Investigation of the effect of astaxanthin on alveolar bone loss in experimental periodontitis

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Background and Objective: Astaxanthin is a keto-carotenoid that has a strong antioxidant effect. The purpose of this study was to evaluate the effects of astaxanthin on alveolar bone loss and histopathological changes in ligature-induced periodontitis in rats.

Material and methods: Wistar rats were divided into four experimental groups: non-ligated (C, n = 6); ligature only (L, n = 6); ligature and astaxanthin (1 mg/kg/day astaxanthin, AS1 group, n = 8); ligature and astaxanthin (5 mg/kg/day astaxanthin, AS5 group, n = 8). Silk ligatures were placed at the gingival margin of lower first molars of the mandibular quadrant. The study duration was 11 days and the animals were killed at the end of this period. Changes in alveolar bone levels were clinically measured and tissues were immunohistochemically examined, osteocalcin, bone morphogenic protein-2, inducible nitric oxide synthase, Bax and bcl-2 levels in alveolar bone and tartrate-resistant acid phosphatase-positive osteoclast cells, osteoblast and inflammatory cell counts were determined.

Results: Alveolar bone loss was highest in the L group and the differences among the L, AS1 and AS5 groups were also significant (P < .05). Both doses of astaxanthin decreased tartrate-resistant acid phosphatase-positive osteoclast cell and increased osteoblast cell counts (P < .05). The inflammation in the L group was also higher than those of the C and AS1 groups were (P < .05) indicating the anti-inflammatory effect of astaxanthin. Although inducible nitric oxide synthase, osteocalcin, bone morphogenic protein-2 and bax staining percentages were all highest in the AS5 group and bcl-2 staining percentage was highest in the AS1 group, values were close to each other (P > .05).

Conclusion: Within the limits of this study, it can be suggested that astaxanthin administration may reduce alveolar bone loss by increasing osteoblastic activity and decrease osteoclastic activity in experimental periodontitis model.

Keywords: antioxidants, astaxanthin, experimental periodontitis, inducible nitric oxide synthase, tartrate-resistant acid phosphatase

1 | INTRODUCTION

Periodontitis is a chronic inflammatory disease of the periodontium and one of the principal causes of tooth loss in humans.1,2 Although

The results of present study revealed that astaxanthin reduced alveolar bone loss, and decreased periodontal inflammation in experimental periodontitis in Wistar rats.

the primary etiologic factor is a bacterial plaque in periodontitis, the host response to this bacterial challenge is responsible for advanced tissue destruction.3,4 Therefore, enhancing host defense is crucial in preventing periodontal tissue destruction.

Neutrophils play major roles in inflammation by killing the invading microorganisms by means of reactive oxygen species (ROS).5,6 ROS cause mitochondrial damage and the damage activates the apoptotic
Astaxanthin is a carotenoid from the xanthophyll family and a natural dietary component, which could be found in red colored aquatic organisms. Astaxanthin has a strong antioxidant effect owing to its lipid-soluble feature. In addition, it was reported to induce proliferation and adipogenic and osteogenic differentiation potential of neural stem cells. Astaxanthin doses of 12.5, 25 and 50 mg/kg was also shown to inhibit cyclophosphamide-induced oxidative stress and DNA damage, decrease malondialdehyde levels and restore glutathione and superoxide dismutase levels in the liver. Astaxanthin with the dose of 10, 20 and 40 mg/kg was also shown to prevent oxidative stress by increasing superoxide dismutase, glutathione peroxidase and decreasing malondialdehyde levels and nitric oxide synthase (NOS) expressions induced by diabetes in Wistar rats. These doses of astaxanthin also prevented inflammation by decreasing the levels of nuclear factor kappa B, tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6. Taken together, astaxanthin seems to have various effects on physiology most probably due to its antioxidant character.

Based on this evidence, the hypothesis of the present study was established as "Astaxanthin administration might have beneficial effects in the inhibition of periodontal inflammation and alveolar bone loss." Therefore, the aim of present study was to evaluate the effects of astaxanthin on periodontal tissues in presence of experimental periodontitis.

2 MATERIAL AND METHODS

The study protocol was approved by the local ethics committee of Cumhuriyet University Faculty of Medicine Ethical Committee of Animal Studies. Experimental procedures were conducted at the Cumhuriyet University Laboratory of Animals Research Center. In total, 28 Wistar male rats (weighing 230-250 g) were used in this study. Rats were kept in individual cages in a 12-hour light/dark cycled room. All rats received water and food ad libitum.

