The effects of breadfruit leaf (Artocarpus Altilis) extract on fibroblast proliferation in the tooth extraction sockets of Wistar rat

Darin Hulwani Rinaldi, David B. Kamadjaja and Ni Putu Mira Sumarta
Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Surabaya - Indonesia

ABSTRACT

Background: A prolonged tooth extraction socket healing process can affect the well-being of the patient and increase the risk of infection. Fibroblast proliferation in the proliferation phase is an important stage in the healing process. Fibroblasts formed from extracellular matrix and collagen fibers support bone formation in the socket. Breadfruit leaves, extremely common in Indonesia, contain polyphenol, flavonoid, tannin and alkaloid substances which accelerate the wound healing process because of their anti-inflammatory, anti-bacterial and anti-oxidant properties. A previous study showed that 16% breadfruit leaf gel extract administered to Wistar rats produced an encouraging anti-inflammatory effect, but its capacity for increasing fibroblast proliferation remains to be fully understood.

Purpose: The aim of this study was to observe the effect of applying breadfruit leaf extract on fibroblast proliferation in the healing process in tooth extraction sockets. A preliminary phytochemical study was undertaken.

Methods: 24 Wistar rats were divided into four groups: two control groups and two experimental groups. 16% breadfruit leaf gel extract was applied to the experimental groups, while none was applied to the control groups. The number of fibroblasts was counted on both the third and fifth days post-extraction. Data was analyzed statistically using an independent T-test.

Results: There were significant differences in the number of post-extraction fibroblasts in Wistar rat tooth sockets on day 3 (p=0.000; p < α=0.05) and day 5 (p=0.000; p < α=0.05).

Conclusion: Breadfruit leaf gel extract application increases fibroblast proliferation during the healing process in the tooth extraction sockets of Wistar rats.

Keywords: tooth extraction; wound healing; fibroblasts; breadfruit leaves

Correspondence: David B. Kamadjaja, Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Jl. Mayjend. Prof. Dr. Moestopo No. 47, Surabaya 60132, Indonesia. E-mail: davidbk65@gmail.com;
anti-oxidant, anti-inflammatory, anti-bacterial and anti-atherosclerotic properties. Another study indicated that 16% breadfruit leaf gel extract administered to Wistar rats induced effective anti-inflammatory activities.

An important factor in the inflammatory process is cyclooxygenase (COX), mainly COX-2, which is formed by macrophages. The presence of this enzyme is extremely strong during the inflammatory process and plays a role in the formation of prostaglandin from arachidonic acid. Another study found that after breadfruit leaf extract application COX-2 expression and activity decreased. This showed that breadfruit leaf extract possesses anti-inflammatory properties with COX-2 expression and activities as the target. It was expected that, with the suppression of COX-2 expression, fibroblast proliferation and differentiation, together with angiogenesis, will ensue.

Fibroblasts constitute extremely important cells in the early stages of wound healing and start to form during the proliferative stage from the third day after injury. Between day 3 and day 5, fibroblasts start to migrate to the wound site and proliferate, resulting in their numbers on the wound site being dominant. Fibroblasts act to break up blood clots, forming the extracellular matrix (ECM) and collagen fibers to support effective new bone formation in the socket.

The ability of breadfruit leaf extract to induce fibroblast proliferation is not yet fully understood. The aim of this study is to observe the effect of its application on this process in tooth socket healing. The effectiveness of breadfruit leaf extract was observed histopathologically, with fibroblast numbers being counted manually.

MATERIALS AND METHODS

This research constituted an in vivo experimental laboratory study with post-test only control group design on Wistar strain Rattus norvegicus obtained from the Biochemistry Laboratory of the Faculty of Medicine at Universitas Airlangga.

Breadfruit leaf samples were cleaned before being dehydrated at room temperature for seven days. The dehydration process was undertaken in order to obtain suitable breadfruit leaf weight for deriving extract. Dehydrated samples were cut into small pieces and subsequently ground in an electric blender. Ten grams of breadfruit leaf powder were macerated through continual mixing with ethanol (mrc, TS-400 orbital shaker) for three days at room temperature. A phytochemical study was conducted using UV-Visible Spectrophotometry to evaluate the chemical composition of the extract which was subsequently centrifuged at 3000 rpm for ten minutes and filtered using Whatman filter grade 1 paper (IKA® RV 065 basic) in a high-pressure vacuum pump. The extract was scanned with a wavelength ranging from 300-1100nm using a Perkin Elmer Spectrophotometer, resulting in the characteristic peaks being detected. The relative percentage of each component was calculated by comparing its average peak area to the total area. The extract was made into gel form at a concentration of 16%.

