Influence of alpha lipoic acid on epithelial apoptosis in experimental periodontitis

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Aim: Alpha lipoic acid (ALA) is a powerful antioxidant. Its antiinflammatory effects and apoptotic mechanism have recently been studied in many diseases. This study aimed to investigate the effects of ALA on apoptotic activity in the treatment of periodontitis.

Materials and methods: Thirty male Wistar rats were randomly divided into 3 groups: control (without ligature), PED (ligature + placebo, saline; oral gavage), and ALA+PED (ligature + alpha lipoic acid at 50 mg/kg/day in saline; oral gavage). Histological and biochemical analyses were performed on the mandibular first molars of sacrificed rats.

Results: Biochemical myeloperoxidase activity in gingivo-mucosal tissue was measured. For immunohistochemical evaluation, tissue sections were stained with Bax, Bcl-2, proliferating cell nuclear antigen (PCNA), and 8-OHdG antibodies by streptavidin-biotin-peroxidase staining. Immunohistochemical results showed that apoptotic activity decreased in the periodontitis group. 8-OHdG reactivity increased in the ALA-administered group.

Conclusion: ALA exhibited proapoptotic and antiinflammatory effects on gingivo-mucosal epithelium in the periodontitis.

Key words: Experimental periodontitis, apoptosis, alpha lipoic acid, oxidative stress

1. Introduction
Periodontal infections occur when biofilm microorganisms initiate a host immune response and produce signs of periodontitis, including loss of connective tissue attachment and alveolar bone resorption (1–3). Bacteria-mediated periodontal diseases generally result in polymorphonuclear leukocyte (PMNL) infiltration. PMNLs are an important part of the immune system, and appear to be functionally activated and exhibit increased production of reactive oxygen species (ROS) (4–6). In the tissue, ROS mediated myeloperoxidase activity can lead to an increase in inflammation severity (7). Once the cells are exposed to these damaging agents, cellular injury may progress, through a reversible stage, to culminate in cell death such as necrosis and apoptosis. Although the bacterial etiology in periodontitis is well known, the pathogenic process is not fully clarified.

Apoptosis is called programmed cell death and occurs in normal living bodies as a physiological process and is regulated by different genes including proapoptotic (Bax, Bcl-xS, Bad, Bak, and Bik) or antiapoptotic (Bcl-2, Bcl-xL, Bag-1, and Mcl-1) gene systems in the cells. It was previously reported that some of the pathogenic microorganisms were able to induce apoptosis in an inflammatory process (8). In this mechanism, reactive oxygen species (ROS) and resultant oxidative stress also play an important role in apoptotic cell death (9,10). However, recently it was demonstrated that the apoptosis process could be impaired and inhibited in chronic inflammatory periodontal diseases (11). In apoptotic regulation, the antioxidative property of Bcl-2 was reported to strongly inhibit the process (12). Moreover, in normal aerobic cell synthesis, antioxidant defense mechanisms counteract the damaging effects of ROS (13). The inhibition of apoptosis was affected by tissue regeneration and healing during chronic inflammatory diseases, such as periodontitis.

Alpha lipoic acid (ALA) is a powerful antioxidant that can alter the cell redox status and interact with thiols and other antioxidants (14). It can scavenge various types of ROS, including superoxide radicals, hydroxyl radicals, peroxyl radicals, and singlet oxygen (15). ALA has been reported to induce apoptosis in various diseases such as...
some kinds of epithelial cancer (16,17). It affects critical survival mediators such as phosphatidylinositol 3-kinase and its effector protein kinase B (PKB/Akt) (18). ALA has recently been thought to be a potential therapeutic agent in the treatment of chronic inflammatory diseases related to its apoptotic potential. The regeneration rate of the gingival epithelium may influence the progression of periodontal diseases (19). Thus, induction of apoptosis in gingival epithelium can change the inflammation characteristic, and inhibit tissue destruction. In many studies, it was also suggested that apoptosis could have a therapeutic role in chronic periodontal inflammation (20–22).

