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The influence of thyroid hormones on periodontitis-related bone loss and tooth-supporting alveolar bone: a histological study in rats

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Background and Objective: Recent studies have pointed to potentially periodontal risk indicators, however no information is available on the impact of changes in thyroid hormone levels on the progression of periodontitis and on the quality of alveolar bone. Thus, the aim of the present study was to evaluate histologically, in rats, the influence of thyroid hormones on the rate of periodontal bone loss resulting from ligature placement and on the quality of tooth-supporting alveolar bone.

Material and Methods: Thirty-six male Wistar rats were randomly assigned to the following groups: healthy (control, $n = 12$), hypothyroidism ($n = 12$) and hyperthyroidism ($n = 12$). Once alterations were confirmed by total serum levels of triiodothyronine and thyroxine, ligatures were randomly placed around one of the first mandibular molars. Thirty days later, the animals were killed and specimens routinely processed for serial decalcified sections. The parameters assessed were periodontitis-related bone loss, quality of tooth-supporting alveolar bone and the number of cells positive for tartrate-resistant acid phosphatase (TRAP), a marker of bone resorption.

Results: At the ligated sites, intergroup analysis revealed that hypothyroidism significantly increased the bone loss resulting from ligature-induced periodontitis ($p = 0.02$) and the number of TRAP-positive cells on the linear surface of bone crest ($p = 0.01$). In addition, no significant differences were detected regarding the quality of the bone ($p = 0.24$) or the number of TRAP-positive cells in the area of the interradicular bone for ligated teeth among the groups ($p = 0.17$).

Conclusion: It may be concluded that decreased serum levels of thyroid hormones may enhance periodontitis-related bone loss, as a function of an increased number of resorbing cells, whereas the tooth-supporting alveolar bone seems to be less sensitive to alterations in hormone levels.

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Periodontal diseases are infections characterized by an imbalance between bacterial challenging and host response (1). The host reaction to microbial insults involves recruitment of inflammatory cells, production of prostaglandins and cytokines, elaboration of lytic enzymes and activation of osteoclasts, leading to alveolar bone resorption and attachment loss (2). Although the dental biofilm is the primary cause of periodontal disease, *in vivo* studies have demonstrated that systemic factors may play an important role in its initiation and progression (3,4).

Triiodothyronine (T_3) and thyroxine (T_4) are hormones secreted by the thyroid gland, and have been shown to be fundamental for normal bone turnover (5). Decreased or increased levels of these hormones may be pathologically secreted to the blood, characterizing the conditions known as hypothyroidism and hyperthyroidism, respectively (6). In hypothyroidism, for instance, bone turnover is slow, bone growth and maturation are retarded in childhood and adults tend to exhibit osteosclerosis, accompanied by increased fracture risk (7,8). In contrast, hyperthyroidism is associated with accelerated bone maturation, high bone turnover, low bone mass and an increased life-time risk for fractures (7–9). Furthermore, the presence of thyroid hormone receptors (TRs) has recently been reported in osteoblasts, suggesting a direct skeletal effect of these hormones (10). It has been proposed that thyroid hormones have an important role in controlling bone resorption through their action on the osteoprotegerin (OPG) and receptor activator of nuclear factor-κB ligand (RANKL) mechanism (11), and on bone regulating factors such as interleukin-6 (IL-6) and interleukin-8 (12,13). Since changes in bone are prominent features of periodontal disease, alterations of thyroid hormone levels may be suggested to be a modulating factor in periodontal disease, as are other systemic conditions, such as smoking and diabetes (14,15).

To date, no information is available on the impact of changes in T_3 and T_4 serum levels on the bone loss resulting from periodontitis and/or the quality of the alveolar bone. Thus, the present study was designed to evaluate the influence of thyroid hormones on the bone loss resulting from experimental periodontitis and on the quality of tooth-supporting alveolar bone around teeth by histometric analysis. Additionally, we aimed to assess the impact of these hormones on the number of cells positive for tartrate-resistant acid phophatase (TRAP), a phenotypic marker of bone resorption, in ligated and non-ligated sites.