Rats were randomly divided into four study groups as:

- non-ligated control group (C, n = 6)
- ligated group (L, n = 6)
- ligated and 1 mg/kg astaxanthin administered group (AS1, n = 8)
- Ligated and 5 mg/kg astaxanthin administered group (AS5, n = 8)

Experimental periodontitis was induced via ligation method. All procedures were performed by the same operator (A.L.A.). Rats were anesthetized with ketamine (Eczacibasi IlacSanayi, Istanbul, Turkey) and the right mandibular first molar teeth were chosen for experimental periodontitis induction. Silk ligatures (4-0) (Dogsan IlacSanayi, Istanbul, Turkey) were firmly sutured around first molars within gingival sulcus. Ligatures were checked after application and every other day for 11 days and any loose or lost ligatures were replaced.

Astaxanthin solutions were freshly prepared daily before administration. For AS1 and AS5 groups, respectively 1 mg and 5 mg AS were dissolved in 1 mL distilled water. Solutions were administered by oral gavage. Rats were kept alive for 11 days and after this period they were killed via anesthetic overdose.

2.1 Measurement of alveolar bone loss

After the scarification, all mandibles were removed and the soft tissues around the first molar teeth were excised. To determine alveolar bone loss, bone levels from the cemento-enamel junction to alveolar crest were measured after mandibles were stained with aqueous methylene blue (1%; Merck & Co., Inc., Whitehouse Station, NJ, USA). Methylene blue was used to identify cemento-enamel junction and visualize bone loss. The alveolar bone loss was measured under a stereomicroscope via digital image software under 16× magnification (Stemi 2000 and Axiovison 4.8, Carl Zeiss, Jena, Germany). The measurements were performed at three points on both the buccal and lingual sides and a mean value for each tooth was calculated. The morphometric measurement of alveolar bone loss was performed by a single examiner (H.T.) who was calibrated and unaware of the identity of samples. Calibration was achieved by measuring 15 different samples three times and the results of the measurements showed $r = .99$ meaning 99% reproducibility. After achieving 99% accuracy, the examiner measured the samples of the present study.

2.2 Histopathological evaluation

Right mandibles were fixed in 10% neutral buffered formalin. The tissues were then decalcified in a fixative-added decalcification solution (GBL Co., Istanbul, Turkey) containing a fixative and ethylenediaminetetraacetic acid with a change twice a week for 10 weeks until decalcification was completed and then the decalcified specimens were dehydrated through an ethanol series and embedded in paraffin. The periodontal tissues in the mesial and distal part of the mandibular first molar tooth were observed. Histological analysis was performed by a single examiner (F.G.) who was also blinded to the identity of samples. Each sample was sliced into 5 μm serial sections and prepared for hematoxylin and eosin and histochemical staining for TRAP and immunohistochemistry staining for iNOS, BMP, osteocalcin, Bax and bcl-2.

Cuboid osteoblast cells bordered with osteoid and neighboring periodontal ligament and visible of active bone formation surfaces were considered as active osteoblast cells and counted in an area
of 10 000 μm² under 1000× magnification. The measurements were performed from three different points as mesial coronal, apical and distal coronal regions in the periodontal ligament area and the means of these three measurements were recorded.

For the evaluation of inflammation, total inflammatory cells in an area of 10 000 μm² (neutrophil, lymphocyte, eosinophil and macrophage cells) were counted with 1000× magnification. The measurements were performed from three different points as mesial coronal, apical and distal coronal regions in the periodontal ligament area and the means of these three measurements were recorded. Cells were counted via a digitalized program (NIS Elements Basic Research; Nikon Instruments Europe BV, Amsterdam, The Netherlands).

2.2.1 | Tartrate-resistant acid phosphatase histochemistry

Deparaffinized sections were subjected to TRAP staining, to identify active osteoclasts, which produce TRAP enzyme. TRAP staining was performed according to the protocol Leong et al. First, rehydrated specimens were treated with 0.2 M acetate buffer, a solution of 0.2 M sodium acetate and sodium tartrate dibasic dehydrate (Sigma-Aldrich, St. Louis, MO, USA). After 20 minutes incubation at room temperature, naphthol AS-MX phosphate and fast red TR salt were added and followed by incubation at 37°C for 1 hour. Bright red staining of the TRAP+ osteoclasts was closely monitored under the microscope. Stained sections were washed in deionized water and sections were counterstained with Gill’s hematoxylin and analyzed using light microscopy (Nikon Eclipse, E 600, Tokyo, Japan). Giant multinuclear cells lining a lacuna in contact with bone were considered as osteoclasts and counted.

