Effect of Lithothamnium sp and calcium supplements in strain- and infection-induced bone resorption

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ABSTRACT

Objective: To investigate the effect of Lithothamnium sp (LTT) supplement, a calcium-rich alga widely used for mineral reposition, on strain-induced (orthodontic tooth movement [OTM]) and infection-induced bone resorption (periodontal disease [PD]) in mice.

Materials and Methods: Mice were divided into two bone resorption models: one with an orthodontic appliance and the other with PD induced by the oral inoculation of Aggregatibacter actinomycetemcomitans (Aa). Both groups were fed a regular diet (vehicle), LTT-rich diet (LTT), or calcium-rich diet (CaCO$_3$). Alveolar bone resorption (ABR), the number of osteoclasts, and the levels of tumor necrosis factor α (TNF-α), calcium, and vitamin D3 were evaluated.

Results: The number of osteoclasts was reduced in LTT and CaCO$_3$ mice, which led to diminished OTM and infection-induced alveolar bone loss. In addition, LTT- and calcium-treated groups also presented decreased levels of TNF-α in periodontal tissues and increased levels of calcium in serum.

Conclusions: These results indicate that the LTT supplement influences ABR, probably due to its calcium content, by affecting osteoclast function and local inflammatory response, thus modulating OTM and PD. (Angle Orthod. 0000;00:000–000.)

KEY WORDS: Orthodontic tooth movement; Calcium; Bone resorption

INTRODUCTION

Orthodontic tooth movement (OTM) and periodontal disease (PD) are deeply related to alveolar bone resorption (ABR). During OTM, the applied forces generate a transient inflammatory process that allows teeth to be moved through the alveolar bone. On the other hand, PD is a chronic infectious and inflammatory disorder caused by oral biofilm bacteria, including Aggregatibacter actinomycetemcomitans (Aa), which...
culminates with ABR and, possibly, tooth loss. Both OTM and PD are strongly influenced by factors such as age, physical activity, genetics, and dietary intake, as well as mediators including growth factors, cytokines, hormones, and calcium availability.

Calcium is an important component of a healthy diet, but it is not produced endogenously, being acquired through dietary intake. Approximately 1% of the body's calcium content serves as a plasma reservoir, which is essential for physiological processes, and the remaining 99% is stored in bones and teeth. Nevertheless, negative calcium balance causes calcium mobilization from bone, leading to bone loss that can modify oral bone metabolism and, consequently, negatively influence OTM and PD.

Several kinds of calcium salts, including calcium citrate, calcium carbonate, and calcium phosphate, are used to provide commercially available calcium dietary supplements (DS). Another potential dietary strategy to increase calcium consumption would be the use of calcium-rich marine algae such as Lithothamnion sp (LTT), a calcium carbonate–rich alga of the Hapalidaceae family.

This study investigated whether LTT supplementation could influence ABR by using two different experimental mice models: strain-induced (OTM) and infection-induced (PD) bone remodeling.

### MATERIALS AND METHODS

#### Experimental Mice

Eight-week-old male C57BL6/J and BALB/c mice were used in the OTM and PD models, respectively. All animals were treated under the ethical regulations for animal experiments, defined by the Institutional Ethics Committee of the Federal University of Minas Gerais, Brazil (protocol number 256/2008). For every set of experiments, five mice were used for each time point (Tables 1 and 2). No significant weight loss was observed throughout the experimental period.

#### Experimental Diet

The OTM and PD groups were divided into three different subgroups and fed with different experimental diets, as follows: vehicle (standard laboratory chow NUVILAB CR1), LTT (1% of LTT algae extract—which is 32% composed by calcium carbonate—mixed in NUVILAB CR1, as described previously), and CaCO3 (NUVILAB CR1 plus the same amount of calcium carbonate found in the algae). NUVILAB CR1 is composed of calcium carbonate (maximum of 1.40%), corn bran, soy bran, wheat bran, dicalcium phosphate, sodium chloride, vitamin premix mineral, and amino acid, per kilogram enriched by vitamins, minerals, and amino acids. The chemical composition of the LTT has been previously described.