Material and methods

Animals

The study included 36 male Wistar rats, aged 60 days and weighing an average of 194.44 ± 27.52 g at study onset. During the experiment, the animals were housed in groups of five in plastic cages. Food and water were given *ad libitum* to all animals. Prior to the experimental procedures, animals were allowed to acclimate to the laboratory environment for 5 days. This protocol was approved by University of Campinas Institutional Care and Use Committee.

Experimental design

The animals were randomly assigned to one of the following experimental groups: G1 (n = 12), control, healthy animals; G2 (n = 12), hypothyroidism, which was induced by the ingestion of a solution containing propylthiouracil (Propilracil®, 100 mg, Biolab Sanus Farmacêutica Ltda., Taboão da Serra, SP, Brazil; 1 g per litre of drinking water), an anti-thyroid drug which blocks thyroid hormone synthesis (16); and G3 (n = 12), hyperthyroidism, which was induced by the ingestion of sodium L-thyroxine (Puran® T_4, 200 μg, Sanofi-Synthelabo Ltda., Rio de Janeiro, RJ, Brazil) and sodium triiodothyronine (90 μg, Drogal Ltda., Piracicaba, SP, Brazil; 800 μg T_4 and 180 μg T_3 per litre of drinking water). The animals were continuously administered with the drugs for hormone changes throughout the experimental period, without interruption, as previously reported (17). Figure 1 schematically illustrates the experimental design adopted.

Biochemical serum analyses and ligature placement

Four months after the beginning of the study and, therefore, induction hormone changes, blood samples were collected for the assessment of total serum levels of T_3 and T_4 by radioimmunoassay (RIA; Active Triiodothyronine (T_3) RIA DSL-3100 and Active Thyroxine (T_4) RIA DSL-3200, Diagnostic System Laboratories Inc., Webster, TX, USA), according to the manufacturer's instructions. This protracted period of induction of dysfunctions was chosen to obtain chronic hormonal alterations. After the establishment of alterations in T_3 and T_4 serum levels, one of the mandibular first molars of each animal was randomly assigned to receive a cotton ligature in a submarginal position to

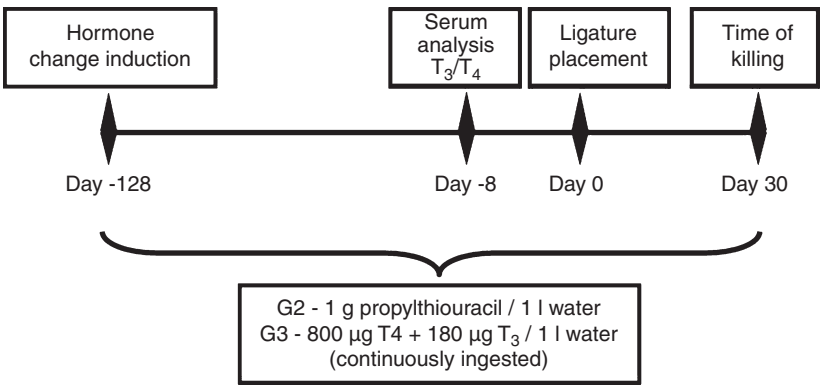

Fig. 1. Illustration of the experimental design.

induce experimental periodontitis, and the contralateral tooth was left unligated to serve as a control.

