The Effect of Papaya Seeds Extracts Nanoliposomes Administration on Osteoclasts Number of Diabetic Periodontitis Animal Model

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Abstract

Periodontitis is the most common complication in diabetes mellitus sufferers with a prevalence rate of up to 75%. The previous study proved that papaya seeds extract can inhibit the osteoclastogenesis process. Nevertheless, generally natural compounds have scarce water solubility, and inappropriate molecular size. The modification of the drug delivery system can optimize the therapeutic effect, such as nanoliposome. The research objective is to evaluate the effect of papaya seeds extract nanoliposome on osteoclasts number in the diabetic periodontitis animal model.

The study used 36 Sprague Dawley rats which were divided into 3 groups on days 3, 7 and 14. Each group was group K (diabetic periodontitis model mice without treatment), group P1 (diabetic periodontitis model mice given ethanol extract of papaya seeds 96 %) and group P2 (diabetic periodontitis model mice given 96% ethanol extract of papaya seed nanoliposome preparations). Both preparations were given 0.5 ml orally and 0.03 ml dropped on the gingival groove once a day. Osteoclasts were counted by HE staining. The data obtained were tested using One Way Anova and Tukey’s Post Hoc test.

Based on the results of the One way Anova test on the 14th day, there was a significant difference in the number of osteoclasts (p <0.05) between groups. Post Hoc test on days 7 and 14 showed no significant difference between groups P2 and P1 (p > 0.05) and there were significant differences between groups K and P1 and P2 (p <0.05).

The administration of papaya seed extract nanoliposome affects reducing the number of osteoclasts in diabetic periodontitis mice, nevertheless there was no significant difference between the effect of nanoliposome and papaya seeds extract administration.


Keywords: Nanoliposome, papaya seeds, diabetes, periodontitis, osteoclasts.

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Introduction

Periodontitis is a complex inflammatory disease characterized by progressive deterioration of the connective tissue and alveolar support of the teeth. In Indonesia, periodontal disease ranks second after caries and is still a problem in society. According to the 2018 RISKESDAS data, the percentage of periodontitis cases in Indonesia reached 74.1%1. The biggest risk factor for periodontitis is systemic diabetes mellitus, which has been shown to increase the inflammatory process in the periodontal tissue and cause alveolar bone destruction2,3. Periodontitis and diabetes mellitus have a two-way relationship, diabetes mellitus is a predisposing factor for periodontitis and periodontitis can worsen the metabolic control of diabetes mellitus 4.

Alveolar bone resorption is a dynamic process in bone tissue due to an imbalance between osteoclast cells as cells that help bone damage and osteoblasts as cells that help bone formation, where the number of osteoclasts is higher than osteoblasts 5. The presence of
osteoeclasts on the periodontal surface of the alveolar bone indicates active resorption and is in the area until the surrounding tissue is damaged. The gold standard for managing periodontal disease is scaling root planning (SRP) as initial therapy with supporting therapy, such as antibiotics and non-steroidal anti-inflammatory drugs for instance ibuprofen, meanwhile for the chronic aggressive periodontitis usually use bone graft therapy. Long-term use of ibuprofen is known to have side effects such as stomach ulcer and hemorrhage. The side effects that can be caused require other safer alternative ingredients. Therefore, efforts were made to develop periodontitis therapy by utilizing natural ingredients such as papaya (Carica papaya L.). The use of papaya seed extract (Carica papaya L.) is based on the results of previous studies where it is known that the phenol and flavonoid compounds in papaya seeds act as anti-inflammatory compounds that inhibit cytokine production and expression. Bioactive components such as chlorogenic acid have been shown to inhibit osteoclast production through cell differentiation and quercetin can increase osteoblast production. However, natural compounds generally have scarce water solubility, low lipophilicity, and inappropriate molecular size. The modification of the drug delivery system can optimize the therapeutic effect by providing specific targets in action one of them is nanoliposome. The main research objective is to evaluate the effect of the papaya seed extract nanoliposomes on the osteoclasts number of the diabetic periodontitis animal models.

