RESEARCH ARTICLES

Antimicrobial photodynamic therapy with erythrosine photosensitizer against immune response in chronic periodontitis model

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ABSTRACT

Chronic periodontitis is a progressive inflammatory disease of the supporting tissues of the teeth caused by dental plaque bacteria with a clinical sign of periodontal pockets. A Gram-negative bacterium that can trigger this inflammatory disease is Porphyromonas gingivalis. Antimicrobial photodynamic therapy with blue LED light irradiation and photosensitizer erythrosine can reduce the survival rate of P. gingivalis. This study aimed to determine the effects of antimicrobial photodynamic therapy (APDT) exposure with blue LED light irradiation and PS erythrosine on the number of macrophages, lymphocytes, and gingival fibroblasts in gingival tissue of Sprague Dawley rats as chronic periodontitis models. This study used a posttest-only control group design to examine 27 Sprague Dawley rats which were divided into P group (healthy rats), N group (untreated chronic periodontitis rats), and PDT groups (chronic periodontitis model given 1 mg/ml PS erythrosine and irradiated with blue LED light for 60 seconds). Cell observation of histologic preparations of rat gingival tissue with hematoxylin-eosin (H&E) staining was carried out on the 1st, 3rd, and 5th days. Histological preparations of gingival tissue with H&E staining was carried out on the 1st, 3rd, and 5th days. Statistical analysis used a one-way ANOVA and the Kruskal-Wallis test, continued with LSD and the Mann-Whitney post-hoc tests. Results showed significant difference in the mean of macrophages in the PDT group compared to the untreated chronic periodontitis group on the 1st, 3rd, and 5th days (p < 0.05). The mean lymphocyte in the PDT group was significantly different from the untreated chronic periodontitis group on the 1st, 3rd, and 5th days (p < 0.05), and significantly lower than that in the healthy group (p < 0.05) but only on the 3rd and 5th days. The mean fibroblast in the PDT group was significantly different compared to the untreated chronic periodontitis group on the 1st, 3rd, and 5th days (p < 0.05). In conclusion, there were significant differences in the number of macrophages, lymphocytes, and fibroblasts in a chronic periodontitis rat model after treatment with APDT exposure with blue LED and erythrosine photosensitizer.

Keywords: antimicrobial photodynamic therapy; chronic periodontitis; fibroblast; lymphocyte; macrophage

INTRODUCTION

Periodontitis is a multifactorial disease that can cause destruction of the periodontium and bone. It is a disease that causes the loss of tissue attachment to the teeth. The prevalence of periodontitis in Indonesia reaches 74.1%, and the prevalence rate increases with age. Chronic periodontitis is one type of periodontitis whose main cause is periodontopathogenic microorganisms such as P. gingivalis bacteria. Inflammation in chronic periodontitis is induced by these bacterial products, such as gingipains and lipopolysaccharide (LPS). Bacterial LPS can cause acute inflammation and induce the migration of neutrophil cells and macrophages as the first line of defense to the area of infection for bacterial phagocytosis. Along with the accumulation of neutrophils, macrophages will reach their peak abundance on day 3 in the area accompanied by inflammatory mediators. Lymphocytes begin to accumulate on day 1 and will reach their peak abundance on day 7. The levels of pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and matrix metalloproteinase (MMP) will increase. Under normal circumstances, fibroblasts will synthesize collagen in the process of tissue healing. The number of fibroblasts in the gingival
connective tissue begins to increase on day 3 to day 5 post-inflammation along with macrophages and peaks on days 7 to 14. Persistence of bacteria in periodontitis can cause chronic inflammation. Release of pro-inflammatory mediators in continuous chronic inflammation can exacerbate tissue damage, collagen degradation, and bone damage, notably alveolar bone resorption that occurs due to the number of tartrate-resistant acid phosphatase (TRAP)-positive cells of osteoclast multinuclear cells.1,2,3

The gold standard therapy for the treatment of periodontal disease is scaling and root planing (SRP) which can reduce the number of pathogenic bacteria by offering additional therapy using antibiotics to increase its effectiveness. Antibiotics can be administered systemically or topically through the gingival sulcus. Antibiotics such as tetracycline can also be proposed as host-modulating agents in host modulation therapy (HMT). However, risks of resistance, contraindications and long-term negative side effects remain a challenge. This is evidenced by the resistance of pathogenic periodontal bacteria to antibiotics which has been reported to have occurred in 74.2% of chronic periodontitis patients. Photodynamic antimicrobial therapy (APDT) can be an effective, efficient, and inexpensive local therapy to overcome this issue.3,4,5