The first aim of the present study was to investigate the possible effects of ALA on apoptosis by inflammatory activity and DNA damage in periodontal disease. Secondly, the potential usefulness of this antioxidant in healing periodontal disease was investigated by histopathologic and biochemical methods.

2. Materials and methods

2.1. Animals and housing

Thirty Wistar male rats weighing about 220–250 g (8 weeks of age) were housed in an air-conditioned room (23–25 °C) with a 12-h light–dark cycle and they received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. They were kept in the animal house at Atatürk University’s Experimental Research Center, Turkey, for 1 week for proper acclimatization before starting the experiment under controlled conditions of illumination (12 h light/12 h darkness) and temperature (23 ± 2 °C). The animals were given standard rat pellets and tap water ad libitum. All experiments in this study were approved by the Local Ethics Board of Animal Care and Use of Laboratory Animals at Atatürk University (25.12.2009/B.30.2.A TA.02385-139).

2.2. Animal periodontitis model

Thirty male Wistar rats were randomly divided into 3 groups:

- Control group (C): without ligature and had no treatment for 50 days.
- Periodontitis group: (PED); ligature + placebo (saline; oral gavage)
- Alpha lipoic acid administered periodontitis group (ALA+PED); ligature + alpha lipoic acid 50 mg/kg/day in saline; oral gavage.

For periodontitis induction, PED and ALA+PED groups rats were anesthetized with xylazine hydrochloride (10 mg/kg) (Rompun*) and ketamine hydrochloride (40 mg/kg) (Ketalar*), and the rats were then subjected to experimental periodontitis by tying 3-0 sterile silk ligatures around the right and left mandibular first molars; these were kept in position for 35 days to promote microbial dental plaque accumulation and inflammation (23,24). After 35 days the ligature was removed. The animals were weighed before the ALA administration at the beginning of the experiment, and ALA dose was improved according to our previous studies (23). Alpha lipoic acid (Sigma Chemical Co., St. Louis, MO, USA) and 0.9% NaCl solution were given for 15 consecutive days after the ligature was removed. At the end of the experimental period, the animals were sacrificed under Rompun and Ketalar anesthesia and then the mandibles were removed. The left side mandible tissues were carefully removed together with the surrounding gingiva for biochemical analyses, according to the delivered standardizes procedure (25). The right side mandibles were fixed in neutral buffer formaldehyde and used for histologic analysis.

2.3. Biochemical analysis

2.3.1. Measurement of MPO activity

For biochemical MPO analysis, all collected samples were obtained from gingivo-mucosal tissues. Then the samples were immediately stored in a deep freeze (at approximately −80°C) for subsequent laboratory analysis. The homogenate was then centrifuged at 10,000 rpm for 15 min, and the supernatant used for the determination of MPO activity according to the method developed by Bradley et al. and was estimated by colorimetric measurement at 460 nm on an ELISA plate reader (μ-Quant, BioTek Instruments, USA) (24). The amount of enzyme necessary to produce a change in absorbance per 1 s was defined as 1 unit of MPO activity.

2.4. Histological analysis

For histological and immunohistochemical analysis, the left mandibular molar regions were removed and fixed in 10% buffered neutral formaldehyde for 3 days. The mandible tissues were decalcified with 6% nitric acid solution for 2 weeks. Fixed and decalcified tissues were dehydrated in ethanol, cleared in xylene, and then embedded in paraffin. The paraffin blocks were cut 5-µm thick and stained with Mallory’s triple stain modified by Crossman for histological evaluation. The inflammation activity was evaluated and photographs taken by a high-power light microscopic (Nikon Eclipse 50i). Inflammation activity evaluation was realized in randomly selected 20 areas of approximately ×20 objective with an image analysis program (Kameram SLR, 1.4.1.0, Mikro Sistem Ltd.). Inflammation activity was microscopically scored by mononuclear and PMNL counts. The scores were derived semiquantitatively by using light microscopy on the 3 slices from each animal and were reported as follows: ++++: numerous, +++: moderate, ++: a few, +: rare, −: not detected.
2.5. Immunohistochemical analysis