Materials and methods

This study used a sample of 36 rats Sprague Dawley and divided into 3 groups with the time series days 3, 7, and 14. The groups were K groups (rats induced diabetic periodontitis-no treatment), P1 groups (rats induced diabetic periodontitis-administered papaya seeds extract), P2 groups (rats induced diabetic periodontitis-administered nanoliposomes papaya seeds extract). Both the extract and nanoliposomes were administered orally 0.5 ml and locally 0.03 ml on sulcus. The rats were sacrificed on day 4, 8, and 15 then the osteoclasts counted by HE stained. The methods used in this study had been approved by the Animal Care and Use Committee Universitas Brawijaya (certificate number: 119-KEP-UB)

Papaya seeds extract nanoliposome synthesis

Extraction of papaya seeds with 96% ethanol solvent and synthesis of nanoliposomes using the same method as the research of Pusporini et al. Diabetic induction

After the rats had fasted for at least 8 hours, they were injected with nicotinamide 150 mg / kg diluted with normal saline intraperitoneally, then 15 minutes later they were injected with STZ (Streptozotocin) at a dose of 50 mg / kg dissolved with citrate buffer (pH = 4.5). The induction procedure was repeated 24 hours later with the same dose. The induction test was done by taking blood from the rats and the glucose level was measured using a commercial glucometer. Rats were declared diabetes mellitus if fasting blood glucose > 126 mg / dl. Induction periodontitis

The rats were anesthetized by injecting ketamine HCL intramuscularly into the hamstrings at a dose of 0.2 ml / 200gram BW. Induction of periodontitis was done by tying 3.0 mm silk ligature in the subgingival area around the RB anterior incisor, then 0.05 ml PBS of 5µg LPS P. gingivalis was dripped into the central buccal incisor once a day for 7 days. This action is modified from Pusporini. The examination of the results of periodontitis induction was carried out in the lower jaw area of experimental animals by looking at the redness, swelling, loss of attachment to the gingiva and the formation of periodontal pockets between 1-2 mm.

Administration of Papaya Seeds Extract nanoliposome

Both the extract and nanoliposomes were administered orally 0.5 ml and locally 0.03 ml on sulcus. The rats were sacrificed on day 4, 8, and 15. The osteoclasts number counted by HE stained.

Results

Observations were made by counting the number of osteoclasts in the alveolar bone in the apical area using a light microscope with a 400x magnification.
magnification carried out on five fields of view of histological preparations with Hematoxylin-Eosin (HE) staining. The results of counting the number of osteoclasts and the mean of osteoclasts on days 3, 7 and 14 in the control, treatment 1 (P1) and treatment 2 (P2) groups are shown in Table 1 and Figure 1.

<table>
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<tr>
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<th>Days-3</th>
<th>Days-7</th>
<th>Days-14</th>
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<tbody>
<tr>
<td>K</td>
<td>20.33±4.04</td>
<td>20.67±2.52</td>
<td>22.00±1.00</td>
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<tr>
<td>P1</td>
<td>17.67±1.15</td>
<td>15.33±1.53</td>
<td>13.67±1.15</td>
</tr>
<tr>
<td>P2</td>
<td>14.33±1.53</td>
<td>13.33±2.08</td>
<td>11.33±1.15</td>
</tr>
</tbody>
</table>

**Table 1.** The mean number of osteoclasts in the control group (K), treatment 1 (P1), and treatment 2 (P2) on days 3, 7 and 14.

**Figure 1.** Diagram of the average number of osteoclasts in the control group (K), treatment 1 (P1), and treatment 2 (P2) on days 3, 7 and 14.

On the day 3, the highest number of osteoclasts in the control group (K) was obtained with an average of 20.33. Treatment group 1 (P1) was more than the treatment group 2 (P2), where the mean osteoclast cells in P1 were 17.67 and P2 was 14.33. The results of observing the number of osteoclasts with a light microscope on the day 3 are shown in Figure 2.

**Figure 2.** Overview of osteoclasts with Hematoxylin-Eosin (HE) staining at 400x magnification on the day 3: (A) Control Group (K), (B) Treatment Group 1 (P1), (C) Treatment Group 2 (P2). The black arrows indicate osteoclasts.