APDT is a non-invasive therapy based on the administration of an exogenous compound in the form of a photosensitizer (PS) which is induced by irradiating the infected area in the gingival pocket using light that has a visible wavelength in the range of 400-700 nm. APDT is considered to be three times more specific at the site of infection because of the greater absorption of PS in target cells compared to non-targets. Thus, toxicity outside the irradiated PS area is absent. Repeated treatment can be carried out safely without the risks of tissue damage and bacterial resistance to PS.6

The newest types of visible light exposure and PS currently being developed for use in APDT are blue light and erythrosine light-emitting diodes (LEDs). Blue LED light have antimicrobial properties and can modulate fibroblast cell metabolism and proliferation in the healing process. Erythrosine has been shown to have low toxicity to oral tissues because it has been used as a disclosing agent in dentistry. Previous studies have shown that the antibacterial effect of erythrosine (22 L) exposed to blue LED light can reduce the survival rate of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* pathogenic bacteria to almost zero percent. Another study was conducted using an in vivo method using male Sprague Dawley rats to determine the effect of APDT with 10 seconds of exposure to LED light per 6 teeth (total 60 seconds per tooth) with 1 mL (1.0 mg/mL) of PS erythrosine. The results of the APDT + SRP group showed lower bone loss than the control group.7,8,9,10

Very little was found in studies on the effect of APDT with LED light and PS erythrosine on the number of inflammatory cells, such as macrophages, lymphocytes, and fibroblasts. This study aimed to determine the effects of APDT exposure with blue LED light irradiation and PS erythrosine on the number of macrophages, lymphocytes, and gingival fibroblasts in gingival tissue of Sprague Dawley rats as chronic periodontitis models.

**MATERIALS AND METHODS**

This study was reviewed and approved by the Ethics and Advocacy Unit of the Faculty of Medicine, Universitas Jenderal Soedirman (No. 042/KEPK/PE/V/2022). This experimental study was conducted with posttest-only control group design. Twenty-seven male Sprague Dawley rats (n = 27) were used as experimental animals in this study. They had the following characteristics: (1) healthy, (2) aged 2-3 months, and (3) weighting 200-300 grams, and were acclimatized to a 12-hour light/12-hour dark cycle room in a temperature of ± 25 °C for 14 days. Throughout the experiment, the animals were fed with a selected solid diet and water ad libitum. The rats were chosen by simple random sampling and were divided into three groups (n = 9/group): a healthy control group (P), periodontitis groups induced with *Porphyromonas gingivalis* bacterial solution without any treatment (N), and APDT with erythrosine (1 mg/ml) and exposed to blue LED (420-480 nm) (PDT). The
samples were divided into smaller groups based on the day of observation (days 1, 3, and 5) (n = 3). *Porphyromonas gingivalis* ATTC33277 were obtained from the Biomedical Laboratory of the Department of Dentistry of the Faculty of Medicine, Universitas Jenderal Soedirman. All samples of periodontitis groups (N and PDT) were induced with *Porphyromonas gingivalis* ATTC33277 sample solution (0.3 ml 1 X10⁸ CFU/ml) in the buccal gingiva between first and second mandibular incisors. The infected rats were then left for 7 days to allow periodontitis to develop. Clinical and periapical radiographic examinations were performed to confirm periodontitis twenty-four hours post-infection administration.

There were several procedures adopted for each treatment group. Each sample of the PDT group received APDT. Erythrosine (1 mg/ml) was applied inside the periodontal pocket and irradiated with dental blue LED curing light (Mini LED SuperCharged Acteon) within the 420-480 nm wavelength, with maximum power density of 2000 mW/cm² and 7.5 mm diameter of active fiber for 60 seconds. The rats were euthanized 24 hours after the 5th day of treatment. Gingival tissue was taken and stored in 10% neutral buffered formalin. Histological preparations were made using hematoxylin-eosin staining. Two observers made histological observations with 400x magnification in 5 fields of view. Cell calculation used Image-J application. The results of the experiment were statistically analyzed by Shapiro-Wilk normality test, Levene’s test, one-way analysis of variance (ANOVA) and Kruskal-Wallis test with a level of confidence of 95% and followed by a post-hoc test with Fisher’s least significant difference (LSD) and Mann-Whitney test using SPSS 23.0.