Histometric analysis

Thirty days after ligature placement, the animals were killed by perfusion under anaesthesia and the macroscopic characteristics of thyroid glands and periodontal tissues were examined. The specimens were then routinely processed, and decalcified serial sections (6 μm) were obtained in a mesio-distal direction and stained with hematoxylin and eosin. Using an image analysis system (Image-Pro®, Media Cybernetics, Silver Spring, MD, USA) and a blinded examiner, the area between the bone crest and furcation roof of ligated and non-ligated teeth was histometrically determined by the point counting technique in 10 sections per specimen (18), which were selected every 30 μm. Therefore, the assessed region represented the area of periodontal ligament and periodontal bone loss for non-ligated and ligated teeth, respectively. In addition, five equally distant sections were assessed regarding the bone quality (proportion of mineralized bone tissue in a 1.000 μm zone under the furcation) in the interradicular area of ligated and non-ligated teeth (19). The histometric parameters are schematically illustrated in Fig. 2.

Staining for TRAP (enzymohistochemistry)

Deparaffinized mesio-distal 6-μm-thick sections were incubated at 37°C for 15 min in a solution prepared by dissolving 4 mg of naphthol AS-BI (Sigma Chemical Co., St Louis, MO, USA) and 24 mg of red violet salt (Sigma Chemical Co.) in 30 mL of acetate buffer (pH 5.2) containing 0.3 mmol/L of tartrate (pH 5.0; Sigma Chemical Co.). Subsequently, sections were washed in distilled water and counterstained with hematoxylin. As a negative control for the TRAP activity, consecutive sections were incubated in substrate-free medium (20). Quantitative analysis of the number of TRAP-positive cells was performed in two regions illustrated in Fig. 2. Firstly, the number of TRAP-positive cells was counted on the linear surface of the bone crest, immediately below the furcation roof in ligated and non-ligated teeth. Secondly, the number of TRAP-positive cells was obtained in the 1000 μm zone of the interradicular bone under the furcation of the first mandibular molars. The TRAP-positive cells were counted in two different sections representative of the mid-portion of the tooth. Total area of the interradicular bone and the extension of the alveolar bone surface in the furcation region were obtained using an image analysis system (Image-Pro®, Media Cybernetics, Silver Spring, MD, USA), and the number of TRAP-positive cells was counted using a light microscope (Axioskop 2 plus®, Zeiss, Jena, Germany) at ×40 magnification. The results are described as the number of TRAP-positive cells/mm and /mm^2 for the bone crest and the 1000 μm area under the furcation of the ligated and non-ligated teeth, respectively.

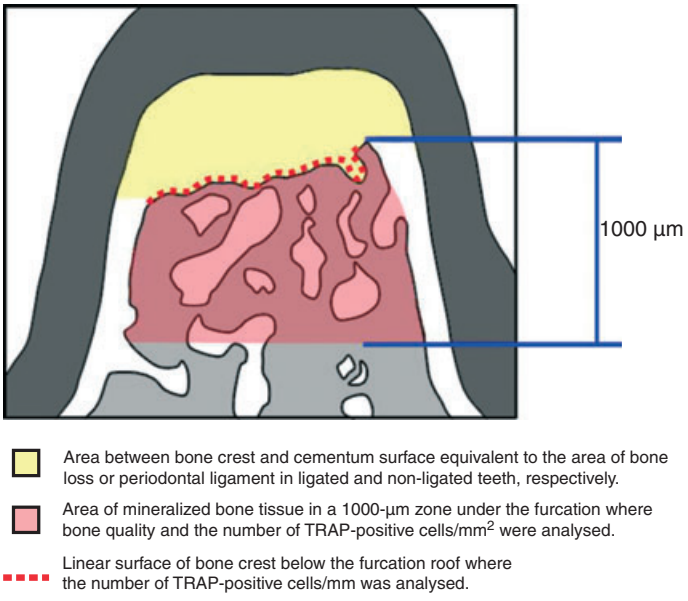

Fig. 2. Schematic illustration of the regions for histometric analysis, including bone loss/periodontal ligament areas and bone quality; and for TRAP-positive cells counted on the linear surface of the bone crest and in a 1000 μm zone under the furcation.