Mice were fed ad libitum with the commercial/experimental diets during 32 days in the OTM model (starting 20 days before mechanical loading up to 12 days of OTM) and 60 days in the PD model (starting on the first day of infection until the end of the experiment, 60 days later).

#### OTM Group

The experimental protocol was based on previous studies. An Ni-Ti 0.25 × 0.76-mm coil spring (Lancer Orthodontics, San Marcos, Calif) was bonded by light-cured resin (Transbond, Unitek/3M, Monrovia, Calif) between the maxillary right first molar and incisors. The force magnitude was calibrated by a tension gauge (Shimpo Instruments, Itasca, Ill) to exert a force of 0.35 N applied in the mesial direction. There was no reactivation during the experimental period. The left side of the maxilla was used as control.

For histopathologic analyses, mice were sacrificed with an overdose of anesthetic after 12 days of mechanical loading. At this time point, a blood sample was obtained to evaluate the serum levels of calcium and vitamin D3. For enzyme-linked immunosorbent assay (ELISA), the animals were sacrificed at 0, 12, or 72 hours.

### Table 1. Sample Size of Each Group Used in the Orthodontic Tooth Movement Model

<table>
<thead>
<tr>
<th>Orthodontic Tooth Movement Model</th>
<th>Histopathological and Serum Levels Analysis</th>
<th>ELISA</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>LTT</td>
</tr>
<tr>
<td>Control</td>
<td>5 ME</td>
<td>5 ME</td>
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<tr>
<td>12 hours</td>
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<td>5</td>
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<td>72 hours</td>
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<td>12 days</td>
<td>5 MD</td>
<td>5 MD</td>
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<tr>
<td>Total mice</td>
<td>5</td>
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</tbody>
</table>

* ME, left hemi maxilla; MD, right hemi maxilla.

### Table 2. Sample Size of Each Group Used in the Periodontal Disease Model

<table>
<thead>
<tr>
<th>Periodontal Disease Model</th>
<th>Alveolar Bone Loss (Hemi Maxilla)</th>
<th>ELISA/MPO (Hemi Maxilla)</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle</td>
<td>LTT</td>
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<tr>
<td>Control</td>
<td>10</td>
<td>10</td>
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<tr>
<td>30 days</td>
<td>5</td>
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<tr>
<td>60 days</td>
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<tr>
<td>Total mice</td>
<td>20</td>
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</tr>
</tbody>
</table>

* ELISA, enzyme-linked immunosorbent assay; LTT, Lithothamnion sp; MPO, myeloperoxidase.
CALCIUM SUPPLEMENTS AND ALVEOLAR BONE RESORPTION

Analysis of OTM and Osteoclasts

The maxillary halves were dissected and fixed in 10% buffered formalin (pH 7.4).1,13–15 After fixation, they were decalcified in 14% EDTA (pH 7.4) for 20 days and embedded in paraffin. Samples were cut into sagittal sections of 5-μm thickness, where the disto-buccal root appeared to be as long as possible, stained for tartrate-resistant acid phosphatase (TRAP; Sigma-Aldrich, Saint Louis, Mo), and counterstained with hematoxylin for histological examination. The Image J software (National Institutes of Health) was used to quantify the OTM through the difference between the distance of the cement-enamel junction (CEJ) from the right first and second molars in relation to the opposite (control) side of the same animal. The mesial side of the first molar distal-buccal root was used for osteoclast counts, which were determined in five consecutive microscopic fields (400×), on five sections per animal.13,14 The slides were counted by two examiners, blinded to group status.