On the day 7, the highest number of osteoclasts in the control group (K) was obtained with an average of 20.67. Treatment group 1 (P1) was more than treatment group 2 (P2), meanwhile the mean of osteoclast cells in P1 were 15.33 and P2 was 13.33. The results of observing the number of osteoclasts with a light microscope on the day 7 are shown in Figure 3.

**Figure 3.** Image of osteoclasts with Hematoxylin-Eosin (HE) staining with 400x magnification on the day 7: (A) Control Group (K), (B) Treatment Group 1 (P1), (C) Treatment Group 2 (P2). The black arrows indicate osteoclasts.

On the day 14, the highest number of osteoclasts in the control group (K) was obtained with an average of 22.00. Treatment group 1 (P1) was more than the treatment group 2 (P2), where
the mean osteoclast cells in P1 were 13.67 and P2 was 11.33. The results of observing the number of osteoclasts with a light microscope on the day 14 are shown in Figure 4.

![Image of osteoclasts with Hematoxylin-Eosin (HE) staining at 400x magnification on the day 14: (A) Control Group (K), (B) Treatment Group 1 (P1), (C) Treatment Group 2 (P2). The black arrows indicate osteoclasts.]

**Figure 4.** Image of osteoclasts with Hematoxylin-Eosin (HE) staining at 400x magnification on the day 14: (A) Control Group (K), (B) Treatment Group 1 (P1), (C) Treatment Group 2 (P2). The black arrows indicate osteoclasts.

**Data analysis**

**One-Way Anova test**

The results of the One-Way Anova test between the Control (K), Treatment 1 (P1) and Treatment 2 (P2) groups on the 3rd day showed a significance result of 0.077, on the 7th day it showed a significance of 0.012, and on the 14th day it showed the result is significant <0.001. On the 3rd day the significance value > 0.05, then there is no significant difference between the Control (K), Treatment 1 (P1) and Treatment 2 (P2) groups. Meanwhile, on the day 7 and 14 the significance value was <0.05, so that with a significance level of 5%, there was sufficient evidence to show that there was a significant difference between the Control (K), Treatment 1 (P1) and Treatment 2 (P2) groups in the 7th and 14th day.

**Post-hoc Tukey test**

The Tukey Post-hoc test was performed after the One-Way Anova test with a significance value of less than 0.05 (p <0.05). The significance value between the control group (K) and treatment 1 (P1) was 0.0046 (p <0.05), between the control group (K) and treatment 2 (P2) was 0.012 (p <0.05) and the treatment group 1 (P1) with the group treatment 2 (P2) of 0.507 (p > 0.05). Therefore, it can be concluded that with a significance level of 5% there is sufficient evidence to show that there is a significant difference in the number of osteoclasts between the control group (K) and treatment group 1 (P1) and the control group (K) with treatment group 2 (P2). However, there is not enough evidence to show a difference between treatment group 1 (P1) and treatment group 2 (P2).

**Discussion**

This study aims to observe the differences in the number of osteoclasts after giving nanoliposomes 96% ethanol extract of papaya seeds in diabetic periodontitis model mice. The results showed that treatment group 1 (P1) and treatment group 2 (P2) had an average reduction in the number of osteoclasts compared to the control group. The number of osteoclasts in the control group (K) showed the occurrence of alveolar bone resorption due to inflammatory reactions as a host response to LPS P. gingivalis in periodontal tissue characterized by polymorphonuclear leukocyte infiltration, production of reactive oxygen species (ROS), and inflammatory mediators such as cytokines and prostaglandins, amplification of lytic enzymes, activation of osteoclasts, edema, and dilation of blood vessels.

The hyperglycemia condition in diabetes mellitus encourages the formation of AGEs (advanced glycation end-products) which when bind to the RAGE receptor will increase inflammation and oxidative stress (OS) which is characterized by the production of reactive oxygen species (ROS) to damage tissue repair.