**RESULTS**

The results of the calculation of macrophages and lymphocytes showed the lowest average number of cells in the APDT group, while fibroblasts showed the highest average number of cells in the APDT group. The one-way analysis of variance (ANOVA) test obtained a significance value of p < 0.05, which may indicate that PDT treatment with erythrosine and blue LED affected the periodontitis inflammation and healing process. The results of the calculation of macrophages, fibroblasts and lymphocytes can be seen based on the histological observations of gingival preparations in Figure 2 and 3. The average of immune cells on each day of treatment can be seen in Tables 1, 2, and fibroblast in Table 3.

In Table 1, the mean of macrophage count in the healthy (P) group showed a consistent pattern, whereas the mean macrophage count in the untreated periodontitis (N) group increased on the 1st, 3rd, and 5th days. The APDT group had not differed significantly with the P group on the 1st, 3rd and 5th days, but it differed significantly with the N group on the 1st, 3rd, and 5th days (p < 0.05). The mean macrophage in the APDT group decreased on each day of treatment and showed a significant difference on the 1st, 3rd, and 5th days.

In Table 2, the mean of lymphocyte count in the P group showed a consistent pattern, whereas the mean lymphocyte count in the N group increased on the 1st, 3rd, and 5th days. The PDT

<table>
<thead>
<tr>
<th>Group</th>
<th>day 1</th>
<th>day 3</th>
<th>day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>P</td>
<td>7.0</td>
<td>3.0</td>
<td>7.77</td>
</tr>
<tr>
<td>N</td>
<td>66.0</td>
<td>20.95</td>
<td>72.44</td>
</tr>
<tr>
<td>APDT</td>
<td>15.33*</td>
<td>2.51</td>
<td>14.0*</td>
</tr>
</tbody>
</table>

P : healthy rats group
N : untreated chronic periodontitis rats group
APDT : chronic periodontitis model given 1 mg/ml PS erythrosine and radiated with 60 second blue LED group
*significantly different with N group (p ≤ 0.05)
group differed significantly with the P group on the 3rd and 5th days, and differed significantly with the N group on the 1st and 3rd days (p < 0.05). The mean lymphocyte in the APDT group decreased on each day of treatment and showed a significant difference on the 1st and 3rd days, but not on the 3rd or 5th days.

In Table 3, the healthy control group (P) had the highest mean number of fibroblasts, whereas the negative control group as the periodontitis model without treatment (N) had the lowest mean number of fibroblasts on the 1st, 3rd, and 5th days. The mean number of fibroblasts in the APDT group increased during the course of the observation (p > 0.05), differed significantly from the untreated negative control group (N) (p < 0.05), and did not differ significantly from the group with healthy condition (P) (p > 0.05).

Table 2. The number of lymphocytes cells in each day

<table>
<thead>
<tr>
<th>Group</th>
<th>day 1</th>
<th>day 3</th>
<th>day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>P</td>
<td>14.3</td>
<td>3.0</td>
<td>14.8</td>
</tr>
<tr>
<td>N</td>
<td>16.2</td>
<td>1.8</td>
<td>17.3</td>
</tr>
<tr>
<td>APDT</td>
<td>13.6*</td>
<td>0.8</td>
<td>7.22*</td>
</tr>
</tbody>
</table>

P : healthy rats group  
N : untreated chronic periodontitis rats group  
PDT : chronic periodontitis model given 1 mg/ml PS erythrosine and radiated with 60 second blue LED group  
*significantly different with N group (p ≤ 0.05)  
**significantly different with P group (p ≤ 0.05)

Table 3. The number of fibroblasts in each day

<table>
<thead>
<tr>
<th>Group</th>
<th>day 1</th>
<th>day 3</th>
<th>day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>P</td>
<td>55.7</td>
<td>4.6</td>
<td>55.0</td>
</tr>
<tr>
<td>N</td>
<td>26.0</td>
<td>1.6</td>
<td>27.5</td>
</tr>
<tr>
<td>APDT</td>
<td>45.5*</td>
<td>5.9</td>
<td>47.7*</td>
</tr>
</tbody>
</table>

P : healthy rats group  
N : untreated chronic periodontitis rats group  
PDT : chronic periodontitis model given 1 mg/ml PS erythrosine and radiated with 60 second blue LED group  
*significantly different with N group (p ≤ 0.05)

Figure 1. Gingival tissue on healthy rats (A); Inflammation on rats with chronic periodontitis (B)
**DISCUSSION**

Inflammation can increase the number of macrophage cells as host defense cells that will produce proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8). When periodontitis is not treated, causing massive infiltration of macrophages compared to the virulence factors of Porphyromonas gingivalis, the number of macrophages in the APDT group showed almost the decreasing number as the day of treatment given to rats. This may suggest that APDT treatment with a combination of blue LED light irradiation and PS erythrosine administration can stimulate the inflammatory process and lead to the healing of periodontal tissue.

