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Expression of receptor activator of nuclear factor κB ligand in ligature-induced periodontitis in osteoporotic and non-osteoporotic rats

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Background and Objective: This study investigated the expression of a key mediator that regulates differentiation of osteoclasts, receptor activator of nuclear factor κB ligand (RANKL), in rats with or without osteoporosis and periodontitis, to provide a better understanding of the association between these two diseases.

Material and Methods: Forty adult Albino rats were divided into four groups: (1) control group; (2) experimentally induced periodontitis group; (3) experimentally induced osteoporosis group; and (4) experimentally induced osteoporosis and periodontitis group. At the end of the experimental period, blood samples were obtained and animals were sacrificed. Serum alkaline phosphatase (ALP) activity levels were measured. Histological evaluation and immunohistochemical detection of RANKL in the periodontal ligament and bone tissues were performed.

Results: There were significantly higher ALP levels in all of the experimental groups than in the control group. The pathology observed in the histological sections from group 4 was more severe than in either group 2 or group 3. The percentage of RANKL-immunoreactive cells in both the periodontal ligament and bone tissues in group 4 (16.8 ± 5.1 and 11.2 ± 5.2%, respectively) was significantly higher ($p < 0.001$) than in the other groups. In the periodontal ligament, the percentage of RANKL-immunoreactive cells in group 2 (10.1 ± 1.9%) was significantly higher ($p < 0.001$) than in group 3 (5.3 ± 2.7%) and the control group (4.12 ± 1.5%).

Conclusion: The increased bone loss observed in group 4 compared with either group 2 or group 3 supports the existence of an additive pathological effect of the two disease conditions. This is consistent with the increased RANKL expression observed in group 4.

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Bone is a specialized type of connective tissue that is continually being remodeled. This remodeling results from the activities of many cell lineages, primarily osteoblasts, osteocytes and osteoclasts (1). Secondary cell types, such as monocytes/macrophages and endothelial cells, also contribute to bone remodeling either by direct contact with osteogenic cells or by the release of soluble factors, such as cytokines and growth factors (2).

The deciphering of the osteoprotegerin–receptor activator of nuclear factor κB ligand–receptor activator of nuclear factor κB (OPG–RANKL–RANK) system and its role in osteoclastogenesis represents a major advance in bone biology by identifying the specific factors produced by preosteoblastic/stromal cells that are both necessary and sufficient for osteoclast development (3). The decisive role played by these factors in regulating bone metabolism has been demonstrated by the findings of extremes of skeletal phenotypes (osteoporosis vs. osteopetrosis) in mice with altered expression of these molecules (4–7).

There is increasing evidence that osteoporosis and the underlying loss of bone mass characteristic of this disease may increase the onset and progression of periodontal disease and tooth loss (8,9). Understanding the association between these two prevalent diseases and the mechanisms underlying bone loss may lead to improved strategies for prevention, diagnosis and treatment.

Unbalanced bone remodeling, leading to the loss of bone tissue observed in osteoporosis and periodontitis, usually results from altered expression of molecules that regulate osteoclast and osteoblast differentiation and function. Alkaline phosphatase enzyme level is the most common serum biochemical marker used for non-invasive assessment of bone disorders. Therefore, this study investigated the expression of RANKL, the level of alkaline phosphatase and resulting histological changes in rat models of osteoporosis and/or periodontitis.