Periodontal Infection (PD Group)

Mice received a direct injection of 1 × 10⁹ CFU/mL of a diluted culture of the periodontopathogen Aa strain FDC Y4, from American Type Culture Collection, in 10 μL of phosphate-buffered saline (PBS) into the palatal gingival tissue close to the second molar.15 Immediately after, 1 × 10⁹ CFU/mL of a diluted culture of Aa in 100 μL of PBS plus 1.5% of carboxymethyl-cellulose was placed in the oral cavity using a micropipette. The protocol was repeated after 48 and 96 hours. The experimental and control groups were evaluated at 30 or 60 days after initial infection (five mice of each group at each time point).

Quantification of Alveolar Bone Loss

The maxillae were hemisected, exposed overnight to 3% hydrogen peroxide, and mechanically defleshed. To distinguish the CEJ, the samples were stained with 0.3% methylene blue.15,16 The palatal faces of the molars were photographed with 20× magnification using a stereomicroscope (Metrimpex Hungary/PZO, Labimex, Hungary) and a digital camera (Kodak EasyShare C743, Rochester, NY). The images were analyzed using the Image J software. Quantitative analysis was used to measure the bone loss area between the CEJ and the alveolar bone crest of the first molar in squared millimeters. Samples were analyzed by a single examiner, blinded to the group status.

Measurement of Cytokine Levels

In the OTM model, a stereomicroscope was used to extract periodontal ligament and surrounding alveolar bone samples from the adjacent areas of the upper first molars.14 In the PD model, periodontal tissues, teeth, and alveolar bone were extracted as previously described.15 The samples were weighed and homogenized in PBS (0.4 mM NaCl and 10 mM NaPO₄ containing protease inhibitors (0.1 mM Phenylmethylsulfonyl Fluoride (PMSF), 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and 0.05% Tween-20 at 1 mg/mL. The mixture was centrifuged (8946 g) for 10 minutes, and the supernatant was stored at −70°C. The levels of tumor necrosis factor-α (TNF-α) were evaluated by sensitive enzyme-linked immunoabsorbent assay (ELISA) according to the manufacturer’s protocol (R&D Systems, Minneapolis, Minn). The results were expressed as picograms of cytokine (±SEM) normalized for 100 mg of tissue.

Myeloperoxidase

Myeloperoxidase (MPO), a neutrophil enzyme marker, was quantified as previously described to verify indirectly the extent of neutrophils in PD.15,16 The MPO activity in homogenized periodontal tissues was evaluated by enzymatic reaction, measured by absorbance at 450 nm. It contents were expressed as relative units calculated from standard curves based on the MPO activity from 5% casein peritoneal-induced neutrophils.

Serum Analysis

The blood samples were collected from the orbital sinus in empty tubes.16 Samples were centrifuged at 2000 g for 5 minutes at 4°C and kept under refrigeration at 4°C. The colorimetric Arsenazo III method was used to analyze calcium levels.17 High-performance liquid chromatograph was used to assess 1.25 dihydroxy vitamin D3 (1.25 [OH]₂D₃).18

Statistical Analysis

Data are expressed as the mean ± SEM. The one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison post hoc test, was used to evaluate and compare the cytokine levels, TRAP activity, number of osteoclasts, the OTM, and quantitative data from PD of each group. The one-way ANOVA, followed by Bonferroni post hoc test, was used for paired comparisons to evaluate differences in serum levels of calcium and 1.25 (OH)₂D₃ among the three groups. Data were processed with GraphPad Prism (version 5.01, GraphPad Software, San Diego, Calif). The level of significance was adjusted at P < .05. To validate the consistency of the evaluations, the intraclass correlation coefficient was performed, and there was a significant positive correlation (P < .001).

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RESULTS

OTM and Number of Osteoclasts Decreased in Mice Fed LTT and CaCO\textsubscript{3} in the OTM Model

Histomorphometric analysis showed a significant reduction ($P < .05$) in the amount of OTM in both the LTT- and CaCO\textsubscript{3}-treated groups after 12 days of force (Figure 1A).

