Published in final edited form as:

J Periodontol Res. 2016 October ; 51(5): 661–668. doi:10.1111/jre.12346.

The effect of an inhibitor of gut serotonin (LP533401) during the induction of periodontal disease

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Abstract

Background and Objective—LP533401 is an inhibitor of tryptophan hydroxylase 1, which regulates serotonin production in the gut. Previous work indicates that LP533401 has an anabolic effect in bone. Thus, we hypothesized that inhibition of gut serotonin production may modulate the host response in periodontal disease. In this study, we aimed to analyze the effects of LP533401 in a rat periodontitis model to evaluate the role of gut serotonin in periodontitis pathophysiology.

Material and Methods—Twenty-four rats were divided into three groups: treated group (T: ligature-induced periodontal disease and LP533401, 25 mg/kg/d) by gavage; ligature group (L: ligature-induced periodontal disease only); and control group (C: without ligature-induced periodontal disease). After 28 d, radiographic alveolar bone support was measured on digital radiographs, and alveolar bone volume fraction, tissue mineral density and trabeculae characteristics were quantified by microcomputed tomography in the right hemi-mandible. Left hemi-mandibles were decalcified and alveolar bone loss, attachment loss and area of collagen in the gingiva were histologically analyzed.

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The authors declare no conflict of interest.

Results—Significant difference between the L and C groups was found, confirming that periodontal disease was induced. We observed no difference between the T and L groups regarding alveolar bone destruction and area of collagen.

Conclusion—LP533401 (25 mg/kg/d) for 28 d does not prevent bone loss and does not modulate host response in a rat model of induced periodontal disease.

Keywords

bone; enterochromaffin cells; periodontitis; serotonin

Serotonin (5-hydroxytryptamine) is a bioamine generated in the brainstem and by enterochromaffin cells in the gut. Importantly, serotonin does not cross the blood–brain barrier, and has unique activity depending on its site of production (1). In the gut, serotonin functions as a hormone, whereas in the brain, serotonin serves as a neurotransmitter (2). The enzyme tryptophan hydroxylase (Tph) initiates the synthesis of serotonin by converting L-tryptophan to L-5-hydroxytryptophan. There are two types of Tph, i.e. Tph1, peripherally expressed, mainly in the enterochromaffin cells, and Tph2 exclusively expressed in the central nervous system (3).

Centrally, serotonin regulates important neurological functions, including mood, behavior, sleep, blood pressure and temperature (4). Peripherally, serotonin is involved in cardiovascular function (5) and intestinal motility (6). Peripheral serotonin represents 95% of all serotonin in the body. It can be carried by platelets or can remain free to interact with cell-specific receptors (3,7). The diversity of serotonin actions is due to the existence of multiple serotonin receptors. Among them, 5-HT_{1A}R, 5-HT_{2A}R, 5-HT_{1B}R and 5-HT_{2B}R are expressed on osteoblasts and 5-HT_{2B}R expression is increased on osteoblasts during differentiation (3,8).

Gut serotonin was linked to bone metabolism after it was shown that low-density lipoprotein receptor-related protein 5 gene (*Lrp5*) inhibits expression of *Tph1*, the rate-limiting biosynthetic enzyme for serotonin in enterochromaffin cells (7). The *Lrp5* loss-of-function mutation seems to have a role in the osteoporosis–pseudoglioma syndrome (9) while gain-of-function mutation in *Lrp5* is linked to high bone mineral density disorders (10). Reduced expression of the gene *Tph1*, and consequent reduction in serotonin stimulates osteoblast proliferation and bone formation (7). Using the drug LP533401 as a pharmacological approach to inhibit *Tph1*, Karsenty and Yadav rescued the bone phenotype of diverse models of osteoporosis (1). However, contradictory results were found by Cui *et al.* (11), who showed no correlation between whole blood serotonin and bone mass in *Lrp5* and *Tph1* mutant mice and using LP923941, an active enantiomer of LP533401. With LP923941, the authors observed a decrease of the whole blood serotonin but no phenotype changes in the animals.