Material and methods

Animals

Forty adult female albino rats, with an average age of 4–6 months and an average weight of 230–250 g, were used in this study. They were housed in specially designed wire cages and maintained on a 12 h–12 h light–dark cycle with a constant room temperature of 23°C. The animals were cared for according to the policies and principles established by the Animal Welfare Act and the US National Institutes of Health *Guide for Care and Use of Laboratory Animals* (http://oacu.od.nih.gov/ac_cbt/guide3.htm). The study was approved by the Ethics Committee, National Research Centre, Cairo, Egypt. The rats were grouped (10 per group) into four groups. Group 1 was the control. Group 2 was the periodontitis group, in which periodontitis was induced by ligature placement under general anesthesia obtained by the intramuscular administration of ketamine (50 mg/kg) and zylazine (10 mg/kg; Phoenix pharmaceuticals, St Joseph, MO, USA). The ligatures were kept in subgingival positions for 4 weeks to cause accumulation of microbial dental plaque during the experimental period (10). Signs of gingival inflammation (e.g. swelling and erythema) were detected in the gingival tissues surrounding the ligatures. Rats in groups 1 and 2 were fed standard diet that had all the nutrient requirements for rodents (11). Induction of osteoporosis was achieved in group 3 by changing the rats' normal diet to bran bread for 45 days (12). This diet lacks the major constituents of a basic diet, thus resulting in an elevated rate of bone resorption and osteoporotic changes as a result of the dietary restriction and calcium and magnesium deficiency. Group 4 consisted of 10 rats in which induction of periodontitis was achieved after the induction of osteoporosis. Bran diet was continued during the 4 weeks following ligament placement in group 4 rats.

Rats from all the groups were weighed before and after the experiments using an XT top-loading balance scale (Fisher Scientific, Pittsburgh, PA, USA). Before sacrificing the animals, blood samples were collected by cardiac puncture under general anesthesia in order to obtain the plasma concentration of alkaline phosphatase (ALP) as a biochemical marker for the turnover rate of bone. The rats were sacrificed by decapitation after 0 days for group 1, 4 weeks for group 2, 6 weeks for group 3 and 10 weeks for group 4, in which osteoporosis and periodontitis were achieved in succession. The lower jaws were dissected, defleshed and prepared for examination. The specimens were fixed in 10% neutral buffered formalin. The tissues were then decalcified in 20% ethylenediamine-tetra-acetic acid (EDTA), pH 7, with a change once a week for 10 weeks until decalcification was completed. After decalcification, the specimens were dehydrated in ascending percentages of ethanol, infiltrated with xylene, embedded in paraffin, sectioned at a thickness of 4 μm and then stained with hematoxylin and eosin (H&E) to study the histopathological changes in the bone and the periodontal tissues.

Immunohistochemistry

From the tissues embedded in paraffin wax, 4 μm thick sections were obtained and collected on 3-aminopropyltriethoxy-silane-coated slides (Sigma Chemical Co., St Louis, MO, USA). The immunohistochemical characterization of the cells was performed using the standard avidin–biotin–peroxidase complex method. The samples were deparaffinized by immersion in xylene and rehydrated through sequential diluted alcohol concentrations followed by incubation with 3% H_2O_2 blocking reagent for 5 min to block endogenous peroxidase. Samples were then immersed in citrate buffer (pH 6.0; Sigma Chemical Co.) for 15 min at 95°C to retrieve the antigen. Soon afterwards, the sections were incubated in 3% normal mouse serum diluted in distilled water at room temperature for 15 min to block non-specific reactivity. Sections were then incubated overnight in a humid chamber with the primary antibody (mouse antihuman RANKL

monoclonal antibody SC-MAB626; R&D Systems, Minneapolis, MN, USA), diluted in antibody diluent provided by the manufacturer at 1:100. Following the incubation period, the sections were washed with phosphate-buffered saline (PBS) and incubated with biotinylated antimouse Immunoglobulin G (CTS003; R&D Systems) for 30 min at room temperature. The sections were then incubated with avidin–biotin-peroxidase complex for 30 min followed by incubation with diaminobenzidine tetraoxide solution before being counterstained with hematoxylin. As negative controls, PBS was substituted for the primary antibody.

Computer-assisted image analyses

The immunostaining was quantitatively analyzed by using a computer-assisted image analysis system. The data from the periodontal ligament and bone tissues were obtained using Leica QWin 500 image analyzer system (Cambridge, UK). The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into units of micrometres. Using the measuring field menu, the percentage area of RANKL immunoreactivity in a standard measuring frame of a standard area equal to 1148.59 μm^2 was chosen from the parameters. In each chosen field, the tissue measured was enclosed inside the standard measuring frame and then the positive cells were masked by a blue binary color to be measured. These measurements were done using an objective lens of magnification 10 (total magnification ×100). Readings from 10 fields were obtained for each specimen and their mean values were calculated.