Twenty-four male Wistar rats aged 2-4 months and weighing 200-250 grams were quarantined for a period of one week. Male specimens were selected in order to avoid the potential hormonal impact affecting female Wistar rats. They were divided into four groups of six subjects comprising two control groups (K1 and K2) and two experimental groups (P1 and P2). K1 represented a control group with no gel extract applied and its fibroblasts being counted on the third day after tooth extraction. K2 constituted a control group with no gel extract applied and the number of fibroblasts calculated on the fifth day after tooth extraction. P1 was an experimental group with gel extract applied and its fibroblasts counted on the third day after tooth extraction and gel extract application. P2 represented an experimental group to which gel extract was applied and whose fibroblasts were computed on the fifth day after extraction and gel extract application. The gel extract used with the experimental groups was applied once, immediately after extraction.

Before the tooth was extracted, the Wistar rats were anesthetized intramuscularly using a mixture of ketamine and xylazine. The left mandibular incisors were extracted with a needle holder and the socket subsequently irrigated with 0.2 ml aquadest in order to eliminate any residual debris in the socket before being dried with sterile gauze. Theretafter, 0.1ml of 16% breadfruit leaf gel extract was applied to the experimental group using a syringe followed by the application of a suture.

The subjects were sacrificed on the third and fifth day post-extraction. Incision of the mandibular body was performed before it was placed in a sterile tube containing 10% formalin solution to inhibit changes in post-mortem tissues. Therefore, the samples did not rot and autolysis was inhibited.

The samples were then processed into histopathological slides for further examination using Harris Hematoxylin and Eosin (HE) staining. These slides were observed under a light microscope at 400x magnification and the fibroblast numbers in five different areas were counted with the average for each group being calculated. The fibroblast numbers in the control and experimental groups were compared with data being analyzed statistically by means of a Kolmogorov-Smirnov Test followed by an independent T-test.

RESULTS

Preliminary phytochemical analysis was performed before the extract was made into a gel in order to evaluate the content of the breadfruit leaves. The phytochemical analysis results are shown in Table 1.
The fibroblast density in H&E staining during examination on day 3 and day 5 is shown in Figure 1. The experimental groups had a denser fibroblast appearance and a higher number of fibroblasts than the control groups on both day 3 and day 5 post-extraction. The fibroblast appearance in each group is shown in Figure 1. A comparison of the average numbers of fibroblasts on day 3 and day 5 is shown in Figure 2.

The data obtained was tested statistically using SPSS. A normality test was conducted by means of a Kolmogorov-Smirnov test. Data from every group showed normal distribution, $p > \alpha$ ($\alpha = 0.05$). An independent T-test was subsequently conducted to compare the difference in the average amount between the study groups. The results showed that there were significant differences between all groups $p = 0.000$; $p < \alpha$ ($\alpha = 0.05$) on both day 3 and day 5.

DISCUSSION

Breadfruit leaf extract was made into gel form in order to ease its application to the tooth socket. A concentration of 16% was selected based on research conducted by Abdassah et al. that found this concentration of breadfruit leaf extract administered to Wistar rats displayed satisfactory anti-inflammatory properties.

These research results showed that control groups had lower fibroblast numbers compared to the experimental group following the administering of 16% breadfruit leaf gel extract. The significant difference in fibroblast numbers between the control and experimental groups was probably caused by polyphenol compounds at a concentration of 4.92%. Polyphenol is the most common compound present in the breadfruit leaf extract used in this experiment indicating that it is more likely to play a key role in fibroblast proliferation.

