The effect of lime (Citrus Aurantifolia Swingle) peel extract in periodontal dressings on the number of fibroblasts in the gingival wound healing process

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ABSTRACT

Background: Periodontal dressing commonly used in dentistry today does not contain compounds that can accelerate wound healing. Lime (Citrus aurantifolia Swingle) peel contains flavonoids that play a role in increasing fibroblast cells so that they can accelerate the healing process. Periodontal dressings supplemented with lime (Citrus aurantifolia Swingle) peel extract are expected to provide an alternative material that can accelerate wound healing in addition to closure. Purpose: The study aims to determine the effect of adding lime (Citrus aurantifolia Swingle) peel extract to periodontal dressings on the increase in the number of fibroblasts in the gingival healing process. Methods: The study was conducted in an experimental laboratory in vivo. The study used a post-randomised control group of 32 rabbits with lesions of the mandibular gingiva using a 2 mm diameter punch biopsy. The experimental animals were divided into 8 groups, namely the control group, which was treated with periodontal dressings without the addition of lime (Citrus aurantifolia Swingle) peel extract, and the treatment group, which was treated with periodontal dressings with the addition of the extract. Histological observations of the tissues were performed with HE staining to count the number of fibroblasts. Results: Statistical test results showed that there was a significant difference in the number of fibroblasts between the control group and the treatment group on day 3 and day 5 (ANOVA, p <0.05). Conclusion: Adding extra lime (Citrus aurantifolia Swingle) peel to the periodontal dressing increases the number of fibroblast cells after gum injury.

Keywords: fibroblasts; lime (Citrus aurantifolia Swingle) peel extract; periodontal dressing; wound healing

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INTRODUCTION

Periodontal dressings are commonly applied to open wounds after periodontal treatment. The periodontal dressings used in dentistry today do not contain compounds that can accelerate wound healing, only protecting wound tissue rather than providing healing factors. Gum damage can occur due to periodontal disease, trauma, tooth extraction or oral surgery. In surgical procedures such as gingivectomy and depigmentation, an incision in the gum tissue is made to provide access and field of view and repair morphological and anatomical damage. Restoring the integrity of damaged tissue and maintaining homeostasis is an important procedure in healing gum wounds. A periodontal dressing is a physical barrier that serves to protect patients from pain due to contact of the wound with food or with the tongue during chewing, provides comfort to the patient, allows the tissues to adapt to the process of wound closure, and minimises postoperative bleeding and the possibility of infection. The healing process takes place in several phases, including the proliferative phase. An indicator of wound healing in the proliferative phase is characterised by an increase in the number of fibroblasts during this phase. Fibroblasts are cellular components commonly found in connective tissue. Fibroblasts are responsible for the formation of collagen, the main constituent of the extracellular matrix, which is useful for strengthening scar tissue, cell contraction, influencing the re-epithelialisation.
process, and forming granulation tissue in the process of angiogenesis. Thus, fibroblast cells play an important role in the wound-healing process.5

The use of natural ingredients is increasing, both in medicine and for other purposes. Natural ingredients have the following advantages: easy to obtain; inexpensive; minimal side effects; and, generally, a plant can have more than one pharmacological effect. Lime (Citrus aurantifolia Swingle) is one of the herbal remedies that can be used as an additional ingredient in periodontal dressings.6

Linden (Citrus aurantifolia Swingle) is a type of herbaceous plant widely cultivated in Indonesia, both in gardens and plantations. Compared to other types of citrus, Citrus aurantifolia has more variations and applications, so it is often referred to as a versatile fruit.7

Lime (Citrus aurantifolia Swingle) peel contains beneficial compounds such as flavonoids, alkaloids, tannins and saponins, which can be used for wound healing.8 The peel has a higher concentration of flavonoids compared to other parts, such as seeds, fruits, and lime juice.9 The flavonoids are believed to have anti-inflammatory properties that inhibit prostaglandins, which are inflammatory mediators, thus reducing the number of inflammatory cells that migrate to the area.10 In addition, the flavonoid compounds have antibacterial and antioxidant properties, so they are effective in accelerating the wound healing process.11

A previous study was conducted on the effectiveness of lime (Citrus aurantifolia Swingle) peel extract in accelerating the healing process of post-extraction alveolar wounds on rats’ teeth. The results indicated a significant increase in the number of fibroblast cells on the third and fifth days after topical application of peel extract gel in the post-extraction socket.12 These findings motivated the authors to investigate the effects of adding lime (Citrus aurantifolia Swingle) peel extract to the periodontal dressing on increasing the count of fibroblasts in the healing process of gum wounds.