Statistical analyses

The data obtained were subjected to statistical analyses using analysis of variance (ANOVA). The mean values of the data and the standard deviation were determined, and the p value was calculated to indicate significance ($p < 0.05$). This was done using SPSS computer system version 9 (SPSS Inc., Chicago, IL, USA).

Results

Differences in body weight

Rats in the experimental groups 3 and 4 lost body weight (Table 1). The reduction in the weight of the rats was significant in groups 3 ($p < 0.01$) and 4 ($p < 0.05$).

Serum alkaline phosphatase level

Serum ALP activity levels are shown in Table 2. There were significantly higher serum ALP levels in groups 2–4 in comparison with the control group ($p < 0.001$). The ALP level was significantly higher in group 3 compared with group 2 ($p < 0.01$).

Histology

Histological sections from the control group showed normal architecture in both the periodontal ligament and the alveolar bone tissues. In the periodontitis group (group 2), there was a disruption of the periodontal ligament fibers accompanied by pronounced vasodilatation and inflammatory cell infiltrate. Alveolar bone resorption was evident, with an increased number of osteoclasts in their Howship's lacunae. Multiple resorption foci were observed along the bone surface, producing a scalloped outline (Fig. 1A,B). Sections from the osteoporosis group (group 3) revealed thinning of the bony trabeculae, with obvious enlargement of the marrow cavities and architectural alterations in terms of the loss of trabecular connectivity. Few osteoclasts in their Howship's lacunae were observed (Fig. 2A,B). In the osteoporosis and periodontitis group (group 4), inflammatory cell infiltrate and increased vasodilatation were more pronounced, with more bone loss than in the other groups. A large number of osteoclasts in their Howship's lacunae were observed along the bone surface. Within the bone, there were marked thinning of bony trabeculae and prominent enlargement in the marrow cavities to the extent that in some areas the trabeculae became fragmented and appeared to form isolated islands embedded in the large bone marrow cavities (Fig. 3A,B).

Immunohistochemistry

Positive RANKL immunoreactivity was detected in the mononuclear spindle-shaped periodontal ligament cells, mononuclear inflammatory cell infiltrate, bone-lining cells and osteoclasts. There were obviously more RANKL-positive cells in all of the experimental groups than in the control group. An intense reaction in inflammatory cells and osteoclasts on the bone surface was detected in sections from group 2 (Fig. 1C,D). There were fewer positively stained osteoclasts in group 3 than in groups 2 and 4 (Fig. 2C). In comparison to the other groups, sections of group 4 revealed the most

Table 1. Body weight of rats before and after the experimental period

Body weight (g)	Group 2	Group 3	Group 4
Before	193 ± 31.04	212 ± 15.32	210.4 ± 20.36
After	192.6 ± 22.74	173.8 ± 30.29**	179.2 ± 22.88*

Values are means ± SD ($n = 10$ per group); *$p < 0.05$ and **$p < 0.01$ vs. before the experimental period.

Table 2. Serum ALP levels (in U/L) in all the groups

Group	Mean ± S.D	Range
Group 1	247.6 ± 54.15	208.8–286.3 U/L
Group 2	459.2 ± 118.84*	374.2–594.2 U/L
Group 3	624.2 ± 209.67*†	474.2–774.2 U/L
Group 4	554 ± 71.82*	502.6–605.4 U/L

$n = 10$ per group; *$p < 0.001$ compared with group 1 and †$p < 0.01$ compared with group 2.