Polyphenol can inhibit pro-inflammatory enzyme activity and increase anti-inflammatory enzyme activity. This statement corresponds to the fact that polyphenol can inhibit pro-inflammatory gene expression such as interleukin (IL) receptors, Toll-like receptors (TLR-4), nuclear factor kappa B (NF-kB), activator protein (AP-1) and c-Jun-N-terminal kinases (JNK), while also increasing the production of anti-inflammatory molecules, for example IL-4, IL-10, IL-13 and adiponectin. Together with polyphenol, flavonoid at a concentration of 2.11% in the breadfruit leaves used in this experiment also acts as an anti-inflammatory. Flavonoid inhibits pro-inflammatory enzymes such as COX-2, lipoxygenase, and inducible nitric oxide synthase (iNOS, TNF $\alpha$, IL-1, NF-kB, AP-1 and MAPK). Pro-inflammatory enzymes inhibited by polyphenol and flavonoids causing stimulation of the phospholipid cell membrane decrease with the result that arachidonic acid cannot be released from the phospholipid cell membrane by phospholipase activation. The inhibited cyclooxygenase and lipoxygenase cycle will suppress prostaglandin, endoperoxidase, thromboxane, hydroperoxidase acid and leukotriene with the result that the inflammatory phase can be reduced and promote more rapid fibroblast proliferation.

Polyphenol and tannin at high concentrations (2.56%) in the extract used, as well as flavonoid compounds, contribute to anti-oxidant effects on the breadfruit leaves

<table>
<thead>
<tr>
<th>No.</th>
<th>Compounds</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Polyphenol</td>
<td>4.92</td>
</tr>
<tr>
<td>2</td>
<td>Alkaloid</td>
<td>3.8</td>
</tr>
<tr>
<td>3</td>
<td>Tannin</td>
<td>2.56</td>
</tr>
<tr>
<td>4</td>
<td>Flavonoid</td>
<td>2.11</td>
</tr>
<tr>
<td>5</td>
<td>Saponin</td>
<td>1.74</td>
</tr>
</tbody>
</table>

Table 1. Phytochemical analysis result of breadfruit leaves extract.

Figure 1. Histopathological appearance on post extraction socket, group A (K1), B (P1), C (K2), D (P2). The arrows point on fibroblast formed in H&E staining, with 400x magnification.

Figure 2. Average amount of fibroblast for each group. The fibroblast number were counted on five different areas using microscope with 400x then averaged on each groups. Fibroblast numbers then compared between control (blue bar) and experimental (red bar) groups on both day 3 and day 5. Experimental group on day 3 and day 5 show a higher number of fibroblast proliferation compared to control group.
used in this experiment. The anti-oxidant effect of breadfruit leaves works by donating one of its electrons to an oxidant substance impeding the activity of that substance.\(^{15}\) Unstable free radicals that bind to the anti-oxidant can reduce cell membrane breakdown and suppress inflammation leading to acceleration of the proliferative phase.\(^{10}\) Another study posited that breadfruit leaves (artocarpone) inhibit nitric oxide (NO) and iNOS production. NO is a free radical substance promoting macrophage cell activation in inflammatory sites.

An antibacterial effect is produced by the alkaloid, tannin and flavonoid in the leaves used. It is believed that the antibacterial effect of breadfruit leaves is caused by their elevated alkaloid content. Alkaloid can disrupt bacteria cell membranes as well as inhibit DNA and RNA synthase, while also being toxic to microorganisms.\(^{16}\) The antibacterial effect of tannin can be achieved in several ways, including creating complex bacterial and fungal compounds which disrupt the metabolism of microorganisms by inhibiting oxidative phosphorylation.\(^{17}\) In contrast, flavonoids exert an antibacterial effect by rendering the microbe adhesion, enzymes and protein cell transport non-active.\(^{7}\) These antibacterial activities can minimize the pathogenic bacteria and their potential interference with the healing process.

The properties mentioned above are supported by the fact that breadfruit leaf extract possesses anti-inflammatory, antibacterial and antioxidant properties.\(^{13}\) These properties are derived from chemical substances contained in the extract such as polyphenol, alkaloid, tannin and flavonoid which promote more rapid fibroblast proliferation.

Based on the findings of the experiment conducted, there are significant differences in fibroblast proliferation between the groups with the application of gel extract and those to which it is applied on the third and fifth days. These findings show that 16% breadfruit leaf gel extract can increase fibroblasts leading to faster than normal wound healing. It can be concluded that 16% breadfruit leaf gel extract increases fibroblast proliferation during the healing process in the tooth extraction sockets of Wistar rats.

**REFERENCES**