Statistical analysis

Intergroup analyses were carried out by using one-way analysis of variance (ANOVA), to statistically assess quality of tooth-supporting alveolar bone and number of TRAP-positive cells at both ligated and non-ligated sites. If statistical differences were detected, a pairwise multiple comparison was additionally performed with the Tukey test. The non-parametric Kruskal–Wallis test was performed for the intergroup comparison, regarding the hormone serum levels and the distance between interradicular bone crest and furcation roof for ligated and non-ligated teeth, respectively. If differences were detected among the groups, Dunn's test was applied. Intragroup analysis was performed using Student's paired *t*-test for all parameters, testing differences between ligated and non-ligated sites. A significance level of 0.05 was adopted for all statistical comparisons.

Results

Clinical observations and biochemical analysis

Macroscopic examination during autopsies suggested successful induction of thyroid dysfunctions. Thyroid glands in the control and hyperthyroidism groups were larger and pink colored, while in hypothyroidism the glands were thin and anemic due to

Table 1. Means and standard errors of serum levels of triiodothyronine and thyroxine achieved in each experimental group

Group	Triiodothyronine (ng/dL)	Thyroxine (μg/dL)
Control	111.53 ± 5.03	2.64 ± 0.58 0.17
Hypothyroidism	78.07 ± 3.50*	1.28 ± 0.17 0.05*
Hyperthyroidism	182.07 ± 9.06*	4.18 ± 0.25*

*Represents statistically significant difference between test (hypothyroidism and hyperthyroidism) and control groups ($p < 0.05$) by the Kruskal–Wallis test and Dunn test.

reduced activity. The glands in hyperthyroidism appeared slightly larger than in the control group. The total serum levels of T_3 and T_4 before ligature placement confirmed hormone changes and are summarized in Table 1. The serum levels of T_3 and T_4 were higher in hyperthyroidism ($p = 0.03$ and $p = 0.04$ for T_3 and T_4, respectively) and decreased in hypothyroidism ($p = 0.02$ and $p = 0.03$ for T_3 and T_4, respectively) compared with the control group.

In addition, at the time of killing, clinical examination of ligated and non-ligated sites revealed signs of gingival inflammation, including color/volume changes and bleeding around the ligated teeth of all groups, with no signs of inflammation at the non-ligated sites.

Histometric analysis

Histometrically, intragroup analysis showed a significant difference in the distance between interradicular bone crest and furcation roof between non-ligated and ligated teeth in all the experimental groups ($p = 0.0001$, $p = 0.0006$ and $p = 0.0001$ for control, hypothyroidism and hyperthyroidism, respectively). Moreover, an intergroup analysis showed an increased distance between interradicular bone crest and furcation roof, representing a greater bone loss in the ligated teeth for the hypothyroidism group compared with the ligated teeth in the control ($p = 0.02$) and hyperthyroidism groups ($p = 0.02$). Furthermore, no statistically significant differences were observed regarding the same area in non-ligated teeth among the groups ($p = 0.08$; Fig. 3A). Figure 4A–C illustrates histological findings, showing that there was an increased bone loss in the furcation region of ligated teeth of the hypo-

