Determination of systemically & locally induced periodontal defects in rats

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Background & objectives: The role of lathyrogens on bone metabolism is unclear, therefore we undertook this study to observe periodontal and systemic alterations in experimental lathyrism in rat and compare these changes to that observed in the locally induced periodontitis group.

Methods: A total of 45 male Wistar rats were equally divided in the lathyritic group (group 1), ligature-induced periodontitis group (group 2), and healthy controls (group 3). Experimental lathyrism was induced by once daily subcutaneous administration of beta-aminoproprionitrile (ß-APN), at a dose of 5 mg/0.4 ml per 100 g of body weight for 40 days. Ligature-induced periodontitis was created by tying silk ligatures on the necks of mandibular molars. After 40 days, blood samples were obtained and the animals were decapitated. Radiographic observations, extraction tests, histologic evaluations were performed, and serum ALP activity and gingival tissue IL-1  levels were measured.

Results: Significant alveolar bone resorption around the mandibular molar teeth (P<0.001); lower extraction force levels (P<0.001); higher numbers of lymphocytes and macrophages (P<0.01) (both in connective tissue and epithelium at the dentogingival junction); decreased ALP activity (P<0.001); and increased gingival tissue IL-1  levels (P<0.001) were observed in groups 1 and 2, compared to those in group 3. ALP activity was higher in group 1 than in group 2 rats (P<0.05).

Interpretation & conclusion: Similar radiographical and histopathological findings and comparable increases in gingival tissue IL-1  levels both in groups 1 and 2 showed that in addition to resorption of alveolar bone, chronic inflammation of periodontium also occurred both in the lathyritic rats as well as in ligature-induced periodontitis group rats.

Key words Alkaline phosphatase - alveolar bone loss - interleukin-1-beta - lathyrism - periodontitis

Periodontitis, a chronic inflammatory disease of the supporting tissues of the teeth, causes loss of connective tissues and resorption of the alveolar bone. Because periodontium has a rapid turnover of soft tissues, especially collagen, during growth and development, it is considered to be a suitable experimental model to study the effects of systemic and local factors on connective tissues. Animal models are widely used in the study of pathologic processes of periodontitis. Pathological changes due to systemic factors, such as intoxication, or local factors, such as the accumulation of microbial dental plaque or related effects, can be achieved in the periodontium of rats.
Lathyrism is characterized by defective collagen synthesis due to the inhibition of lysyl oxidase and is caused by ingestion of the *Lathyrus* species and their toxic constituents. The lathyritic agent, beta-aminopropionitrile (β-APN), isolated from *Lathyrus odoratus* (sweet pea), is toxic and responsible for the predilection of tissues with a high collagen content. β-APN, by inhibiting cross-linking of the collagen molecule, reduces any contraction occurring during collagen maturation. This phenomenon may affect the resistance of the periodontal ligament fibers to tension.

Experimental lathyrism mostly affects the tissues which have high collagen content, such as cartilage, bone, and fibrous and elastic connective tissue. The effect of lathyrogens on bone metabolism is not well understood though their effects on the organic portion of the bone are well known.

Alkaline phosphatase (ALP) plays an important role in the regulation of bone formation and may increase in areas where high bone turnover occurs. Evaluation of the relationship between locally induced experimental periodontitis and systemically induced experimental lathyrism and serum ALP activity may be important to detect the systemic role of both β-APN and ligature-induced periodontitis.

In sites with periodontal destruction, total numbers of inflammatory cells, plasma cells, and lymphocytes increased when compared with stable sites. When microbial dental plaque accumulates, monocytes/macrophages, lymphocytes, fibroblasts, and vascular endothelial cells express cytokines, which exhibit pro-inflammatory activities. Cytokines participate in the initiation and progression of chronic inflammatory diseases like periodontitis and arthritis. Interleukin-1 (IL-1) production is important in Gram negative infections like periodontitis. IL-1β, a pro-inflammatory cytokine, is produced by macrophages, fibroblasts, keratinocytes, and polymorphonuclear leukocytes. Increased IL-1β level of gingival tissue and gingival crevicular fluid (GCF) in periodontitis sites has been observed. No information is available on the role of this marker in the experimental lathyrism model.

Local and systemic determination of the role of experimental lathyrism and periodontitis may also be important in evaluating the local effects of lathyrogens on the periodontium and the systemic effects of the experimental periodontitis on bone metabolism.

The present study was undertaken to observe periodontal and systemic alterations in experimental lathyrism in rat and compare these changes to that seen in the ligature-induced periodontitis group.