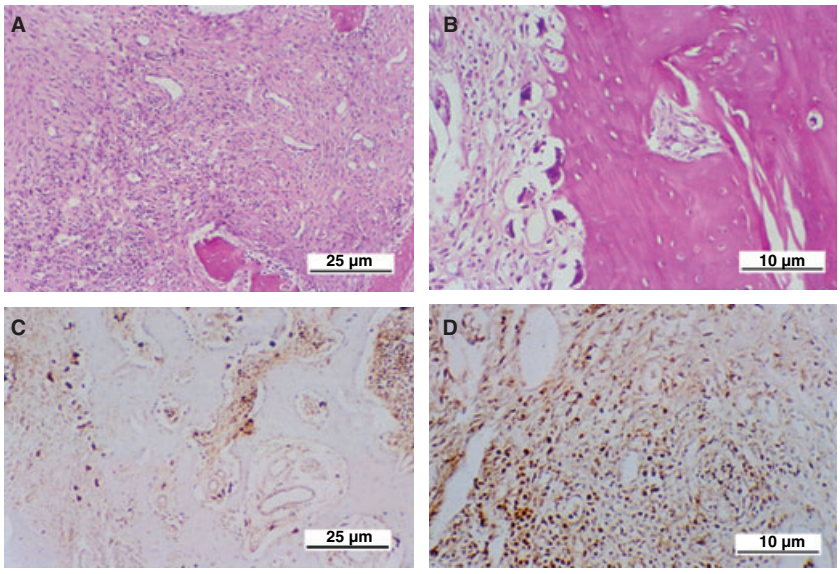

Fig. 1. Photomicrographs of sections from group 2 showing intense inflammatory cell infiltrate and dilated blood vessels (A; H&E ×40), osteoclasts in their Howship's lacunae with multiple resorption foci (B; H&E ×100), positive reaction in osteoclasts along the bone surface (C; anti-RANKL antibody ×40) and positive inflammatory cell infiltrate (D; anti-RANKL antibody ×100).

intense RANKL immunoreactivity (Fig. 3C–F). The results were quantified using the image analyzer Leica QWin 500 software and are presented in Figs 4 and 5. The percentage area of RANKL immunoreactivity in both the periodontal ligament and the bone tissues in group 4 (16.8 ± 5.1 and 11.2 ± 5.2%, respectively) was significantly higher ($p < 0.001$) than in the other groups. In the periodontal ligament, the percentage area of RANKL immunoreactivity in group 2 (10.1 ± 1.9%) was significantly higher ($p < 0.001$) than that in group 3 (5.3 ± 2.7%) and the control group (4.12 ± 1.5%).

Discussion

The present study examined RANKL expression in experimentally induced periodontitis and osteoporosis in an attempt to understand the relationship between these two common osteolytic diseases. The body weight of rats before and after the experimental period and the serum alkaline phosphatase levels were recorded. Histological examination and immunohistochemical detection of RANKL were performed on tissue sections.

Analyses of the data concerning body weight of the animals of groups 2, 3 and 4 before and after the experimental period showed that loss of weight in groups 3 and 4 was statistically significant. This might have been caused by nutritional insufficiency in groups 3 and 4, in addition to the periodontal tissue destruction in group 4.

Alkaline phosphatase is the most common serum biochemical marker used for non-invasive assessment of bone disorders. It plays an important role in the regulation of bone formation and turnover rate (13). Evaluation of the relationship between locally induced periodontitis, systemically induced osteoporosis and serum ALP levels may be important to detect the systemic roles of both diseases. In the present study, it was demonstrated that there were significantly higher serum ALP levels in groups 2–4 in comparison with the control group. Group 3 showed the highest increase in the ALP level. Higher serum ALP levels are often associated with systemic metabolic or hormonal diseases affecting the skeleton. It might be that levels of ALP were lower in group 2 because ligature-induced periodontitis did not damage any other bones in the body, since its effects were localized to the area of the ligatures.

In the present study, immunohistochemical sections were examined using the image analyzer. The image analyses have several advantages over standard counting. First, the threshold for positivity was held constant by the computerized system, unlike the human observer, who has a potential for variance from field to field. Second, a larger area can be analyzed than by manual methods. Third, the operator could focus on any area and selectively eliminate other areas from evaluation. The image analyzer provides a higher degree of specifity in tissue evaluation (14).