For immunohistochemical staining, 5-μm thickness sections were deparaffinized, dehydrated, and immersed in a phosphate buffer with 3% hydrogen peroxide to eliminate endogenous peroxidase activity. For antigen retrieval, the sections and all antibodies were treated by heating in a heater at 121 °C in an EDTA buffer (pH 8.0) for 20 min. The sections for Bax, Bcl-2, proliferating cell nuclear antigen (PCNA), and 8-OHdG immunostaining were treated with normal serum for 20 min to block nonspecific binding; the sections were incubated with Bax (1/50 dilution; Abcam, Cambridge, UK), Bcl-2 (1/50 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), PCNA (1/100 dilution; Thermo Fisher Scientific, Fremont, CA, USA), and 8-OHdG (1/50 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) primary antibodies at 37 °C for 60 min; and the standard biotinylated secondary antibody kit (DAKO-Universal LSAB Kit-K0690) was used for immunohistochemical staining according to the manufacturer's instructions. Nuclei were lightly counterstained with Harris hematoxylin. The binding sites of the antibody were visualized with DAB (Sigma), and photographed using a high-power light microscopic (Nikon Eclipse 50i).

2.6. Stereological analyses

In all group sections, the numerical density of cells (according to the antibodies’ positivity) was evaluated via the optic fractionator frame method. Stereological analyses were performed in a stereology workstation, consisting of a modified light microscope (Leica DM4000 B) and stereology software (Stereo Investigator version 9.0, Microbrightfield, Colchester, VT, USA). The unbiased counting frame-fractionator combination is a stereological method for counting cells in tissue sections (23,26). In the present study, we used the unbiased counting frame and fractionator methods to estimate the numerical density of positive cells according to staining with Bax, Bcl-2, and PCNA antibodies in the gingival epithelium. Five section slides from each animal were sampled using the fractionator principle of the stereology software. Cells were counted using a 40× Leica Plan Apo objective (NA = 1.40), which allowed accurate recognition (Figure 1). The positive cells density was estimated according to the formula given below:

\[
PCD = \frac{NPC}{(CFA \times NSS)},
\]

where \( PCD \) is positive cell density per µm² area, \( NPC \) is number of positive cells, \( CFA \) is counting frame area (XY) (µm²), and \( NSS \) is number of sampling sites. The data were expressed as a group mean.

2.7. Statistical analysis

The histopathologic and biochemical results were expressed as mean ± SEM of 10 animals in each group. For statistical analysis, differences between the groups were tested by analysis of variance using SPSS software, version 17.0 (SPSS Inc.). Statistical significance was accepted for all tests at P < 0.05.

3. Results

3.1. Biochemical results

3.1.1. MPO activity

We used the MPO activity in our study as an indicator of inflammation activity. The MPO activity of the PED group showed significantly increased inflammatory activity compared with that of the ALA treatment and control groups (P < 0.05; Table).

3.2. Histologic results

3.2.1. Determination of inflammatory activity

The histological examination showed that the control group exhibited normal histologic tissue structures. An increase in mononuclear and PMNL cell infiltration was

<table>
<thead>
<tr>
<th>Epithelial positive cell number/1000 µm² area</th>
<th>Inflammation activity</th>
<th>Myeloperoxidase (MPO) (Units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>Bcl-2</td>
<td>PCNA</td>
</tr>
<tr>
<td>Control (n = 10)</td>
<td>0.3721</td>
<td>0.4974</td>
</tr>
<tr>
<td>PED (n = 10)</td>
<td>0.2731b</td>
<td>0.8271b</td>
</tr>
<tr>
<td>ALA+PED (n = 10)</td>
<td>0.5942c</td>
<td>0.6315bc</td>
</tr>
</tbody>
</table>

