinensis* group and hyaluronic acid group groups on days 3, 5, 7, and 14; and between *C.sinensis* group and hyaluronic acid group groups on days 5 and 14.

Fibroblasts

The histological image of fibroblast in this study can be seen in Fig.2. According to Table II, the number of fibroblasts increased from day 3 to day 7 in each group, before decreasing on day 14 in *C.sinensis* group and hyaluronic acid group. However, it continued to increase in CMC-Na group. ANOVA with two variables revealed a significant effect ($p<0.05$) followed by a Post Hoc test using the Least Significant Difference (LSD) method. On days 3, 5, and 7, the Post Hoc LSD test revealed a significant difference ($p<0.05$) between CMC-Na group, *C.sinensis* group and hyaluronic acid group, as well as between *C.sinensis* group and hyaluronic acid group on day 14. However, no significant difference ($p>0.05$) was observed between *C.sinensis* group and hyaluronic acid group on days 3, 5, and 7, or between CMC-Na group, *C.sinensis* group and hyaluronic acid group on day 14.

Density of collagen fiber

As viewed on Table III, the density of collagen fiber increased from day 3, 7, and 14 in each of the groups. The highest density of collagen fiber was found on day 3 in the *C.sinensis* group, and on days 7 and 14 in the hyaluronic acid group. The two-

Table I : Comparison of the number of blood vessel among three groups on days three, five, seven, and fourteen

Day	Number of blood vessel (Mean ± SD)			P	Intergroup comparison (using LSD test)		
	I	II	III		I-II	I-III	II-III
3	0.70 ± 0.86	1.70 ± 0.66	1.80 ± 1.01	< 0.05	0.035*	0.23*	0.817
5	1.75 ± 0.85	2.70 ± 0.80	2.75 ± 1.29	< 0.05	0.044*	0.35*	0.908
7	2.85 ± 1.39	3.85 ± 1.31	4.35 ± 1.14	< 0.05	0.035*	0.004*	0.259
14	1.80 ± 1.20	2.75 ± 1.25	2.85 ± 0.93	< 0.05	0.044*	0.028*	0.817

I: CMC-NA; II:*Citrus sinensis* peel extract gel; III:Hyaluronic acid gel; *statistically significant

Table II : Comparison of the number of fibroblast among three groups on days three, five, seven, and fourteen.

Day	Number of Fibroblast (Mean ± SD)			P	Intergroup comparison (using LSD test)		
	I	II	III		I-II	I-III	II-III
3	0.05 ± 0.22	12.07 ± 6.24	14.45 ± 5.404	< 0.05	0.004*	0.001*	0.465
5	5.55 ± 1.63	17 ± 11.04	18.05 ± 6.18	< 0.05	0.005*	0.003*	0.761
7	8.2 ± 4.29	31.05 ± 17.26	31.95 ± 15.98	< 0.05	0.000*	0.000*	0.795
14	20.25 ± 11.76	17.55 ± 5.07	27.05 ± 11.61	< 0.05	0.440	0.067	0.016*

I: CMC-NA; II:*Citrus sinensis* peel extract gel; III:Hyaluronic acid gel; *statistically significant.

Table III : Comparison of the collagen density among three groups on days three, seven, and fourteen

Day	Collagen density (Mean ± SD)			P	Intergroup comparison (using LSD test)		
	I	II	III		I-II	I-III	II-III
3	24.760 ± 2.765	28.567 ± 2.121	30.599 ± 3.039	< 0.05	0.141	0.198	0.827
7	29.282 ± 5.593	36.228 ± 4.644	38.758 ± 1.201	< 0.05	0.023*	0.008*	0.515
14	28.651 ± 6.743	38.126 ± 4.180	42.100 ± 3.712	< 0.05	0.017*	0.003*	0.263

I: CMC-NA; II:*Citrus sinensis* peel extract gel; III:Hyaluronic acid gel; *statistically significant.

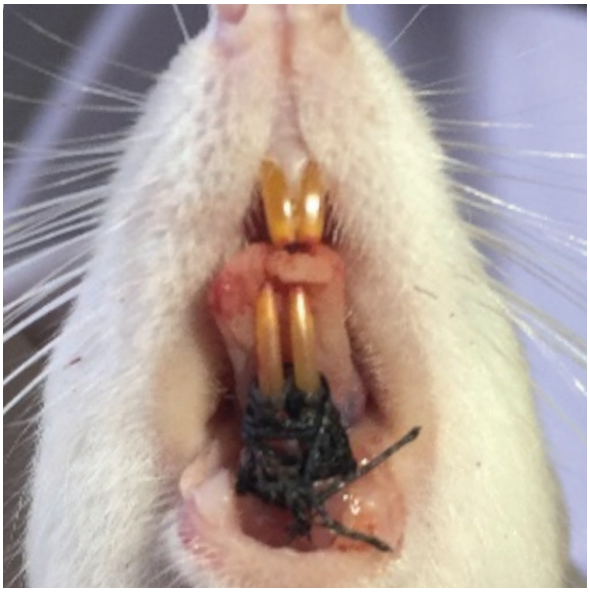

Fig. 1 : Periodontitis induction in rat incisor. Silk suture was placed around the lower incisor to induce periodontitis.