The control group sections revealed weak RANKL immunoreactivity, which was detected in the spindle-shaped mesenchymal periodontal ligament cells localized mostly at a perivascular location near the alveolar bone surface. RANKL was also detected in osteoblasts, odontoblasts and some osteocytes. The finding that RANKL-positive stromal cells/osteoblasts were detected in sections from the control group with an absence of positive osteoclasts demonstrates the crucial role of stromal cells/osteoblasts in osteoclast development. Liu *et al.* (15) demonstrated that receptors for the bone-resorbing hormones (parathyroid hormone and vitamin D_3) were present not in osteoclasts or in their precursor cells but in osteoblasts. This supports the fact that osteoblasts are responsible for the process of osteoclastogenesis by sending a second signal to the osteoclast precursors in response to the primary osteolytic signals that stimulates the fusion of osteoclast precursors into multinucleated cells capable of resorbing bone through RANKL expression.

An increase in the number of osteoclasts and in the overall RANKL immunoreactivity was obvious in sections of group 2 compared with groups 1 and 3. The dense infiltrates of inflammatory cells seen in these sections might cause such a significant increase. It was demonstrated that antigen activation of $CD8^+$ and $CD4^+$ T cells resulted in expression of

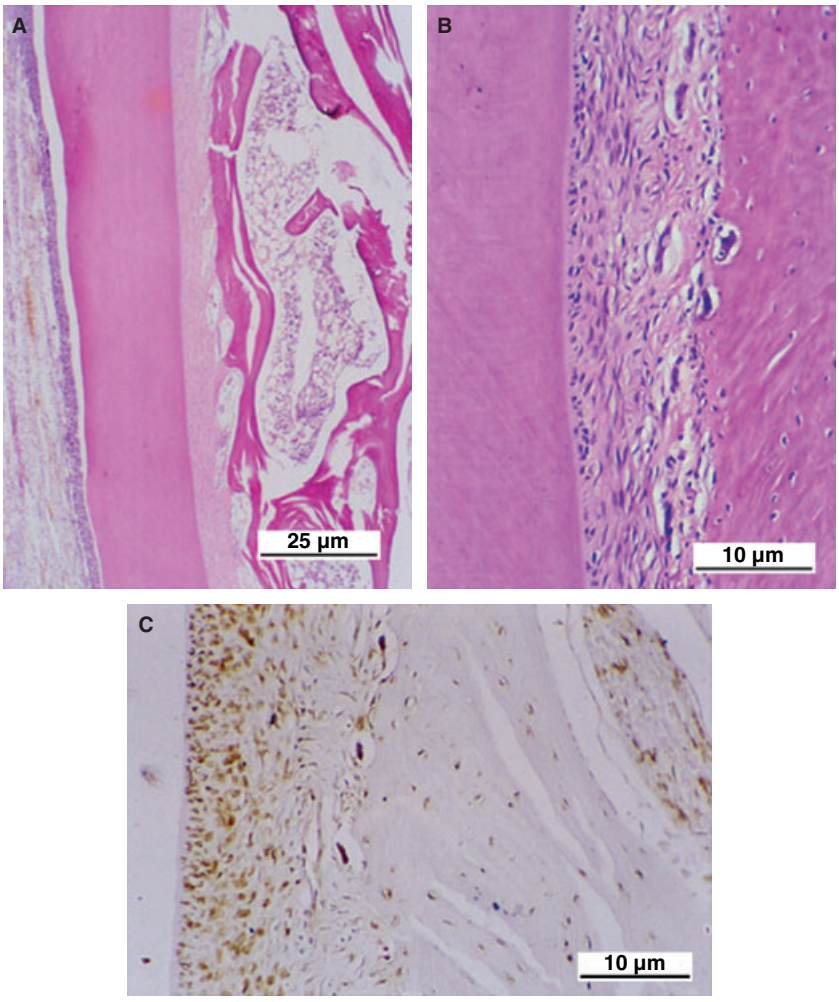

Fig. 2. Photomicrographs of sections from group 3 showing the characterstic features of osteoporotic bone with thin bony trabeculae and excessive widening of marrow cavities (A; H&E ×40), a few osteoclasts in their Howship's lacunae (B; H&E ×100) and positive reaction in periodontal ligament cells and osteoclasts along the bone surface (C; anti-RANKL antibody ×100).