The number of osteoclasts significantly diminished ($P < .05$) in mice fed with LTT or CaCO\textsubscript{3} (Figure 1B). In the control side (with no force), TRAP activity was found on the distal side, and no activity was observed in the mesial region of the periodontium (Figure 2A,B). After 12 days of force, TRAP activity appeared to decrease more extensively in mice fed with LTT (Figure 2E,F) or CaCO\textsubscript{3} (Figure 2G,H), which presented less ABR than mice fed with vehicle (Figure 2C,D).

ABR Decreased in Mice Fed LTT or CaCO\textsubscript{3} in the PD Model

Aa-infected mice fed with LTT (Figure 3A,D) or CaCO\textsubscript{3} (Figure 3A,E) presented significantly less ABR ($P < .05$) when compared with infected mice fed with vehicle (Y4; Figure 3A,C).

Mice Fed LTT Had Decreased Levels of TNF-\textalpha in Both OTM and PD Models

As ABR was reduced in both groups, we evaluated whether such DS could have altered the expression of TNF-\textalpha in periodontal tissues. In OTM (after 72 hours of force) and PD models, mice fed with LTT showed significantly lower levels ($P < .05$) of TNF-\textalpha (Figure 4A,B). In addition, the presence of neutrophils after PD induction was affected, as the MPO activity decreased in Aa-infected mice after treatment with LTT (noninfected mice, 0.048 ± 0.03; Aa-infected mice [Y4] fed with vehicle, 0.104 ± 0.03; infected mice [Y4] fed with LTT, 0.055 ± 0.02; $P < .05$).

Serum Levels of Calcium and 1.25-Dihydroxy Vitamin D3 in Mice Fed LTT or CaCO\textsubscript{3}

The serum levels of calcium were significantly higher ($P < .05$) in both LTT- and CaCO\textsubscript{3}-fed mice (Figure 5A). In contrast, such calcium-rich diets did not affect the levels of 1.25 (OH)\textsubscript{2}D\textsubscript{3} (Figure 5B).

DISCUSSION

Calcium DS is one of the most common therapies of bone diseases, including osteopenia and osteoporosis\textsuperscript{19}. This study investigated whether a higher dietary calcium intake, such as on the LTT supplementation, could influence ABR associated with OTM or PD, two of the most relevant dental conditions involving bone remodeling. Herein, the treatment with LTT and CaCO\textsubscript{3} decreased periodontal levels of TNF-\textalpha, the number of osteoclasts, and, consequently, ABR. Other studies\textsuperscript{7,8} have suggested that calcium intake may alter bone remodeling, but this is the first one that clearly shows that calcium carbonate from mineral or marine alga sources may significantly influence ABR during OTM or after infection.

Human adults require 1000 to 1300 mg of calcium in their daily diet, which is often prescribed as a DS for...
Figure 2. Histological changes after 12 days of force. Close-up view in A (B), in C (D), in E (F), and in G (H). Small arrows indicate TRAP-positive osteoclasts. (A, B) Control group (no force). (C, D) Vehicle-fed, (E, F) LTT-fed, and (G, H) CaCO₃-fed mice experimental groups (12 days after orthodontic force). DB indicates distal alveolar bone; LTT, Lithothamnium sp; MB, mesial alveolar bone; PL, periodontal ligament; R, root; TRAP, tartrate-resistant acid phosphatase. Arrow bar = 100 µm.

prevention and treatment of bone disorders. Indeed, several studies have attempted to elucidate the role of calcium supplementation and deficiency on the mechanisms that control osteoclast activity and their consequences on human health and diseases. Calcium might be associated with osteoclast recruitment, differentiation, activation, function and, consequently, bone remodeling. Accordingly, this study
indicated that a higher dietary calcium intake, either with LTT or CaCO$_3$, significantly decreased ABR in both OTM and PD models.