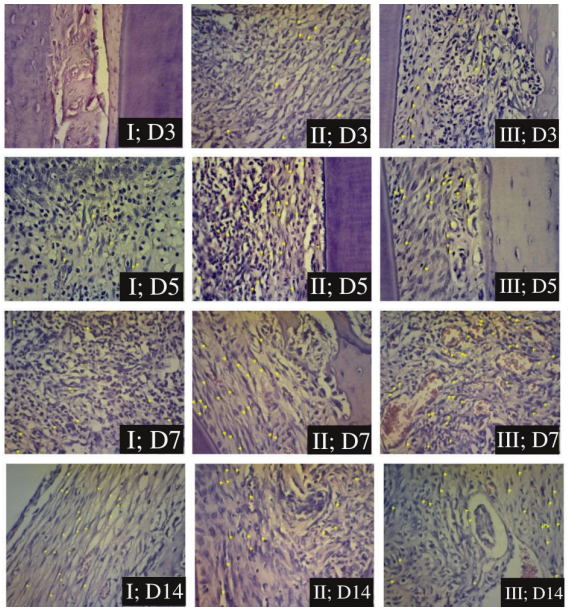

Fig. 2 : Samples of histological image of fibroblast among day three, five, seven, and fourteen from all groups. I: CMC-NA; II: *Citrus sinensis* peel extract gel; III: Hyaluronic acid gel; D: day. Yellow arrow indicating fibroblast.

way Anova test revealed a statistically significant effect ($p<0.05$). On days 7 and 14, the Post Hoc LSD test revealed a significant difference ($p<0.05$) between *C.sinensis* group and hyaluronic acid group. On days 3, 7, and 14, no significant difference ($p>0.05$) was observed between *C.sinensis* group and hyaluronic acid group, as well as between CMC-Na group, *C.sinensis* group and hyaluronic acid group on day 3.

Osteoclasts

Based on Table IV it can be concluded that the number of osteoclasts decreased from day 3 to day 14 in each of the groups. The histological image of osteoclast in this study can be seen in Fig.3. The CMC-Na group had the most osteoclasts, while the hyaluronic acid group had the fewest. The Anova two-way test revealed a significant effect ($p<0.05$), followed by a Post Hoc test using the Least Significant Difference (LSD) method. On days 3 and 7, the Post Hoc LSD test results revealed a significant difference ($p<0.05$) between the CMC-Na, *C.sinensis*, and hyaluronic acid group, as well as between the *C.sinensis* group and the hyaluronic acid group, and between the *C.sinensis* group and the hyaluronic acid group on day 14. However, there was no statistically significant difference ($p>0.05$) between the CMC-Na group observed on day 14, the *C.sinensis* group observed on day 7, or the three groups observed on day 14.

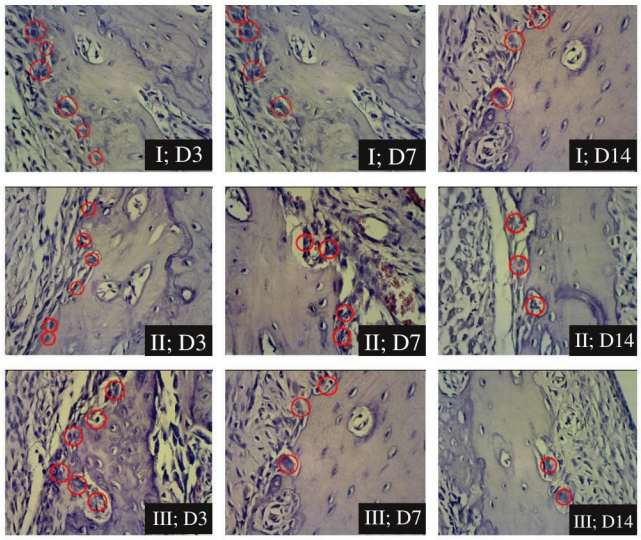

Fig. 3 : Samples of histological image of osteoclast among day three, seven, and fourteen from all groups. I: CMC-NA; II: *Citrus sinensis* peel extract gel; III: Hyaluronic acid gel; D: day; Red circle indicating osteoclast.