2.2.2 | Inducible nitric oxide synthase, bone morphogenetic protein, osteocalcin, Bax and bcl-2 Immunohistochemistry

iNOS immunohistochemistry was performed to evaluate NO activity. BMP and osteocalcin were evaluated to determine osteoblastic activity and bax-bcl immunohistochemistry was performed to evaluate the apoptotic state. Bax is a member of the bcl family and the bax/bcl ratio is considered an indicator of apoptosis or survival in the cell. After deparaffinization and dehydration of the sections, antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) for 2 hours at 70°C. Then the sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After incubation with normal rabbit serum for 30 minutes, samples were incubated with primary antibodies overnight. The antibodies and conditions used were as follows: goat polyclonal anti-iNOS antibody (1:100), anti-BMP antibody (1:50), anti-osteocalcin antibody (1:50), anti-bax antibody (1:100) and anti-bcl-2 antibody (1:100), all from Abcam plc (Cambridge, UK). After washing five times with phosphate-buffered saline the sections were incubated with biotinylated immunoglobulin G for 30 minutes, washed several times with phosphate-buffered saline and reacted with streptavidin-horseradish peroxidase conjugated reagent for 30 minutes. Following the 5 minute washes, three times with phosphate-buffered saline, samples were incubated with AEC chromogen (Abcam plc) to visualize the immunoreactivity. Sections were counterstained with hematoxylin and analyzed using light microscopy.

2.3 | Immunohistochemical semiquantitative H score analysis

Five areas were selected randomly from the sections belonging to each animal to be examined, under a light microscope with 1000× magnification. Categorical enumeration of the cells within these areas was made according to their immune staining intensity. All cells in an area of 10 000 μm² were counted considering the staining intensities. During these counts, both the number of the cells showing positive immunoreactivity and immunoreactivity intensity degrees of these cells and all cells that were stained and not stained were considered. No staining was considered as score “0,” slight staining intensity was “1,” mild staining intensity was “2” and severe staining intensity was “3.” The average of the results of a blind study was taken. For estimating the results of the counts, the H score formula $\sum P_i(i+1)$ was used. In this formula, $i$, shows the staining intensity score and $P_i$, shows the percentage of the stained cells.

2.4 | Statistical analysis

Data were presented as mean ± SD or percentage as appropriate. Results and statistical analysis were performed via a statistical program SPSS v. 20.00 (IBM Corp., Armonk, NY, USA). Alveolar bone loss, osteoclast numbers, osteoblast numbers and H scores of iNOS, BMP, osteocalcin, Bax and bcl-2 were evaluated with ANOVA followed by Tukey test for pair-wise comparisons. $P < .05$ were considered statistically significant.

3 | RESULTS

3.1 | Morphometric analyses

Experimental periodontitis was successfully achieved and ligation caused periodontal destruction and alveolar bone loss around mandibular first molar teeth. The highest alveolar bone loss was observed in the L group and the difference was statistically significant compared to the other groups ($P < .05$) (Figure 1). Both doses of astaxanthin decreased bone loss to a significant level compared to the L group ($P < .05$), but not to the level of the control group ($P > .05$). In addition, there was no significant difference between AS1 and AS5 groups ($P > .05$).

3.2 | Histopathological analyses

Representative images of histological slides were shown in Figure 2. Like alveolar bone loss, the highest inflammatory cell count was observed in the L group and lowest counts were found in the C group. The differences among the groups were statistically significant.
compared to the other groups \( (P < .05) \). Inflammatory cell numbers in AS1 group was slightly higher than AS5 group but the difference was not significant \( (P > .05) \) (Table 1).

Osteoclast cells stained with a red color were counted and the results showed that counts were lower in the C group than L and AS1 groups \( (P < .05) \) (Figure 3). The difference between C and AS5 group was not significant \( (P > .05) \). L group had highest osteoclast cell counts and astaxanthin decreased active osteoclast cell numbers in a dose-dependent manner.

The L group had the lowest osteoblast cell counts compared to the other groups \( (P < .05) \) and both 1 and 5 mg/kg astaxanthin administration increased osteoblast cell counts \( (P > .05) \) (Figure 4).

### 3.2.1 Inducible nitric oxide synthase, osteocalcin, bone morphogenetic protein, Bax and bcl-2 immunohistochemistry

INOS scores of the periodontitis group were significantly lower than those of the control, AS1 and AS5 groups \( (P < .05) \). C, AS1 and AS5 groups had a similar INOS expression \( (P > .05) \) (Figure 5).