Figure 2. Histological observation of macrophages (M) fibroblasts (F), and lymphocytes (L) on healthy rats P group (A); rats with induced chronic periodontitis N group (B)

Figure 3. Histologic images of macrophages (M) fibroblasts (F), and lymphocytes (L) on rat gingival tissue of each group stained in Hematoxylin-eosin (HE). (A) Healthy control group (P); (B) Untreated negative control group (N); (C) APDT group (PDT)
DISCUSSION

APDT treatment with a combination of blue LED light irradiation and PS erythrosine administration showed an antibacterial effect as measured by the effect on the gingival immune response model of chronic periodontitis. The mean number of macrophages in the N group showed a pyramid pattern where macrophages increased on the 3rd day and started to decrease on 5th day. This pattern may indicate an inflammatory process in the chronic periodontitis rat model. Inflammation can increase the number of macrophage cells as host defense cells that will produce proinflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8. Porphyromonas gingivalis induced chronic periodontitis in rats is related to the virulence factors of Porphyromonas gingivalis bacteria such as lipopolysaccharide (LPS), gingipain, and fimbriae. LPS can induce a host immune response; therefore, it can stimulate the release of cytokines TNF-α, PGE-2, and IL-6 which are produced by inflammatory cells. The inflammation can continue when periodontitis is not treated, causing massive infiltration of macrophage cells as one of the body's defenses against inflammatory agents. The pattern of the number of macrophages in the APDT group showed almost the decreasing number as the day of treatment given to rats. These numbers may suggest that the treatment can decrease the number of macrophages compared to no treatment (N). This may indicate a healing process in the inflamed periodontal tissue.

The results of the APDT group showed no significant difference from the P group on days 1, 3, and 5 of the treatment. These results indicated that the APDT treatment could reach the number of normal macrophages in the healthy group. Fracalossi et al found that APDT combined with erythrosine as a photosensitizer can effectively inactivate bacteria that cause dental plaque. It can also significantly reduce the number of other pathogenic bacteria and prevent the possibility of bacteria to develop resistance to small treatments which are unlike the use of antibiotic therapy. Indrawati et al showed that the method to inactivate bacteria that cause periodontitis is a photodynamic reaction of APDT which works by damaging the permeability of cell membranes and mitochondrial membranes of bacteria/ target cells, so the chance of bacterial resistance in APDT is small. Habiboallah et al state that APDT as adjuvant therapy for chronic periodontitis needs to be balanced with the gold standard therapy, scaling and root planning, so the therapy can be more effective. The results of this study are in line with the research of deOliviera et al which found that the use of complete PPE can reduce the number of bacteria that cause periodontitis. APDT has been shown to produce an antibacterial effect that can reduce the number of bacteria that cause periodontitis.

The mean lymphocyte in the N group increased gradually, indicating an inflammatory process following the induction of chronic periodontitis. One of the bacteria that causes periodontitis is P. gingivalis which has a cell wall consisting of one component, namely lipopolysaccharide (LPS). LPS is a bacterial virulence factor that can cause the destruction of gingival epithelium and activate immune cell chemotaxis. Immune cells migration in chemotaxis will cause an increase in the number of immune cells circulating in the tissue, one of which is lymphocytes.

In the healthy control group (P), the number of lymphocytes had a stable pattern on days 1, 3, and 5. The healthy control group became the benchmark for the number of lymphocytes in healthy conditions. Previous studies have shown that mean lymphocyte in healthy conditions is lower than in the periodontitis-induced group. The periodontitis model that was given the intervention, the APDT group, had a decrease in the average number of lymphocytes in the 1st, 3rd, and 5th days of treatment. Wound healing in tissues can occur by eliminating the etiology, one of which is by killing plaque bacteria such as P. gingivalis. APDT treatment can remove plaque bacteria by increasing the levels of ROS in the tissue, causing damage to bacterial cells. The absence of bacteria can stop the induction of cytokines in tissues, thereby stopping the migration and chemotaxis.
of immune cells. Immune cells in the tissue will undergo apoptosis in the absence of antigens. This apoptotic mechanism can lead to differences in the number of lymphocytes in different groups and on different days. This is a key factor in the decrease in the number of lymphocytes in the treatment group.