DISCUSSION

Angiogenesis

On the third day after tissue damage, the formation of blood vessels by Basic Fibroblast Growth Factor (bFGF) starts and continues taking place until the fifth day (13). Angiogenesis reaches its peaks on the seventh day, resulting in a large number of blood vessels (23) . On day 14, there is a decrease because the formation is already complete and stable, so excess cells undergo apoptosis (23, 24).

As shown in Table I, there was a significant difference between the CMC-Na group, *C.sinensis* group, and hyaluronic acid groups on days three, five, seven, and fourteen. The *C.sinensis* group contained flavonoids which have anti-inflammatory activity, thus decreasing the number of inflammatory cells and accelerating proliferation phase (25, 26). The hesperidin content has angiogenic factors which stimulate the formation of new blood vessels by inducing VEGF (10, 11). The tannin and saponin contents also have strong angiogenic factors which increase VEGF thus increasing endothelial cell proliferation (27). The vitamin C content could increase the proliferation of endothelial cells thus increasing the blood vessel count (14). 0.2% hyaluronic acid has anti-inflammatory effect, increases cell proliferation, accelerates angiogenesis, and accelerates the regeneration of new healthy tissue (21, 28). It is unlike the CMC-Na which acted as a stabilizer, emulsifier, thickening agent, gelling agent, and did not have active compounds to increase the number of blood vessels (29).

Table IV : Comparison of the number of osteoclast among three groups on days three, seven, and fourteen.

Day	Number of osteoclast (Mean ± SD)			P	Intergroup comparison (using LSD test)		
	I	II	III		I-II	I-III	II-III
3	6.70 ± 1.42	5.10 ± 0.78	4.50 ± 1.05	< 0.05	0.003*	0.000*	0.165
7	3.82 ± 0.59	2.90 ± 0.553	2.20 ± 0.52	< 0.05	0.040*	0.002*	0.112
14	2.25 ± 0.62	1.95 ± 0.826	1.85 ± 0.49	< 0.05	0.469	0.314	0.807

I: CMC-NA; II: *Citrus sinensis* peel extract gel; III:Hyaluronic acid gel; *statistically significant.

On days five and fourteen, there was no significant difference between the CMC-Na, *C.sinensis* group, and hyaluronic acid groups. This is because the formation of blood vessels has stabilized and the tissue's oxygen requirements have been met, resulting in a decrease in the number of blood vessels on day fourteen and apoptosis of excess cells. (24, 25).

Fibroblasts

Fibroblast recruitment starts on day three, then fibroblast proliferation increases until day five, and reaches its peak on day seven. On day fourteen the number of fibroblasts decreases and the remodeling phase starts (30). There was a significant difference between the groups of CMC-Na, *C.sinensis* group, and hyaluronic acid group. The *C.sinensis* group received a gel containing 10% *Citrus sinensis* peel extract which contains antioxidant and anti-inflammatory effects, and capable of increasing growth factor production, such as fibroblast stimulating factor, fibronectin, and collagenase, which are able to increase the proliferation of fibroblasts (12, 31). The positive control group was given the application of 0.2% hyaluronic acid (Gengigel® gel) which is able to inhibit the expression of IL-1β so it has an anti-inflammatory effect and is able to stimulate growth factors for the proliferation of fibroblasts (32). Meanwhile, the CMC-Na group group was given the aplication of 2% CMC-Na which does not have any active compounds that could increase the number of fibroblasts (29).

On day fourteen, there was no significant difference between the CMC-Na group, *C.sinensis* group, and positive control groups. The *C.sinensis* group and the positive control groups could well stimulate the proliferation of fibroblasts, thus producing quite-dense collagen fibers to start the remodeling phase, thereby lowering the production of growth factor and stopping the proliferation of fibroblasts (33, 34). Nonetheless, the number of fibroblasts in the CMC-Na group still increased due to slow healing, making collagen deposition insufficient to start the remodeling phase, so the proliferation of fibroblasts still took place (35).

Density of colagen fiber

Collagen formation starts on day three (36). It can be clearly seen on day seven because the number of fibroblasts increases significantly and collagen synthesis can also be seen significantly (34, 37). On day fourteen, collagen synthesis reaches its peak in the remodeling phase (34).