**Material & Methods**

Male, adult Wistar rats (n=45) with an average weight of 150-250 g, were used in the study. They were housed separately in plastic cages and kept in an temperature-controlled room with a standard 12:12 h light-dark illumination cycle. In the lathyritic group (n=15, group 1) experimental lathyrism was induced by once daily subcutaneous administration of β-APN (Sigma-Aldrich Chemie., Taufkirchen, Germany), at a dose of 5 mg β-APN/0.4 ml distilled water per 100 g of body weight for 40 days. Ligature-induced periodontitis was created in another group of 15 rats by tying sterile silk ligatures (Dogsan, Istanbul, Turkey) on the necks of mandibular molars, under systemic anaesthesia obtained by the intraperitoneal administration of ketamin-HCl (Warner Lambert, Pfizer Inc. Istanbul, Turkey), at a dose of 60 mg/kg (group 2). The ligatures were kept in position to cause accumulation of microbial dental plaque during the experimental period (40 days). Ligature-induced periodontitis was created in another group of 15 rats by tying sterile silk ligatures (Dogsan, Istanbul, Turkey) on the necks of mandibular molars, under systemic anaesthesia obtained by the intraperitoneal administration of ketamin-HCl (Warner Lambert, Pfizer Inc. Istanbul, Turkey), at a dose of 60 mg/kg (group 2). The ligatures were kept in position to cause accumulation of microbial dental plaque during the experimental period (40 days). Control rats (n=15, group 3) received daily subcutaneous injections of 0.4 ml/100 g body wt of saline. All rats were fed a powdered diet and water *ad libitum*.

Blood was drawn by cardiac puncture at the end of 40 days. All animals were weighed and then decapitated. Mandibles were removed and gingival tissue samples were extracted from the buccal region of the mandibular right first molars (3 mm x 3 mm). Radiographs were obtained by long cone technique at 70KvP, 8mA (Trophy Dental Radiography, Istanbul, Turkey). Radiographic observations were performed on the molars.

After taking the radiographs, extraction tests were performed on the right first molars of all rats.
The jaws were kept in saline solution at 4°C and the right first molars were extracted for 2 h, using a computer-assisted tensile strength testing machine (Universal Instron Lloyd LRX, Lloyd Instruments TIC, UK). First molars were extracted at a rate of 5 mm/min.

Histopathological evaluations were performed on the left mandibular molars. The left mandibles were fixed with 10 per cent buffered formalin. The samples were decalcified by 10 per cent ethylenediaminetetraacetic acid (EDTA) for 4 wk and washed with water for 24 h and were embedded in parafin and blocked in the mesio-distal direction. Thin sections (5 µm), were cut and stained by hematoxylin and eosin (H&E) and analyzed by light microscopy. The level of the alveolar bone was determined by histometric measuring the distance from the cementoenamel junction to the alveolar bone crest14,15. These measurements were performed under a microscope fitted with a micrometer eyepiece (each unit = 4 µm) at 25 (objective magnification) X 0.8 (tube magnification) X 10 (eyepiece magnification). Inflammatory cells were counted in a 36 µm² area separately, including connective tissue and epithelium at the dentogingival junction, by a light microscope transferred to a monitor with a camera apparatus (Panasonic F10 CCD Camera, Objective X 3.3) at 4165 X magnification.

Blood samples were centrifuged at 500 X g for 10 min to obtain serum. Serum ALP activity was assessed by the optimized standard method16 using the Roche-Hitachi MODULAR system. Diethanolamine was used as a buffer solution. In the presence of magnesium and zinc ions, p-nitrophenyl phosphate was hydrolyzed by phosphatases to form phosphate and p-nitrophenol. The p-nitrophenol released is proportional to the ALP activity and can be measured photometrically. Two point calibrations were performed.

The gingival tissues obtained were immediately weighed, placed in a sterile saline solution and stored in liquid nitrogen. Gingival biopsies were transferred in a phosphate buffer solution (4°C, pH 7.0) for homogenization. The samples were homogenized at maximum rotational speed for 30 seconds by homogenisator (Ultra Turrax T25, IKA LABORTECHNIK, Staufen, Germany). The homogenate was sonicated three times by ultrasonicator (MSE Soniprep 150, Sanyo Gallenkamp PLC, UK) at 15-20 µ for 30 seconds at 20 seconds intervals. All these processes were performed at 0-4°C. Gingival tissue IL-1β levels were measured by standard ELISA apparatus at 450-550 nm using Endogen ELISA Kits (Endogen Rat Interleukin-1beta ELISA, Endogen Inc. Woburn, MA, USA).