MATERIALS AND METHODS

This research is an in vivo laboratory experiment using the post test-only control group research method. The study was approved by the animal care and use committee, Brawijaya University, Malang, with the ethics committee number 051-KEP-UB-2021. The sample used in this study was the New Zealand white rabbit. The sample crown was selected using the simple random sampling technique. The samples were of 32 individuals and were divided into 8 groups. The control groups (K1, K2) had no peel extract added to the periodontal dressing. The treatment groups (P1, P4) had 5% peel extract added to the periodontal dressing; the treatment groups (P2, P5) had 10% peel extract added to the periodontal dressing, and the treatment groups (P3, P6) had 15% peel extract added to the periodontal dressing.

Inclusion criteria for the sample were: healthy, male New Zealand white rabbits, 4-5 months old, with body weights of 3–4 kg, without previous treatment or exposure, and not disabled. The exclusion criteria were rabbits that died during the study. This research was conducted for about four months in the stem cell laboratory of the University of Airlanga.

The experimental animals were selected according to the criteria of the sample and then adapted for 7 days. The production of lime (Citrus aurantifolia Swingle) peel extract employed the maceration method using 70% ethanol solvent and a thick extract preparation was obtained.

A periodontal dressing According to Baer’s formulation consists of a powder and a paste. On a paper stirrer using a wooden spatula, make a powder dressing by mixing 28.5 g of rosin and 21.5 g of zinc oxide until homogeneous. Additionally, make a paste dressing by mixing 47.5g of hydrogenated fat and 2.5g of zinc oxide until blended on a paper stirrer using a wooden spatula. Then gradually mix 50 mg of powder and 50 mg of paste until homogeneous and 100 mg of each group is obtained (Figure 1A).13 Then Baer’s formulation of periodontal dressing (mg) was mixed with lime (Citrus aurantifolia Swingle) peel extract (mg) into groups as shown in Table 1.

Then the operator performed surgery on the test animals, first disinfecting the operating area with 70% alcohol. They were then anaesthetised by injecting a combination of ketamine and xylazine intramuscularly. The dose of ketamine used was 25 mg/kg body weight, followed by an injection of xylazine at a dose of 3 mg/kg body weight. Each rabbit underwent surgery on the lower incisor region of the attached labial gingival mucosa with

Figure 1. The manipulation of the periodontal pack with lime peel extract (A) and the performed punch biopsy on the rabbit gingiva (B).
the same control treatment, namely: using a punch biopsy tool with a diameter of 2 mm and pressing the depth of the wound to reach the alveolar bone but not to damage it, so as not to cause bone injury (Figure 1B). Then, the wound was cleaned with a solution of 0.9 ml NaCl and 3% H2O2.13

This was followed by the application of a periodontal dressing to the wound area (Figure 2A). The periodontal dressing had lime (Citrus aurantifolia Swingle) peel extract added for the treatment group and was without added peel extract for the control group. The periodontal dressing is shaped according to the shape of the wound. After application, the periodontal dressing was gently squeezed to cover the gum wound area using an excavator. To increase the retention of the periodontal dressing so that it would not come off, sutures were made between the lips and the gingival lining of the lower jaw using a 5.0 floss thread (Figure 2B).

Sampling was done on the third and fifth days to evaluate the number of fibroblast cells in the gingival granulation tissue of each experimental group. Experimental animals were euthanised under anaesthesia using a lethal dose of ketamine, i.e. 200 mg/kg body weight, by intramuscular injection. Then, the gingival granulation tissue was placed in a container with a 10% formalin solution.14 Tissue preparations were made with eosin haematoxylin staining. Observation of fibroblast cells was performed with an Olympus digital optical microscope at 400x magnification from 5 different fields of view.15 Shapiro Wilk’s statistical test and Levene’s test were performed because the data results were homogeneous and normal and then continued with one-way ANOVA, post-hoc Tukey, and independent T tests.