There was statistically significant difference between the groups of CMC-Na groups, *C.sinensis* group, and hyaluronic acid group. The *C.sinensis* group received a gel containing 10% *Citrus sinensis* peel extract which contains hesperidin which can inhibit the activity of MMP-8 which is collagenase against collagen and increase the synthesis of collagen fibers (38). Rutin and quercetin could help the proliferation of fibroblasts, thus accelerating collagen synthesis (38). The vitamin C content accelerates the formation of collagen triple helix which then accelerates collagen synthesis (16). Hyaluronic acid 0.2% (Gengigel® gel) has an anti-inflammatory effect which could accelerate the proliferation phase to stimulate collagen secretion (3, 28, 35). CMC-Na 2% is only a gelling agent which does not have any therapeutic effect (29). On day 3, there was no significant difference between the CMC-Na group, *C.sinensis* group, and hyaluronic acid group because overlapping between the inflammatory phase and the proliferation phase of periodontitis healing takes place, resulting in only a few number of collagen fibers synthesized (36).

Osteoclasts

The number of osteoclasts decreases from day three to day fourteen. Regarding periodontitis condition, there is an increase in the mediators of bone resorption from excessive chronic inflammatory cells in the bone such as T lymphocytes, B lymphocytes, and macrophages. The mediators of bone resorption such as interleukin (IL-1β), IL-6, and Tumor Necrosis Factor-α (TNF-α) increase the expression of Receptor activator of nuclear factor kappa-B ligand (RANKL) which then bind to Receptor activator of nuclear factor kappa-B (RANK) on the surface of pre-osteoclasts, then induce these cells to differentiate into osteoclasts which serve as bone resorption cells. Inflammatory mediators increase directly due to periodontitis induction administered, and the highest number of cells is found on day 3 but decreases gradually thereafter. Osteoclasts last 8-10 days according to their lifespan. Consequently, the osteoclasts on day three after *C.sinensis* group are histologically higher in number than those on the other days (30, 40, 41).

A significant difference occurred among the CMC-Na group, *C.sinensis* group, and hyaluronic acid group. The group of positive controls was given the application of 0.2% hyaluronic acid (Gengigel® gel) which could lower IL-6 and Activator Protein 1 (AP-1). The *C.sinensis* group group received a gel containing 10% *Citrus sinensis* peel extract which contains hesperidin, rutin, and quercetine which can lower NF-kB and AP-1. Mediators of bone resorption such as IL-6 and activation of AP-1 and NF-kB could increase the expression of RANKL which then bind to RANK on the surface of pre-osteoclasts, which then

induce these cells to differentiate into osteoclasts which serve as bone resorption cells. Meanwhile, the CMC-Na group group was given the application of 2% CMC-Na which does not have any therapeutic effect (9, 20, 21, 42, 43).

On day 14, there was no significant difference among the CMC-Na group, *C.sinensis* group, and positive control groups, owing to the fact that alveolar bone damage does not occur continuously in periodontitis. On day 14, the osteoclast count is lowest. The reduction in the number of osteoclasts is a result of cells undergoing apoptosis or osteoclast release from the resorption area caused by the expression of calcitonin, interferonγ, and TGFβ as negative-feedback mechanism in the bone remodeling phase. In fact, bone density starts to increase, characterized by an increase in the number of osteoblasts in the area of resorption (40, 44).

On each variable, there was no significant difference between the *C.sinensis* group and hyaluronic acid groups, proving that 10% *Citrus sinensis* peel extract gel has therapeutic effectiveness which is almost the same as that in 0.2% hyaluronic acid (Gengigel® gel) as anti-inflammation, stimulating the production of growth factors, increasing the proliferation of endothelial cells and fibroblasts, accelerating collagen deposition during the remodeling phase, and accelerating the healing of bone damage by lowering IL-6, NF-kB, and AP-1 (21, 28, 39, 42, 43, 44, 45). Thus, 10% *Citrus sinensis* peel extract gel has the potential to be an ingredient for an adjunctive therapy after curettage that supports the healing of periodontitis. Based on the study that had been conducted, future studies are recommended to observe other variables in the healing of periodontitis such as neutrophil count, macrophages, epithelial thickness and attachment of periodontal ligaments; perform toxicity testing and release profile of active substances in 10% *Citrus sinensis* peel extract gel in terms of half-life and bioavailability and; use Tartrate-Resistant Acid Phosphatase (TRAP) stain in the making of histological preparations to count the number of osteoclasts.

CONCLUSION

The administration of 10% *Citrus sinensis* peel extract gel into periodontal pockets following curettage improved the number of fibroblasts, angiogenesis, and collagen fiber density, and accelerating the reduction of osteoclasts count during healing process of periodontitis in *Rattus norvegicus*.

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