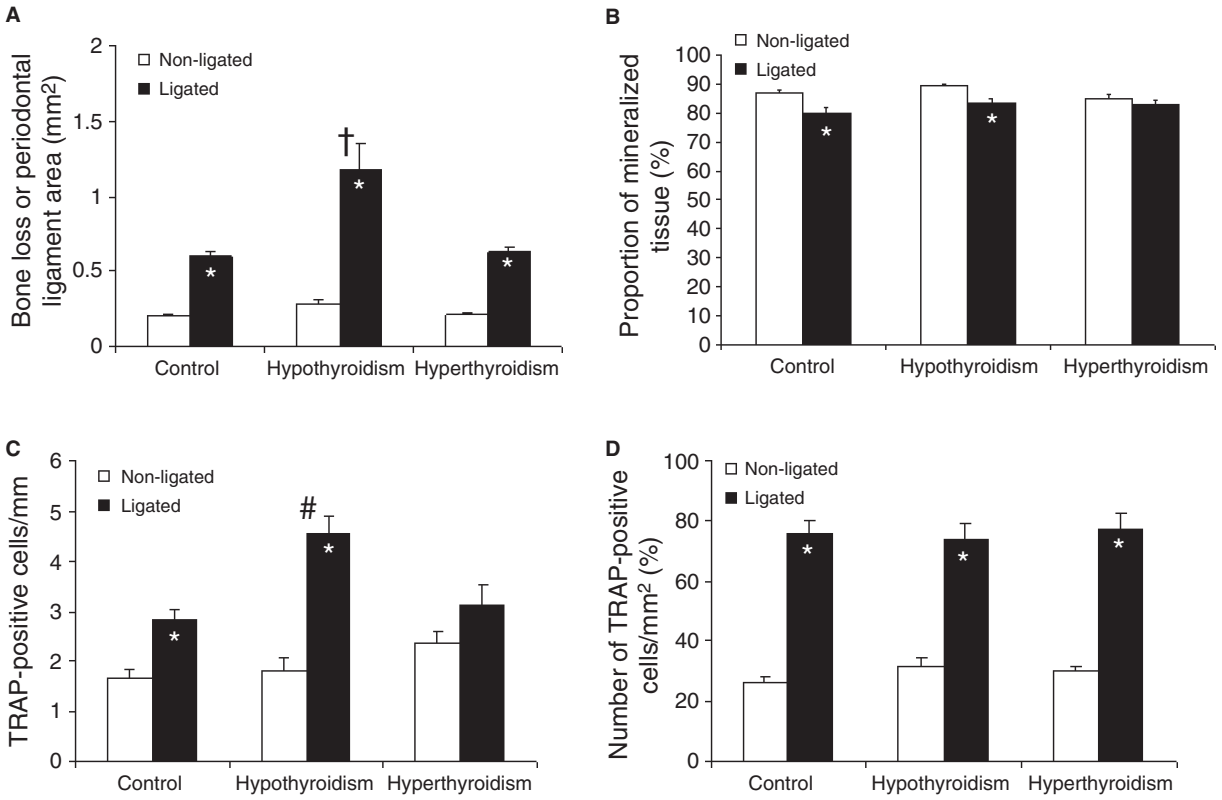

Fig. 3. The illustration graphically represents the means and the standard errors of the study parameters achieved in ligated and non-ligated sites for control, hypothyroidism and hyperthyroidism groups. (A) Bone loss and periodontal ligament area for ligated and non-ligated teeth, respectively (mm^2). (B) Proportion of mineralized tissue (%). (C) Number of TRAP-positive cells/mm in a linear surface on the bone crest. (D) Number of TRAP-positive cells/mm^2 in a 1000 μm zone under the furcation. *Statistically different by an intragroup comparison, ligated vs. non-ligated teeth ($p \leq 0.05$), by Student's paired t test. †Statistically different by an intergroup comparison, control vs. hypothyroidism vs. hyperthyroidism ($p \leq 0.05$), by the Kruskal–Wallis test followed by Dunn's test. #Statistically different by an intergroup comparison, control vs. hypothyroidism vs. hyperthyroidism ($p \leq 0.05$), by one-way ANOVA followed by the Tukey test.