RESULTS

This study showed that the number of fibroblast cells on day 3 (Figure 3) and day 5 (Figure 4) in the control group was lower than in the treatment group. This is because the treatment group received lime (Citrus aurantifolia Swingle) peel extract to speed up wound healing.

In Figure 5, it can be seen that in a peel extract of 5% and 10%, the average number of fibroblasts in the gum healing process on day 3 showed an increase, but it showed a decrease in a concentration of 15%. Results on day 5 were the same. Overall, the administration of the 10% peel extract showed the highest average number of fibroblasts on day 3 and day 5 compared to other groups.

Table 1. Baer’s formulation periodontal dressing with and without added lime peel extract

| Group | Baer’s formulation of periodontal dressing (mg) | Lime peel extract (mg) |
|-------|---------------------------------------------|----------------------|
| Day-3 |                                            |                      |
| K1 0% | 100                                         | 0                    |
| P1 5% | 95                                          | 5                    |
| P2 10% | 90                                         | 10                   |
| P3 15% | 85                                         | 15                   |
| Day-5 |                                            |                      |
| K2 0% | 100                                         | 0                    |
| P4 5% | 95                                          | 5                    |
| P5 10% | 90                                         | 10                   |
| P6 15% | 85                                         | 15                   |

Figure 2. Application of periodontal pack on the gingival wounds (A) and wound closure with sutures (B).
Figure 3. Day 3 Fibroblast cells with HE staining, magnification 400x. a) (K1) periodontal dressing without lime peel extract, b) (P1) periodontal dressing with 5% lime peel extract, c) (P2) periodontal dressing with 10% lime peel extract, d) (P3) periodontal dressing with 15% lime peel extract.

Figure 4. Day 5 Fibroblast cells with HE staining, magnification 400x. a) (K2) periodontal dressing without lime peel extract, b) (P4) periodontal dressing with 5% lime peel extract, c) (P5) periodontal dressing with 10% lime peel extract, d) (P6) periodontal dressing with 15% lime peel extract.

Figure 5. The average of fibroblast cells by preparation.
The results of the one-way ANOVA test (Table 2) on days 3 and 5 obtained sig 0.000 (sig < 0.05), which indicates the effect of adding lime (*Citrus aurantifolia* Swingle) peel extract to periodontal dressings by the increase in the number of fibroblasts in the gingival healing process on day 3 and day 5. The post-hoc Tukey test was performed separately between time points due to the sheer effect of each day of treatment. The results of the post-hoc Tukey test—the difference in the number of fibroblasts in the gingival healing process on the 3rd and 5th days—indicated that each group showed a significant difference (sig < 0.05), which can be seen in Table 3. This proves that adding lime (*Citrus aurantifolia* Swingle) peel extract to the periodontal dressing significantly increased the number of fibroblasts in the gingival healing process on day 5, with a concentration of 10% resulting in the greatest increase in the number of fibroblasts, followed by a concentration of 5%, and then by a concentration of 15%.

To find out if there was a difference in the number of fibroblasts in the gingival healing process between observations, an independent t test was performed. The results of the independent t test at concentrations of 5%, 10% and 15% of lime (*Citrus aurantifolia* Swingle) peel extract showed a significant increase in the number of fibroblasts (sig < 0.05) from day 3 to day 5 (Table 4).

**Table 2.** Data of one-way ANOVA fibroblast cells

|                | Number of fibroblast cells | Sig. |
|----------------|-----------------------------|------|
| Day 3          | Between groups              |      |
|                | Within groups               |      |
|                | Total                       |      |
| Day 5          | Between groups              |      |
|                | Within groups               |      |
|                | Total                       |      |

**Table 3.** Post-hoc Tukey (HSD) test day 3 and 5

| Day test | Group treatment | Sig  |
|----------|-----------------|------|
| Day 3    | K1 – P1         | 0.000|
|          | K1 – P2         | 0.000|
|          | K1 – P3         | 0.000|
|          | P1 – P2         | 0.016|
|          | P1 – P3         | 0.000|
|          | P2 – P3         | 0.000|
| Day 5    | K2 – P4         | 0.000|
|          | K2 – P5         | 0.000|
|          | K2 – P6         | 0.000|
|          | P4 – P5         | 0.000|
|          | P4 – P6         | 0.000|