Prolonged exposure to endotoxins in the host, particularly to fibroblasts, can also result in chronic inflammation. Resident fibroblasts are responsible for modulating the resolution of inflammation and the beginning of healing process by signaling various other proinflammatory cells to resolve the infection. Since fibroblasts cannot withstand prolonged contact to \textit{P. gingivalis} LPS bacterium, they may continue to send out persistent proinflammatory signals. Fibroblasts in periodontal tissues maintain the generation of matrix metalloproteinases (MMPs) by type 1 macrophages (M1) that are active in chronic periodontitis. In this study, the number of fibroblasts in the negative control group (N) remained very low until day 5. Reactive oxygen species (ROS), which are created by other immune cells and accumulate in tissues with chronic inflammation, can cause oxidative stress in fibroblasts. Prolonged high ROS concentrations in tissues can harm cells and cause fibroblast apoptosis. In the healing phase of chronic periodontitis, a lack of fibroblasts and the degradation of newly generated collagen in inflamed tissue can cause a delay in inflammation resolution and tissue regeneration.

Exposure to APDT with 0.2 ml of erythrosine and blue LED light irradiation for 60 seconds (PDT) could increase the number of fibroblasts on days 1, 3, and 5. The findings of this study are consistent with those of Astuti et al, which showed that the number of fibroblasts in the APDT group was higher than in the periodontitis group without treatment (N). Previous studies have demonstrated that APDT using PS erythrosine and blue LED light irradiation can kill pathogenic microorganisms in the oral cavity, including \textit{P. gingivalis} and \textit{C. albicans}. Additionally, it has been demonstrated that APDT using PS erythrosine and blue LED light irradiation is safe for host tissues, does not harm fibroblast cells, and can boost their proliferative capacity in vitro via elevation of its metabolism activities.

Reduction in the amount of periodontopathogenic bacteria allows the transition of host tissues from the inflammatory to the proliferative phase. After the inflammation has reduced, the proliferative phase of wound healing would start on day 3 and peaks on day 5. In this study, the number of periodontitis rat fibroblasts rose on the third day after exposure to APDT, indicating that the tissue had started to transition to the proliferative phase. A decrease in proinflammatory cytokines is one indicator of the transition from the inflammatory to the proliferative phase. Proinflammatory cytokines including IL-1, IL-6, TNF-, and IL-8 can also be decreased by APDT, which allows inflammation resolution. The process of migration and proliferation will then result in an increase in the number of fibroblasts in the inflammatory area. Numerous growth factors, such as basic fibroblast growth factor (bFGF), transforming growth factor (TGF-), vascular endothelial growth factor (VEGF), and others, promote the proliferation of fibroblasts.

On the fifth day of observation of the chronic periodontitis rats receiving APDT, the number of fibroblasts peaked and equaled that of healthy controls without any significant difference. The rise in the number of fibroblasts may suggest that the tissue was at the peak proliferative phase and the inflammatory phase had passed. Astuti et al found that the number of fibroblasts of chronic periodontitis rats after APDT peaked on day 5. The increase in the number of fibroblasts of chronic periodontitis rats after APDT is followed by an increase in collagen production and the development of new blood vessels, or angiogenesis, in the tissue healing process. Vasodilation and angiogenesis promote periodontal tissue repair because fibroblasts secrete collagen when oxygen levels in the tissues are high. Fibroblasts will continue to produce type III collagen, which over time will be strengthened into type I collagen. In vivo, APDT with erythrosine and blue LED light irradiation combined with scaling and root planing...
(SRP) can reduce bone damage, the RANKL/OPG ratio, and the amount of TRAP positive cells better than SRP treatment alone.\textsuperscript{10} The limitation of this study is that there is no clinical observations of the periodontal tissue of rats in each group on each day of treatment. These observations should be done to further see the effect of APDT on the clinical healing process. Further research is also needed to further evaluate the efficacy of APDT with blue LED light irradiation and PS erythrosine on cytokine levels in periodontitis models with various irradiation durations.

CONCLUSION
There were significant differences in macrophages, lymphocytes, and fibroblasts between chronic periodontitis rats with the treatment of APDT and untreated chronic periodontitis rats.

ACKNOWLEDGMENT
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CONFLICT OF INTEREST
The authors declare no conflict of interest with the data contained in the manuscript.

REFERENCES
10. de Oliveira PG, Silveira e Souza AM, Novaes AB, Taboa M, Messora MR, Palito DB, Grisi MF, Tedesco AC, de Souza SL. Adjunctive effect of antimicrobial photodynamic therapy in


25. Longo M, Garcia VG, Ervolino E, Alves ML, Duque C, Wainwright M, Theodoro LH. Multiple