AS1 and AS5 groups had a significantly increased osteocalcin score than the C and L groups \( (P < .05) \). Despite being insignificant, osteocalcin levels were higher in the AS5 group than AS1 group \( (P > .05) \). In addition, osteocalcin staining was lowest in the L group, but the difference was not significant \( (P > .05) \) (Figure 5).

Although BMP staining was slightly higher in the AS5 group, no significant differences were observed among the groups \( (P > .05) \).

The results of the Bax and bcl-2 staining were expressed as a bax/bcl ratio obtained by dividing the bax H score and bcl-2 H score. The Bax/bcl-2 ratio was considered a good indicator of a cell's apoptosis/survival situation. The Bax/bcl staining ratio was lowest in the AS5 group compared to the other groups \( (P < .05) \). Control and AS1 groups also had a lower bax/bcl ratio than the L group but the difference was not significant \( (P > .05) \).

### 4 DISCUSSION

The present study is the first to evaluate any beneficial effect of astaxanthin on experimental periodontitis and alveolar bone tissue. The results showed that both doses of astaxanthin provided significant improvements regarding alveolar bone loss and periodontal inflammation in a rat model of periodontitis.

The effects of astaxanthin on periodontitis were evaluated via ligature-induced periodontitis model, which is the most preferred
method for inducing experimental periodontitis.\textsuperscript{20-24} To induce periodontitis, ligation provides a retentive area around the tooth, causes dental plaque accumulation and ulceration in the sulcular epithelium facilitating the bacterial invasion into connective tissue and causing an acute periodontal inflammation and alveolar bone destruction.\textsuperscript{4,25-27} As a limitation, this acute course of periodontitis differs from human periodontitis but this method still provides advantages such as low cost, easy handling, availability and a similar anatomic structure and response to periodontal treatment as humans.\textsuperscript{4,25,27} In the present study, it was observed that ligature caused an intense alveolar bone loss in the L group and both doses of astaxanthin prevented ligature-induced bone loss.

Through various biological activities, astaxanthin has a great potential in the treatment of numerous diseases such as cancer, hepatitis, Alzheimer’s disease and diabetes.\textsuperscript{14,28-30} The most studied doses of astaxanthin are varied starting from 3 to 50 mg/kg.\textsuperscript{13,30-32} As a dietary component, no genotoxic or mutagenic effect of astaxanthin was reported with higher doses of 40, 200 and 1000 mg/kg/day.\textsuperscript{33} In addition, astaxanthin caused no adverse effects on fetal development in rabbits with 100, 200 and 400 mg/kg/day doses.\textsuperscript{34} As a result of these studies, astaxanthin could be considered a safe agent even at the higher doses. The present study used two doses of astaxanthin, 1 and 5 mg/kg. Both are below the aforementioned doses and no adverse effect of astaxanthin was found during the study.

<table>
<thead>
<tr>
<th>Groups/staining density (%)</th>
<th>C group</th>
<th>L group</th>
<th>AS1 group</th>
<th>AS5 group</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>42.83 ± 3.38</td>
<td>38.28 ± 7.85*</td>
<td>46.77 ± 1.67**</td>
<td>49.41 ± 5.00**</td>
</tr>
<tr>
<td>OCN</td>
<td>37.88 ± 4.94</td>
<td>35.60 ± 3.65</td>
<td>42.17 ± 6.25**</td>
<td>47.03 ± 5.27**</td>
</tr>
<tr>
<td>BMP-2</td>
<td>48.74 ± 4.03</td>
<td>46.23 ± 7.68</td>
<td>49.69 ± 7.58</td>
<td>55.23 ± 6.19</td>
</tr>
<tr>
<td>Bax</td>
<td>39.20 ± 0.84</td>
<td>39.10 ± 0.78</td>
<td>39.53 ± 3.69</td>
<td>44.80 ± 3.80</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>46.17 ± 13.33</td>
<td>46.55 ± 4.08</td>
<td>55.08 ± 2.44</td>
<td>54.93 ± 8.49</td>
</tr>
<tr>
<td>Inflammatory Cell Infiltration (ICI)</td>
<td>0.33 ± 0.50</td>
<td>41.33 ± 5.29*</td>
<td>21.00 ± 11.32**</td>
<td>13.33 ± 1.58**</td>
</tr>
</tbody>
</table>

AS1, ligated and 1 mg/kg astaxanthin administered group; AS5, ligated and 5 mg/kg astaxanthin administered group; BMP, bone morphogenetic protein; C, non-ligated control group; iNOS, inducible nitric oxide synthase; L, ligated group; OCN, osteocalcin. Despite slight differences in all immunohistochemical parameters, no significant differences were observed among the study groups. For inflammatory cell counts *P < .05 vs control group and **P < .05 vs ligature group.