RANKL and that co-culture of such cells with osteoclast progenitor cells gave rise to formation of mature osteoclasts by a mechanism sensitive to inhibition by OPG (16). B-lymphocytes may also participate in osteoclast formation, either by expressing RANKL or by serving as osteoclast progenitor cells (17).

Osteoclast number and the percentage area of RANKL immunoexpression in both the periodontal ligament and bone were less obvious in sections from group 3 than in those from groups 2 and 4. This suggests that the bone loss associated with osteoporosis is mainly due to increased osteoclast resorptive activity rather than the slight increase in osteoclast number. Jevon *et al.* (18) demonstrated that the proportion of circulating osteoclast precursors in the peripheral blood of primary osteoporosis patients is not increased relative to normal control subjects. The study suggested that the osteoclasts produced in osteoporosis are somehow more active and possess a functional advantage over normal osteoclasts in terms of resorptive activity. The study stated that it appears to be possible that the increased resorptive activity of these cells could be due to the action of one or more of the cellular/humoral factors, which are required for their formation.

Sections from group 4 showed the most significant ($p < 0.001$) increase in RANKL immunoexpression. This demonstrates that, in rats, the additive bone destructive effect between osteoporosis and periodontitis correlates with an increased level of RANKL expression and with the increase in both osteoclast number and bone resorptive activity. These results also provide evidence that osteoporosis alone cannot induce significant changes in the number of osteoclasts.

A link between systemic osteoporosis and periodontal bone loss has been suggested by the fact that osteoporosis results in a significant up-regulation of bone-resorptive cytokines in bone and bone marrow cells. Increases in the circulating levels of interleukin-1, interleukin-6 and tumor necrosis factor-α ensue. Even small elevations in the levels of these cytokines initiate the cytokine–prostaglandin (prostaglandin–matrix metalloprotienase–RANKL) cascade locally in the periodontium, especially when co-induced by bacterial factors such as lipopolysaccaride (19). In addition, the evidence for an interaction between local inflammatory disease, such as periodontitis, and systemic osteoporosis has been strengthened by several human clinical studies demonstrating a relationship among osteoporosis, tooth loss and alveolar bone loss (20–27). In support of this, Golub *et al.* (28) found that treating osteoporotic rats with CMT8 (a chemically modified non-microbial analogue of doxycycline) reduced, in a dose-dependent fashion, the severity of systemic osteoporosis and reduced local periodontal destruction, including a normalization of both pathologically excessive host collagenase activity in the gingiva and alveolar bone loss.

In summary, the results of this study show that osteoporosis alone did not result in a significant increase in osteoclast number, while ligature-induced periodontitis resulted in marked up-regulation of osteoclast formation. In addition, ligature-induced periodontitis increased alveolar bone loss resulting from the osteoporotic state. Thus, an additive effect was observed. This was indicated by the increased