**Table 4.** Independent t test

| Group                | Observation time | Mean  | Sig  |
|----------------------|------------------|-------|------|
| 5% Lime peel extract | Day 3            | 111.95| 0.000|
|                      | Day 5            | 138.65|      |
| 10% Lime peel extract| Day 3            | 119.55| 0.000|
|                      | Day 5            | 164.25|      |
| 15% Lime peel extract| Day 3            | 67.50 | 0.000|
|                      | Day 5            | 79.85 |      |

**DISCUSSION**

This study indicates that compared to the control group, the treatment group that received a periodontal dressing with the addition of lime (*Citrus aurantifolia* Swingle) peel extract in concentrations of 5%, 10% and 15% was able to accelerate the healing response in rabbits previously treated with the labial gingiva of the lower jaw. The increased acceleration of the wound healing response was indicated by the increased number of fibroblast cells in the wound area in the treatment group.

In observation of the histological preparations of the post-injured rabbit gingival granulation tissue, fibroblast cells were observed on the 3rd and 5th days. This is consistent with Chasya et al.’s statement that active fibroblast cells proliferate or experience a significant increase from day 3 to day 7 after injury. In a study conducted by Nguyen et al., it was found that fibroblast cell proliferation occurred for 7 days and reached its peak on day 5. After which, the more the number of days, the more fibroblast proliferation decreases, indicating that there has been progress in the healing process.

In the control group, the number of fibroblasts was the least because the control group had not received any stimulus that could increase the activation of macrophages to stimulate growth factor components, so the group self-healed, unlike the treatment group, which showed an increase in the number of fibroblasts caused by the presence of active substances contained in the lime (*Citrus aurantifolia* Swingle) peel extract to speed up the healing process of gum wounds by, for example, the intermediary role of macrophages that stimulate growth factors.

In this study, Baer’s periodontal dressing formula was applied because it is commonly used for research purposes and is a pure composition of periodontal dressing without the addition of other ingredients. The advantage of this periodontal dressing is that it is safe to use during the wound healing process as it does not contain eugenol that can cause soft tissue irritation or necrosis, so it does not interfere with fibroblast formation.

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Based on the study’s phytochemical tests, Krisnay et al. showed that the highest saponin content in lime (*Citrus aurantifolia* Swingle) peel was 3.05, which had antiseptic, antioxidant and antibacterial properties. Next is the flavonoid content of 2.78% with its anti-inflammatory, antioxidant and antibacterial properties. In addition, lime (*Citrus aurantifolia* Swingle) peel also contains 2.14% of tannic compounds with antioxidant and antibacterial properties, and 1.86% of alkaloids with antibacterial properties. So that the most dominant chemical activity is saponins and flavonoids.

The ability of the active substances in lime (*Citrus aurantifolia* Swingle) peel extract to increase the average number of fibroblast cells in the treatment group is due to the anti-inflammatory effect of the flavonoids that work by directly inhibiting the activity of COX enzymes and lipoxygenase, which cause release inhibition of a...
number of inflammatory mediators such as prostaglandins, thromboxane, and leukotrienes, leading to a decrease in the inflammatory response in the wound area. Flavonoids have the ability, as immunomodulators, to activate macrophages to perform phagocytosis, tissue repair, and work to activate T cells to differentiate and proliferate into TH1, TH2, and TH3. TH3 cells produce growth hormones (growth factors) such as the Epidermal Growth Factor (EGF), Transforming Growth Factor-β (TGF-β) and Fibroblast Growth Factor (FGF), which play a role in stimulating fibroblast proliferation and increasing the migration of fibroblasts, smooth muscle cells, and endothelial cells in the wound area.\(^\text{15}\)