The MPO activity is expressed as mean ± S.D. and remaining values are expressed as means. The footnote letters in the same column indicate significant differences between groups (P < 0.05). For statistical analysis, differences between the groups were tested by analysis of variance followed by the Duncan post hoc test (ANOVA).
observed in the PED group and the densities of PMNL and MNL were lower in the ALA treated and control groups. In the ALA-treated group tissues, a small number of inflammatory cells were seen and these tissues exhibited a nearly normal, noninflamed structure. The inflammation scores and figures for all the groups are shown in the Table and Figure 2.

3.3. Immunohistochemical results

3.3.1. Determination of Bax and Bcl-2 expression

To investigate the effect of ALA on apoptosis in periodontal tissues, we determined Bax and Bcl-2 expression. The immunohistochemical results show that low-level, positively stained Bcl-2 cells were observed in the ALA+PED and control groups. Conversely, the immunohistochemical results for the PED group reveal high-level, positively stained Bcl-2 cells in the gingivo-mucosal tissues and the increase in Bcl-2 activity was significant compared to the control group (P < 0.05). In the immunohistochemical analysis of Bax expression, all groups were significantly different from each other (P < 0.05) and the degree of positive staining of Bax positive cells was dramatically reduced in the PED group. An increase in the degree of Bax positive cells was observed in the gingivo-mucosal tissues of the ALA-administered group. The control group exhibited a normal level of positive staining of Bax cells. The Bax and Bcl-2 analyses results and figures for all the groups are summarized in the Table and Figures 3 and 4.

3.3.2. Determination of the expression of proliferating cell nuclear antigen (PCNA)

The immunohistochemical findings show that PCNA expression decreased in the PED group compared with ALA+PED and control groups. However, there was no significant difference in the comparisons of the groups (P > 0.05). Stereologic estimation of PCNA immune reactivity results and figures for all the groups are shown in the Table and Figure 5.

3.3.3. Determination of 8-OHdG expression

To test whether ALA administration affects DNA damage in gingivo-mucosal epithelial tissues during periodontitis, we determined 8-OHdG expression. The immunohistochemically stained tissue sections did not show a significant increase in 8-OHdG-immunopositive cells in the periodontitis group compared with the ALA and control groups (P > 0.05). The ALA+PED group had higher 8-OHdG immune-positive staining than did the control (P < 0.05). 8-OHdG immune reactivity results for all the groups are shown in the Table and Figure 6.

4. Discussion

The present study revealed that ALA administration showed potential effects on oxidative stress-mediated apoptosis, cell proliferation, and DNA damage. In the current work, ALA administration increased gingivo-mucosal epithelial apoptosis during periodontitis because of the increased expression of Bax and the decreased expression of Bcl-2, respectively. The PCNA protein level verified our hypothesis that PCNA activity and apoptotic activity occur in parallel with each other. Ascertaining the effective use of ALA necessitates gathering evidence on whether a treatment agent has pronounced apoptotic effects on gingivo-mucosal epithelial tissue during periodontitis. Further experiments will be required for the comparison of such tissues with periodontitis-affected gingival epithelial cells. This study was performed to...
investigate the effects of ALA treatment on epithelial apoptosis in rats.

ALA has previously been recognized to provide protection against the oxidative stress mediated damages in experimental studies, both in vivo and in vitro (24,25). Periodontal diseases are related to an imbalance between oxidant and antioxidant levels due to oxidative stress (27). These antioxidant and oxidant imbalances could impair apoptosis (programmed cell death) and promote higher release of ROS by phagocytes; decrease in catalase and superoxide dismutase activity could stimulate accumulation of ROS and result in further tissue destruction. In previous studies, ALA treatment of animals has been shown to protect tissues against oxidative damage and observed to act as an antioxidant towards hydroxyl radicals (28) and to inhibit the oxidation of lipids and proteins (29). In the present study, we used the ALA antioxidant substance, which can neutralize free radicals by donating an electron without becoming unstable themselves, to promote the apoptotic activity in the damaged tissues.