### Table I. Body weights of rats in the three groups before and after the experimental period

<table>
<thead>
<tr>
<th>Body weight (g)</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before experimental period</td>
<td>193.20±14.06</td>
<td>192.13±17.42</td>
<td>190.53±9.75</td>
</tr>
<tr>
<td>After experimental period</td>
<td>187.53±16.40*</td>
<td>180.67±18.20**</td>
<td>211.73±12.63**</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=15 in each group)

\( *P<0.05 \), \( **P<0.001 \) compared to respective group before experimental period (Paired T-test)

### Table II. Numbers of inflammatory cells counted in the connective tissue and epithelium

<table>
<thead>
<tr>
<th>Connective tissue</th>
<th>Lymphocytes</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>2.60±0.51*</td>
<td>1.07±0.88*</td>
</tr>
<tr>
<td>Group 2</td>
<td>2.47±0.52*</td>
<td>1.27±1.03*</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.40±0.51</td>
<td>0.13±0.35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Epithelium</th>
<th>Lymphocytes</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>2.00±0.76*</td>
<td>1.00±0.76*</td>
</tr>
<tr>
<td>Group 2</td>
<td>2.20±0.68*</td>
<td>0.93±0.80*</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.13±0.35</td>
<td>0.13±0.35</td>
</tr>
</tbody>
</table>

Values are mean±SD (n=15 in each group)

\( *P<0.01 \) compared to group 3 (Kruskal Wallis test and Mann-Whitney U test)
**Fig. 1** (a) The distance from cementoenamel junction to the alveolar bone crest in the periodontium of healthy rats in control group (Magnification 12.5 x 0.8 x 10). (CEJ, cementoenamel junction; AC, alveolar crest). (b) Lymphocytes and macrophages both in connective tissue and epithelium at the dentogingival junction of the control group (Magnification 100 x 0.8 x 10) (DGJ, dentogingival junction; E, epithelium; CT, connective tissue; CEJ, cementoenamel junction).
Fig. 2 (a) Alveolar bone loss in the lathyritic group (Magnification 12.5 x 0.8 x 10). (CEJ, cementoenamel junction; AC, alveolar crest). (b) Inflammatory cells both in connective tissue and epithelium at the dentogingival junction of the lathyritic group (Magnification 100 x 0.8 x 10) (DGJ, dentogingival junction; E, epithelium; CT, connective tissue).
**Fig. 3** (a) Alveolar bone loss in the ligature-induced periodontitis group (Magnification 12.5 x 0.8 x 10). (CEJ, cementoenamel junction; AC, alveolar crest). (b) Inflammatory cells both in connective tissue and epithelium at the dentogingival junction of the ligature-induced periodontitis group (Magnification 100 x 0.8 x 10) (DGJ, dentogingival junction; E., epithelium; CT, connective tissue).
The study protocols were approved by the Animal Experiment Ethics Committee of Ondokuzmayis University, Turkey.

Data were analyzed using paired T-test, one-way analysis of variance (one way ANOVA), Post Hoc Tukey test, Kruskal Wallis test, and Mann-Whitney U test. For the statistical comparison of the groups one way ANOVA and Post Hoc Tukey parametric tests were used. Comparisons between groups regarding the numbers of inflammatory cells were made using Kruskal Wallis and Mann-Whitney U non-parametric tests. $P<0.05$ was considered as significant.

**Results**

Rats in lathyritic and ligature-induced periodontitis groups lost body weight contrary to the increase in the control group (Table I).

In groups 1 and 2, alveolar bone resorption was radiographically observed around the mandibular molar teeth. There was no resorption in the control group.

The extraction force required for group 1 rats was 17.09±3.83N, 16.95±5.66N for group 2, and 39.56±5.93N for group 3. Teeth of groups 1 and 2 rats were extracted by a lower force compared to those of the controls ($P<0.001$). There was no significant difference between the groups 1 and 2.

The histometric alveolar bone level measurements were 1091.2±182.97, 1308.8±141.98 and 391.2±81.28 µm in groups 1, 2 and 3 respectively. The distance from the cementoenamel junction to the alveolar bone crest both in groups 1 and 2 was significantly higher than the control group ($P<0.001$). There was also a significant difference between the groups 1 and 2 ($P<0.001$). Significant alveolar bone loss was detected in both lathyritic and ligature-induced periodontitis groups compared to the control group ($P<0.001$).

The number of inflammatory cells (lymphocytes and macrophages) both in the connective tissue and epithelium, was significantly higher in groups 1 and 2 compared to the control group ($P<0.01$). There was no significant difference between the groups 1 and 2 (Table II). The levels of alveolar bone and inflammatory cells in connective tissue and epithelium at the dentogingival junction in the three groups are shown in Figs 1a, 1b; 2a, 2b; and 3a, 3b.