On the day 3, there was a decrease in the mean number of osteoclasts, but there was no significant difference in the number of osteoclasts between the Control (K), Treatment 1 (P1), and Treatment 2 (P2) groups based on the results of the One-Way Anova test. Significant differences in the number of osteoclasts between groups occurred on days 7 and 14. These results are consistent with research conducted by Kwak et al in 2013 which stated that chlorogenic acid can inhibit bone destruction mediated by inflammation. In that study, there was a significant improvement in bone damage induced by LPS on day 8. Chlorogenic acid is a phenolic component in papaya seeds that functions as an antioxidant needed to prevent further tissue damage due to the high production of reactive oxidative stress (ROS), reduces the number of...
osteoclasts, and inhibits bone resorption through decreasing RANKL regulation.19–21

On the day 7, there was a decrease in the average number of osteoclasts between the control group (K), treatment group 1 (P1), and treatment group 2 (P2). The results of Tukey's Post-Hoc Test show that there is sufficient evidence that shows a significant difference between the control group (K) and the treatment group 1 (P1) and the treatment group 2 (P2). This shows that the active compound content in papaya seeds (Carica papaya L.) functions as an anti-inflammatory and can inhibit the alveolar bone resorption process which is evident from a decrease in the number of osteoclasts. Polyphenol content can reduce inflammatory mediators and influence bone formation mechanisms by inhibiting osteoclastogenesis, inhibiting osteoclast activity by blocking the production of RANKL, and decreasing the production of pro-inflammatory factors such as COX-2.22 Flavonoids are polyphenolic compounds contained in papaya seeds and quercetin is a subclass of flavonoid compounds.23 Quercetin can act as an anti-inflammatory and antioxidant. The mechanism of action of quercetin is related to inhibition of the production of pro-inflammatory cytokines (IL-1β and TNF-α), reduced expression of inflammatory molecules, inhibition of intracellular signaling pathways such as mitogen-activated protein kinases (MAPK) and NFκB resulting in reduced inflammation and oxidative stress.24

In the process of bone resorption by osteoclasts, there is a release of bone matrix derived factors which can regulate the formation of osteoblasts.25,26 Osteoblast cells influence the regulation of osteoclastogenesis through the regulatory system of OPG / RANKL / RANK. The RANKL / OPG ratio is a determinant of the bone resorption process.11 Inhibition of bone resorption. Based on the results of Tukey's Post-Hoc Test on the day 14, there is sufficient evidence that shows a significant difference between the control group (K) and the treatment group 1 (P1) and the treatment group 2 (P2). The number of osteoclasts in the control group (K) was the highest of all days. and the number of osteoclasts in treatment group 2 (P2) was the lowest of all days. This proves that there is an inhibition of the bone resorption process in alveolar bone damage after administration of 96% ethanol extract of papaya seeds nanoliposomes on treatment on the day 14 through inhibition of RANKL production so that RANKL binding by the RANK receptor (RANKL-RANK) is the initial process of bone destruction reduced.

Based on the results of Tukey's Post-Hoc Test on days 7 and 14, there is not enough evidence to show a significant difference between treatment group 1 (P1) and treatment group 2 (P2). However, there was a difference in the average number of osteoclasts between groups, where the number of osteoclasts in treatment group 2 (P2) was lower than the number of osteoclasts in treatment group 1 (P1). This could be related to the nanoparticle synthesis process for liposomes as a drug delivery system. Some of the problems that often arise in nanoparticle preparation are the occurrence of fast aggregation and uneven particle size, so that the stability of the dispersion system becomes difficult to control.27,28 The problem can be understood by carrying out a comprehensive characterization of nanoparticles, apart from the particle size, it is necessary to know the morphological characters of the particles and the zeta potential value.29,30

Conclusions

Based on the results of the discussion, it can be concluded that there is an effect of the administration of papaya seed extract nanoliposomes on the number of osteoclasts in diabetic periodontitis animal model, nevertheless there was no significant difference between the effect of nanoliposome and papaya seeds extract administration. Therefore, it is necessary to do further research on the toxicity and characterization of papaya seeds extract nanoliposomes in order to obtain the correct dosage for use as a supportive therapy for diabetic periodontitis condition.

Declaration of Interest

The authors report no conflict of interest.