ROS-mediated oxidative stress causes cell damages during periodontitis. Damaged cells die by either necrosis or apoptosis (9). When cells die by apoptosis, soluble factors that recruit phagocytes are secreted. The cells express adhesive glycoproteins and are recognized by phagocytes and macrophages as binding apoptotic cells (but not live cells). This binding facilitates dead cell clearance before these cells undergo secondary membrane damage and release their cellular contents (which can result in inflammation). This apoptotic phagocytic process is highly effective in preventing dead cells from disappearing without leaving a trace and in inhibiting inflammatory activity. In chronic oxidative stress-mediated cell damage, the cell membrane releases the enzymes that leak out of lysosomes, thereby resulting in necrosis. Cellular contents also induce host inflammatory response, which increases oxidative stress and causes cells to experience potential cellular damage (30). Various antioxidants have protective mechanisms that minimize chemiotaxis effects, as well as oxygen metabolites and cytokine toxicity. Such antioxidants include catalase, superoxide dismutase, and glutathione. In
some cases, however, cellular antioxidants do not prevent inflammatory damage. Depending on cell type and cellular oxidative status, ALA can be a potent antioxidant (31). It can regenerate other cell protective antioxidants, such as ascorbic acid, vitamin E, and glutathione (32). Its potential underlying apoptotic pathway is through catalysis of the formation of intramolecular disulfides in certain signaling proteins that are responsible for oxidant detection (33).

In many apoptosis models, an alteration in cell redox status induces the final stage of caspases activation (10). In the present study, ALA can inactivate the Akt (PKB) pathway, which can result in caspase-9 activation. Akt
activity is consistent with the negative regulatory role of phosphatase and tensin homolog (PTEN) in Akt signaling. Therefore, PTEN activity is associated with the degree of Akt activity, indicating that ALA-mediated apoptosis induction is realized through the Akt/PTEN pathway. Because of apoptosis induction, the antioxidant effects of ALA are generally observed under oxidative stress conditions, such as inflammation inhibition (10). However, Bcl-2 protein is known as a potent apoptosis inhibitor, whose activity increases in periodontal disease (34). In contrast, Lucas et al. suggested other factors in the inhibition of apoptosis in periodontitis (35). Bcl-2 associated transcription factor 1 (BCLAF1) is a transcriptional repressor that is related to several members of the Bcl-2 family and triggers apoptosis. These proapoptotic factors are Bax, Bag3, and BID proteins, and they participate in the intrinsic pathway in the regulation of apoptosis in periodontal ligament cells (36). In the present study, ALA administration can reduce Bcl-2 expression and induce apoptosis by increasing Bax protein activity.

In the present study, we used the PCNA antibody for evaluating the cells’ kinetic, normal, and pathologic situation indicators, as per Casasco et al. (37). In a normal cell cycle, PCNA is a nuclear protein that has an important function in DNA synthesis. PCNA protein level is directly related to cell proliferation and PCNA functions are controlled by p21CIP1/WAF1, which is also controlled by the p53 gene (33,38,39). The P53 gene contributes to the inhibition of cell growth and can actively arrest the cell cycle and trigger apoptosis (40). In the present study, ALA administration can reduce Bcl-2 expression and induce apoptosis by increasing Bax protein activity.

In summary, the results of this study reveal that ALA administration can induce apoptosis of gingival epithelial cells in the intrinsic apoptotic signaling pathway. While the process of apoptosis in periodontal diseases is very complex, this study demonstrated that the apoptotic process can accelerate the treatment of periodontitis because it does not allow inflammation or chemiotaxis in periodontal disease. These data suggest that use of this antioxidant agent may be considered as therapeutic for the treatment of human chronic periodontitis.

References