The serum ALP activity levels were 572.40±65.21 U/l in group 1, 497.80±84.88 U/l in group 2, and 934.27±81.59 U/l in group 3 rats. In both groups 1 and 2, there was a significant decrease in serum ALP activity ($P<0.001$) compared to the control group. The ALP activity was significantly higher in the group 1 compared to group 2 ($P<0.05$).

Gingival tissue IL-1 beta levels were 48.27±10.18 pg/ml in group 1, 44.45±10.93 pg/ml in group 2, and 22.99±6.25 pg/ml in group 3 rats. Levels of IL-1 beta in groups 1 and 2 were significantly ($P<0.001$) higher than those in the control group.

**Discussion**

Animal models have been used to investigate the pathologic processes of infectious diseases such as periodontitis. Ligature-induced periodontitis has been used in primates, dogs, and rats to study factors affecting the severity of periodontitis.

A high dose of the lathyrism agent, b-APN, leads to acute intoxication. Acute lathyrism is not an appropriate model for periodontitis which has a chronic origin. In the present study, a low dose of b-APN was administrated for 40 days to achieve a chronic lathyrism condition in the rats. Animals in both the lathyrism and ligature-induced periodontitis groups lost body weight which might have caused by insufficient chewing capacity resulting from periodontal destruction.

Alveolar bone resorption in both groups 1 and 2 was observed, radiographically and histopathologically. In addition, the teeth from lathyrism animals were extracted with forces similar to the ligature-induced periodontitis teeth in non-lathyrism animals. b-APN is reported to produce lathyrism changes on periodontal ligament and alveolar bone in an average period of 3 wk at any dosage. Significant resorption of the alveolar crest is observed histopathologically at 6 wk. Had the study period been longer, animals in both groups 1 and 2 might have
spontaneously lost their teeth due to either lathyrogen-induced or ligature-induced alveolar bone resorption.

In the gingival tissues of individuals with periodontitis, inflammatory infiltrate develops and is primarily composed of lymphocytes and macrophages. Mononuclear cell infiltration and trans-migration of mononuclear phagocytes and lymphocytes are important characteristics of chronic gingival inflammation. In our study, the number of lymphocytes and macrophages significantly increased in both groups 1 and 2. Therefore, it may be concluded that chronic inflammation of periodontium was present in both the groups. During the progression of periodontal diseases spaces within the tissues are created due to destruction of collagen fibers, these spaces are then quickly filled by inflammatory cells and loose periodontal connective tissue. We may speculate that in our study, following the destruction of collagen fibers, inflammatory infiltrate developed and connective tissue was lost in the lathyritic rats which might have caused the formation of retention areas for microbial dental plaque accumulation in the periodontium. This phenomenon could have increased the severity of periodontal inflammation.

Radiographically, in groups 1 and 2, alveolar bone resorption was observed in the molar region. The lathyritic group may also have been involved in systemic bone resorption. Serum ALP activity in the ligature-induced periodontitis group was found to be lower than the lathyritic group. Higher serum ALP levels are often associated with increased bone formation. We may conclude that during the 40 days experimental period, lathyrogens did not damage other bones in the body due to the higher serum ALP activity. In ligature-induced periodontitis group serum ALP activity was lower than that in lathyritic group, and histopathological results showed higher alveolar bone loss in this group.

It has been reported that bone loss affects the jaw bones (particularly the alveolar bone), cranial bones, ribs, vertebrae, and long bones in descending order. The alveolar bone is affected first due to the highest rate of remodelling. It appears from the present findings that the lathyritic agent injected in a low dose for a short duration presumably did not cause systemic bone resorption in rats.

Increase in gingival tissue IL-1b levels is a sensitive and reliable marker of chronic inflammatory disease activity. In the present study, gingival tissue IL-1b elevation may demonstrate tissue destruction in both lathyritic and ligature-induced periodontitis groups. Significant increase in IL-1b levels in active periodontitis sites compared to inactive periodontitis and healthy sites has been reported. Both IL-1b and IL-6 levels are shown to be increased in diseased tissues of periodontitis patients.

To conclude, similar radiographical and histopathological findings and comparable increases in gingival tissue IL-1b levels both in groups 1 and 2 showed that in addition to resorption of alveolar bone, chronic inflammation of periodontium also occurred in the rats of both lathyritic and ligature-induced periodontitis groups. This model may be used for studying the effects of different agents on periodontal defects.

References


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