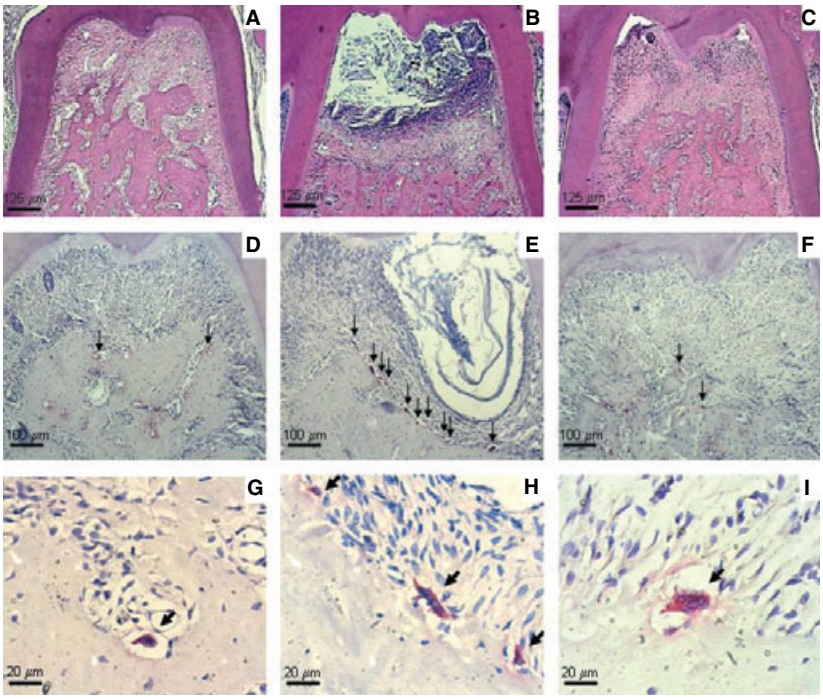

Fig. 4. Photomicrographs illustrating the histological aspects of bone loss in the furcation region and TRAP-positive cells on the linear surface of the bone crest immediately below the furcation roof in the ligated teeth for control (A,D), hypothyroidism (B,E) and hyperthyroidism groups (C,F). Hematoxylin and eosin or TRAP counterstaining with hematoxylin. Photomicrographs G–I show high-magnification details of TRAP-positive cells (TRAP counterstaining with hematoxylin.

thyroidism group compared with the control and hyperthyroidism groups.

Regarding the assessment of tooth-supporting alveolar bone, data analysis showed, despite the changes in T_3 and T_4 serum levels, a similar proportion of mineralized tissue among the experimental groups with the same ligature status ($p = 0.24$ and $p = 0.08$, for ligated and non-ligated teeth). In contrast, an intragroup analysis showed a significant decrease in the proportion of mineralized tissue in the ligated teeth vs. the non-ligated teeth in the control ($p = 0.02$) and hypothyroidism groups ($p = 0.002$). Figure 3B graphically illustrates the results described above.

Staining for TRAP

The number of TRAP-positive cells was analysed in two different regions. Firstly, for the linear surface of the bone crest, an intergroup analysis showed an increase in the number of TRAP-positive cells per millimetre in the ligated teeth for the hypothyroidism group compared with the control ($p = 0.01$) and hyperthyroidism ($p = 0.025$) groups. In addition, intragroup analysis demonstrated a significant increase in the number of TRAP-positive cells in ligated teeth than in non-ligated teeth for control ($p = 0.01$) and hypothyroidism groups ($p = 0.001$). Figure 3C illustrates the results.

When the number of TRAP-positive cells was evaluated in the 1000 μm zone of the interradicular bone under the furcation of both ligated and non-ligated sites, there was no significant difference among the groups ($p = 0.17$ and $p = 0.91$ for ligated and non-ligated teeth, respectively). However, a significant increase was observed in the number of TRAP-positive cells/mm^2 in ligated compared with non-ligated teeth for each group ($p = 0.0001$ for control, hypothyroidism and hyperthyroidism). Figure 3D graphically illustrates the reported data. Figure 4D–F illustrates the TRAP-positive cells in a linear surface on the interradicular bone crest. It is possible to observe that there is an increased number of TRAP-positive cells in the micrograph of the hypothyroid rat (Fig. 4E) compared with the control and hyperthyroid rats (Fig. 4D,F, respectively). Figure 4G–I shows TRAP-positive cells in detail.