The increase in the average number of fibroblast cells in the treatment group can also be caused by antibacterial properties. Alkaloids play an important role because they have antibacterial properties that work by inhibiting the formation of protein synthesis so that they can interfere with bacterial metabolism, while flavonoids act as antibacterial with bacteriostatic properties. Flavonoid compounds can form complex compounds with proteins through hydrogen bonds, so that the tertiary structure of the protein is disrupted and the protein can no longer function in accelerating wound healing.\(^\text{17}\) Tannins also have antibacterial properties that work by binding to any of the membrane proteins possessed by bacteria and can then damage the availability of receptors on the surface of bacterial cells or interfere with cell permeability so that it interferes with cellular metabolic processes and cells can suffer death.\(^\text{18}\) In addition, saponins also have antibacterial properties by binding to sterols (bacterial proteins) on the surface of the bacterial cell membrane, which can increase the permeability of the bacterial cell membrane so that it can change the structure and function of the membrane causing protein denaturation, so that the cell membrane will be damaged and lysis.\(^\text{18}\) Bacterial death results in a reduction in bacterial phagocytosis by PMN leukocytes. This result in a brief inflammatory phase so that the proliferative phase takes place earlier.\(^\text{19}\)

The increase in the average number of fibroblast cells in the treatment group can also be caused by the antioxidant capacity of tannins. Tannins have an antioxidant feature that acts as an anti-inflammatory by inhibiting the production of oxidants (O2) by neutrophils, monocytes and macrophages. This high antioxidant activity can accelerate wound healing as it can stimulate the production of endogenous antioxidants at the wound site and provide a conducive environment for wound healing.\(^\text{19}\)

Compared to previous studies, testing the periodontal dressing with the addition of cinnamon extract that showed that the average number of fibroblasts on days 3 and 5 respectively in the control group was 21 and 16, whereas in this study the average number of fibroblasts in the control group on days 3 and 5 respectively was 41 and 59. In the treatment group, the cinnamon extract added at 5% showed average results of 22 and 20 while this study obtained averages of 111 and 138. In the treatment group, 10% of added cinnamon extract showed average scores of 26 and 23, while in this study averages of 119 and 164 were obtained. In the treatment group, when 15% of cinnamon extract was added, it showed average scores of 32 and 28, while in this study averages of 67 and 79 were obtained.\(^\text{19}\) This shows that adding lime (\textit{Citrus aurantifolia Swingle}) peel extract to periodontal dressings is more effective in accelerating wound healing compared to adding cinnamon extract to periodontal dressings, because the active content of 1 lime (\textit{Citrus aurantifolia Swingle}) peel extract can inhibit and significantly decrease the inflammatory response. This is in line with the statement by Sloane et al.\(^\text{21}\) on the efficacy test of lime (\textit{Citrus aurantifolia Swingle}) peel extract cream at a concentration of 10% that was found to be 96% effective in healing burns in the skin of white mice.

In this study, the results showed that the average increase in the number of optimal fibroblast cells was obtained in the treatment group that received the periodontal dressing with Baer’s formula with the addition of lime (\textit{Citrus aurantifolia Swingle}) peel extract to a concentration of 10%, while at a concentration of 15%, the average number of fibroblasts was the lowest. Indeed, the mixture of materials is not as homogeneous as in the mixture of a periodontal dressing with the addition of extract concentrations of 5% and 10%. With the addition of a concentration of 15%, the preparation of the periodontal dressing becomes more humid and difficult to position, so that the substances contained in the peel do not work optimally at a concentration of 15%. Meanwhile, adding an extract with a 5% concentration increased the number of fibroblast cells, which was less than optimal compared to adding a 10% concentration. This is probably caused by the lack of active lime (\textit{Citrus aurantifolia Swingle}) peel content added to the periodontal dressing, causing less than optimal results.\(^\text{13}\) We can conclude that adding lime peel extract (\textit{Citrus aurantifolia Swingle}) to the periodontal dressing has the effect of increasing the number of fibroblasts in the gum healing process. The number of fibroblast cells in the periodontal dressing treatment group with the addition of lime (\textit{Citrus aurantifolia Swingle}) peel extract was higher than that in the group without the addition of the extract, and there is a relationship between the number of fibroblasts and the lime (\textit{Citrus aurantifolia Swingle}) peel extract in the healing process of gum wounds.

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