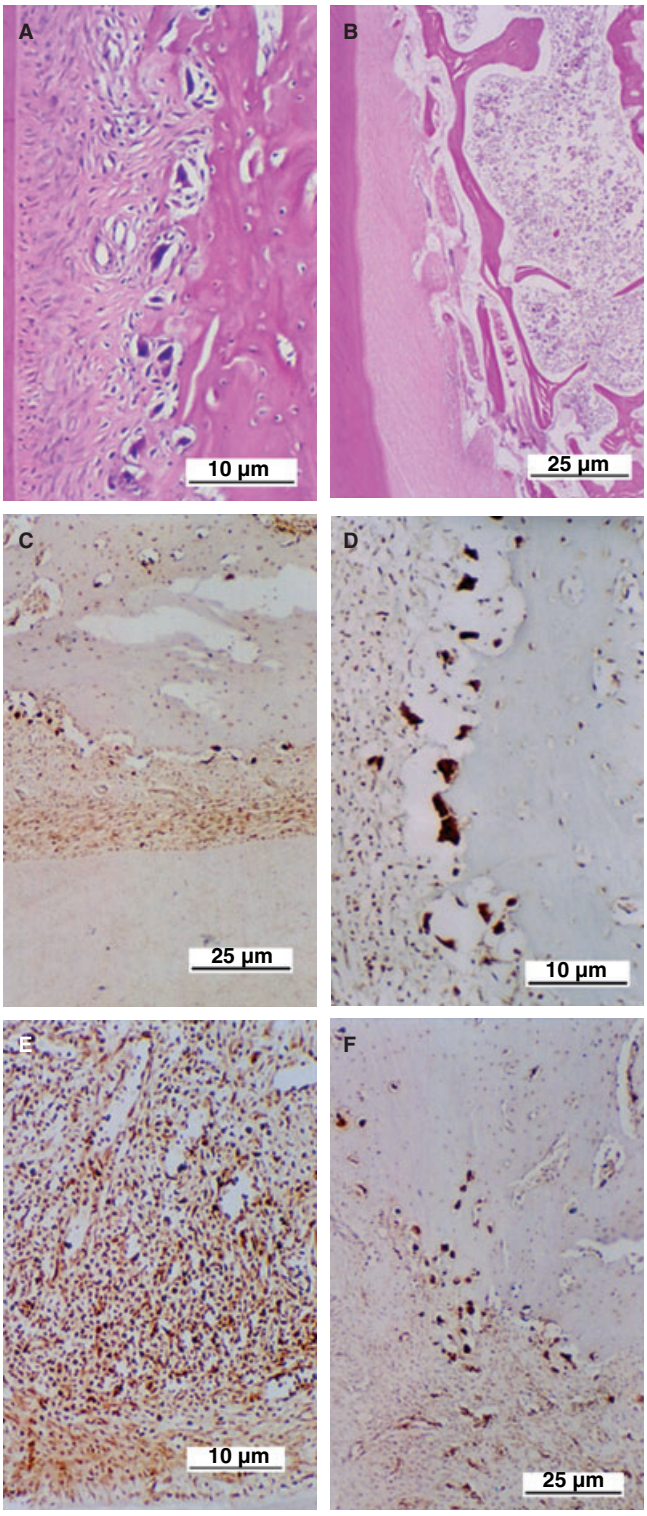

Fig. 3. Photomicrographs of sections from group 4 showing prominent increase in the number of osteoclasts along the whole length of bone surface (A; H&E ×100), excessive thinning of the bony trabeculae and widening of marrow cavities (B; H&E ×40), positive periodontal ligament cells and osteoclasts along the bone surface (C; anti-RANKL antibody ×40), a higher-power views of C (D; anti-RANKL antibody ×100), positive inflammatory cell infiltrate within the periodontal ligament (E; anti-RANKL antibody ×100) and positive reaction in an area of osteoclastic aggregation on the bone surface (F; anti-RANKL antibody ×40).

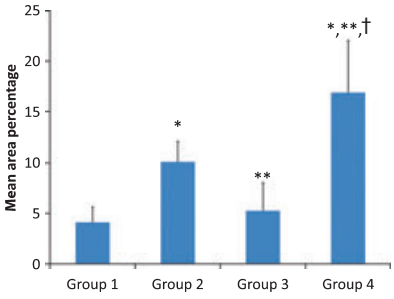

Fig. 4. Mean percentage area of RANKL immunoreactivity in the periodontal ligament. *Significant compared to group 1; **Significant compared to group 2; †Significant compared to group 3.

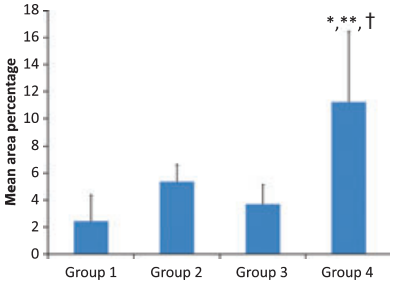

Fig. 5. Mean percentage area of RANKL immunoreactivity in the bone tissue. *Significant compared to group 1; **Significant compared to group 2; †Significant compared to group 3.

number of osteoclasts and RANKL immunoreactivity. It can be concluded that RANKL expression levels correlate significantly with disease progression and could thus serve as a diagnostic marker, a predictive index for bone disease progression and a possible therapeutic target for local and systemic osteolytic diseases.

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