In line with this, the number of osteoclasts has also been reduced by DS with LTT or CaCO$_3$. In fact, some reports have already demonstrated that calcium might be important in receptor activator of nuclear factor kappa-B ligand–mediated osteoclastogenesis.$^{21}$ The data showed that increased circulating serum levels of calcium, due to LTT or CaCO$_3$ supplements, is associated with reduced numbers of osteoclasts and, consequently, reduced amount of ABR in both OTM and PD models. In contrast, the levels of 1,25(OH)$_2$D$_3$, one of the most known active metabolites in regulating

Figure 3. (A) Alveolar bone resorption (ABR) in the periodontal disease model during 60 days. Macroscopic evaluation of ABR in (B) mice without infection and infected mice (Y4) fed with (C) vehicle, (D) Lithothamnium sp (LTT), or (E) CaCO$_3$. The arrow shows interdental ABR. $^*P < .05$ when comparing the control group (noninfected mice), one-way analysis of variance (ANOVA). $^*P < .05$ when compared with vehicle-fed mice, one-way ANOVA. The values (means ± SEM) were obtained from five animals in two independent experiments.
calcium and phosphorus metabolism, have not been significantly affected. It is known that excessive dietary calcium intake can suppress circulating 1,25(OH)\(_2\)D\(_3\) levels, although these data suggested that LTT supplements did not affect the serum levels of vitamin D3.

We also investigated whether such DS had any effect on the inflammatory response (or could interfere with the production of inflammatory mediators), given that some types of algae present anti-inflammatory properties. The periodontal tissue levels of TNF-\(\alpha\), an important proinflammatory cytokine involved in bone remodeling associated with OTM and PD, were evaluated and showed a significant decrease in LTT-fed mice after strain or infection stimuli. In accordance, the MPO levels were also reduced after

Figure 4. (A) Expression of tumor necrosis factor \(\alpha\) in the orthodontic tooth movement model after 0, 12, and 72 hours of mechanical loading, and (B) in the periodontal disease model after 30 days of infection (Y4). *\(P < .05\) vs control group (no force or no infection), one-way analysis of variance (ANOVA). *\(P < .05\) vs vehicle-fed group, one-way ANOVA. The values (means ± SEM) were obtained from five animals at each point in two independent experiments.

Figure 5. (A) Amount of calcium and (B) 1,25 dihydroxy vitamin D3 serum levels. *\(P < .05\) when compared with the control group (no force), one-way analysis of variance (ANOVA). *\(P < .05\) vs vehicle-fed mice, one-way ANOVA. The values (means ± SEM) were obtained from five animals in two independent experiments.
LTT treatment during PD. TNF-α is also important in neutrophil recruitment to inflammatory sites. This cell line plays a role in the progression of PD, being the main mechanism responsible for host defense against bacterial periodontal infection and tissue damage. Indeed, sulfated polysaccharides derived from red microalgae, such as LTT, seem to significantly inhibit neutrophil recruitment toward inflammatory stimuli. In contrast, other evidence suggests that some multivitamin/mineral supplementation may not induce any effect on inflammatory cytokine production. This study suggests that the effects of LTT go beyond its calcium content, since its potential anti-inflammatory properties may also be important in explaining its bone-protective effects.

Altogether, within the limitations of this study, it can be suggested that the calcium-rich LTT DS may be useful in preventing inflammatory ABR in both strain-induced bone remodeling and infection-induced bone loss. Nevertheless, the extrapolation of these findings to humans deserves caution. Although this supplement is currently marketed worldwide, it is known that it can present potential toxicity, given that calcium saturation may be associated with bloating, constipation, and a slightly increased risk of kidney stones. As daily calcium intake continues to expand steadily, new studies should be conducted to evaluate the possible consequences of calcium supplementation on the orthodontic treatment of patients, as well as the possibilities of periodontal calcium therapy. Further studies are now required to understand the effects of a high-calcium diet on important regulators of calcium homeostasis and their indirect effects on OTM and PD.

CONCLUSIONS

- This study suggests that the CaCO3 from LTT DS decreases the number of osteoclasts and inflammatory mediators and, consequently, reduces ABR.
- This is a demonstration that dietary intake might play a role in OTM and PD bone resorption.

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