![Figure 3](image-url)  
**Figure 3** Osteoclast counts of the groups. AS1, ligated and 1 mg/kg astaxanthin administered group; AS5, ligated and 5 mg/kg astaxanthin administered group; C, non-ligated control group; L, ligated group. *P < .05 vs control group and †P < .05 vs ligature group

![Figure 4](image-url)  
**Figure 4** Osteoblast counts of the groups. AS1, ligated and 1 mg/kg astaxanthin administered group; AS5, ligated and 5 mg/kg astaxanthin administered group; C, non-ligated control group; L, ligated group. *P < .05 vs control group and †P < .05 vs ligature group
expressions in the spleen and liver tissues. Conversely, one study found that astaxanthin had no effect on iNOS levels with the doses 2 and 5 mg/kg. In the present study, astaxanthin decreased osteoclast cell numbers in a dose-dependent manner. However, we evaluated bone expressions of iNOS by immunohistochemistry and found more intense staining in AS groups, but this difference did not reach statistical significance. In the present study, increased inflammatory cell infiltration was observed with ligature placement, but systemically AS administration decreased inflammation in both AS groups. The anti-inflammatory effect of astaxanthin was also shown in liver injury as astaxanthin inhibited production of TNF-α, IL-6, IL-1β and interferon-γ in an autoimmune hepatitis model of mice. In regards to immunomodulation, Sakai et al have shown that astaxanthin suppressed mast cell degranulation and signal transduction. Yoshihisa et al reported that astaxanthin decreased mRNA expression of iNOS, cyclooxygenase-2, IL-1β and TNF-α, and release of prostaglandin E2.

Several reports have demonstrated that osteoblast differentiation can be inhibited by oxidative stress, induced by exogenous stimuli such as hydrogen peroxide. Kim et al showed that astaxanthin induced proliferative capacity, osteogenic and adipogenic differentiation potential of neural stem cells. They also reported that astaxanthin treatment caused calcium deposits and increased expressions of osteonectin, retinoid X receptor, osteopontin and peroxisome proliferator-activated receptor-γ. In our study, we found that administration of astaxanthin increased osteoblastic activity and this result was supported by the increase in osteocalcin expressions in rats. Despite being insignificant, a decrease in BMP-2 levels in the L group and an increase in the AS5 group were also observed.

Apart from the antioxidant effect, astaxanthin has the ability to alter proliferation, cell viability and apoptosis. Bax is a member of the bcl family and the bax/bcl ratio is considered an indicator of apoptosis or survival in the cell. Yan et al have shown that astaxanthin prevented acetaldehyde toxicity by decreasing p38 mitogen-activated protein kinase and preventing Bak protein and apoptosis while increasing the level of activated extracellular signal-regulated kinases and supporting bcl-2 production and survival. However, Wen et al have found that astaxanthin decreased caspase-3, -8 and -9 expressions, accumulation of endogenous ROS and mitochondrial bcl-2 expressions while increasing mitochondrial bax expressions in neural cells. In this study, bax-bcl immunohistochemistry was performed to evaluate the apoptotic state in osteoblast cells and a ratio of bax to bcl-2 was calculated to evaluate apoptotic protein levels against anti-apoptotic protein levels. The results of present study revealed a similar bax expression among the groups except for the AS5 group, which had the lowest bax/bcl ratio. Ligature placement...
caused an increase in the bax/bcl ratio and AS successfully improved the apoptotic state.

Immunohistochemistry in histological slides is a widely used histological procedure in basic research to understand the involvement of various biomarkers in the pathogenesis of periodontitis. Immunohistochemistry provides advantages such as requiring small samples of tissues, wide availability, application in frozen or fixed samples, and evaluation of biochemical events in cell level. However, the response to antibody might be variable in different samples and reproducibility of results may be variable. In addition, interpretation of the results might be subjective. Therefore, using more quantitative and less subjective analysis along with immunohistochemistry would increase the accuracy and reliability of the results.

5 | CONCLUSION

In conclusion, astaxanthin is a strong antioxidant and has a great potential in preventing diseases associated with oxidative stress. Astaxanthin successfully prevented disease progression by both inhibiting osteoclastic activity and increasing osteoblast cells. However, the effect of astaxanthin in the present study was based on histological findings of osteoblast cells, which were determined based on the morphological evaluation. Further studies with the involvement of advanced biochemical analysis would be beneficial in evaluating the effect of astaxanthin on bone.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this study.

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