Discussion

Thyroid dysfunctions are common health problems in the population, and the determination of the influence of thyroid hormone imbalance in periodontitis may be important for the prevention of morbidity related to this condition when the association is present. Thus, the present study aimed to investigate the impact of thyroid hormones on the alveolar bone loss resulting from experimental periodontitis in rats and on the quality of the tooth-supporting alveolar bone. Moreover, the number of TRAP-positive cells on the linear surface of bone crest, immediately below the furcation roof, and in a 1000 μm area of the interradicular bone under the furcation of the ligated and non-ligated teeth was also assessed. In the present investigation, biochemical analysis demonstrated that the experimental perturbation used was able to induce changes in the serum levels of T_3 and T_4, as previously reported (17). Furthermore, data analyses demonstrated that a thyroid-hormone-deficient state may significantly increase the bone loss resulting from ligature-induced periodontitis, whereas no effect was observed for the opposite condition (increased levels of thyroid hormones). Finally, data analyses further demonstrated that the quality of the tooth-supporting alveolar bone under the furcation was not affected by the thyroid hormone serum levels. Although several studies have reported an association between thyroid hormone levels and skeletal bone mass (9), changes in the microtrabecular alveolar bone following changes in thyroid hormones have rarely been reported (21). Thyroid hormones regulate various leucocytic actions, such as activation (22) and proliferation of different cellular lineages, including T- and B-lymphocytes

(23). Moreover, thyroid hormones also participate in the release of cytokines, such as interferon γ (24) and IL-6 (25). Thus, the decrease in the levels of these hormones may promote a less competent immunogenic response to the infection induced by the experimental periodontitis (26) and, therefore, it may be suggested that the influence of thyroid hormones on the progression of periodontitis may be related to a relationship between the immune system and the thyroid axis, and not related to the effect of hormone changes on the alveolar bone quality (27). However, further studies should be considered in order to specifically address the effect of thyroid hormone changes on the immune system in the local microenvironment of the periodontium.

Data analysis further demonstrated that the number of TRAP-positive cells may also be affected by lower serum levels of the hypothyroid hormones in sites with periodontitis and, therefore, provides support for the data obtained by histometric analysis in both regions evaluated. Tartrate-resistant acid phosphatase is an iron-containing enzyme whose biological function is not fully understood. This enzyme may be involved in the degradation of bone constituents by osteoclasts. Recently, a role for TRAP has been suggested in regulating intracellular vesicular trafficking in resorbing cells (28). High amounts of TRAP are expressed in bone-resorbing cells, and changes in bone resorption are usually associated with changes in the number of resorbing cells, suggesting that secreted TRAP may be a useful marker of bone resorption (29). On a molecular basis, in gingival tissue with periodontal disease, bone resorption is characterized by a primary production of an osteoclast differentiation factor, RANKL, by B- and T-cells, which induces differentiation and activation of resorbing cells (30) and may support the increased number of TRAP-positive cells in the ligated vs. non-ligated sites in the present study. Moreover, it can be speculated that the increased number of TRAP-positive cells in the linear surface of bone crest and, consequently, more pronounced bone loss in the hypothyroidism group, may also be associated with the upregulation of RANKL in the RANKL–OPG system by altered concentrations of prostaglandins and IL-6. These levels of inflammatory molecules are altered as an additional consequence of hypothyroidism or secondary to inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-1, which regulate osteoclast differentiation and function independently of the RANKL–RANK interaction (31). However, it remains unclear which factors may trigger the increased differentiation and activity of the resorbing cells *in vivo* when hypothyroidism is correlated with an infectious process.

In conclusion, within the limits of the present study, in rats, a thyroid hormone-deficient state may potentiate the bone loss resulting from ligature-induced periodontitis, whereas no significant impact of thyroid hormone changes was observed in non-inflamed sites. The findings of the present study suggest that an increased number of TRAP-positive cells in the hypothyroid condition may play an important role. Therefore, in addition to the importance of thyroid hormone deficiency in general health status, hypothyroidism may also constitute a critical state with respect to the periodontium, and controlled clinical studies should be considered in order to provide information concerning the best approach to deal with this condition.

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