In addition to the controversial results associating gut serotonin and bone, the role of serotonin in periodontal disease remains unclear, although a number of studies suggest its potential involvement in this condition (12–15). Serotonin transporter polymorphisms for instance are associated with aggressive periodontitis (12). Furthermore, rat with induced

periodontal disease treated with fluoxetine, a selective serotonin reuptake inhibitor, showed reduced alveolar bone loss (13,14) and inflammatory markers (13). While serotonin in the central nervous system seems to have a positive effect on periodontal disease, the role of gut serotonin, to the best of the authors' knowledge, has not been studied in a periodontitis model. Therefore, we aimed to evaluate the role of gut serotonin in periodontitis pathophysiology. We hypothesized that the inhibition of gut serotonin would modulate host response during periodontal disease induction, reducing alveolar bone and periodontal collagen loss.

Material and methods

Induction of periodontal disease

Twenty-four 90 d old rats (male, *Rattus norvegicus albinus*, Wistar) were caged and fed with standard diet and water ad libitum. The Institutional Ethics Committee for Research Involving Animals approved these experiments (2/2012-PA/CEP).

All animals were weighed and anesthetized using an intramuscular injection of ketamine (13 mg/kg)/2% xylazine (33 mg/kg) (Ceva, São Paulo, SP, Brazil). Periodontal disease was induced by inserting two loops of cotton thread (3-0) around the lower first molars of the rat, with the knot placed on the mesial surface of the teeth. After 15 d, ligatures were checked. If the ligatures were missed in this first check or at the moment of death, the animals were excluded and replaced. In the control group, periodontal disease was not induced; however, the animals were anesthetized and manipulated in the same fashion as the other groups, with the exception of the ligature. Medication, according to each group, started the day after ligature placement.

Drug

We used LP533401 HCl (Dalton Pharma, Toronto, ON, Canada) diluted in polyethylene glycol (Sigma-Aldrich, St. Louis, MO, USA) with 5% dextrose (Sigma-Aldrich) (40 : 60) (16). The solution was prepared daily by mixing the LP533401 and polyethylene glycol solution overnight, and adding the 5% dextrose just before administration. The control groups received only the vehicle. Drug solution was administered by gavage in a dose of 25 mg/kg/d. This dose was based on the minimum concentration sufficient to revert ovariectomy (OVX)-induced bone loss in rats (17).

Experimental groups

For the experiments, animals were randomly divided into three groups: (i) treated group (T) consisted of animals with ligature-induced periodontal disease that were treated with LP533401 (25 mg/kg/d) by gavage for 28 d ($n = 8$); (ii) ligature group (L) consisted of animals with ligature-induced periodontal disease that were treated with vehicle by gavage for 28 d ($n = 8$); and (iii) control group (C) consisted of animals with no periodontal disease that received vehicle by gavage for 28 d ($n = 8$). Sample size was calculated using data from a similar previous study (13), considering a power of 0.8 and an alpha of 0.05 (G-Power, Version 3.1.9.2, Heinrich University Dusseldorf, Dusseldorf, Germany).

Tissue preparation

After 28 d of treatment, animals were anesthetized and killed by decapitation. Both hemi-mandibles were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4; 0.1 M). After 48 h, the hemi-mandibles were washed in tap water. The right hemi-mandibles were stored in 70% ethanol at 4°C and the left hemi-mandibles were treated with 10% ethylenediaminetetraacetic acid (Merck KGaA, Darmstadt, Germany) at room temperature for decalcification.

X-ray two-dimensional analysis

The right hemi-mandibles were radiographed on the lingual surface using dental digital X-ray equipment (Gendex 765 DC, Gendex Dentals, De Plaines, IL, USA), at 65 kVp and 7 mA, with a focal-object distance of 30 cm and 0.05 s exposure time. The mandibles were positioned such that the lingual and buccal cusps were parallel, and the radiographic images of the cusps and roots were superpositioned. One blinded and calibrated observer performed the data analysis.

The linear bone support percentage was calculated according to the following formula

$$\text{Bone support(\%)} = AC \cos(\hat{CAB}) \times 100 / AB,$$

where AC is the distance from the top of the bone crest to the distal root apex, AB is the distance between the distal cusp top to the distal root apex and $\hat{CAB}$ is the angle between these two segments (Fig. 1). The linear measures were performed using image analysis software (ImageJ 1.31p; National Institutes of Health USA; available from <http://imagej.nih.gov/ij/>) (18).

Microcomputed tomography

The right hemi-mandibles were scanned in a Scanco μ CT40 (Scanco Medical AG, Brüttisellen, Switzerland) at 70 kV and 114 μ A X-ray source, 500 projections/180° rotation and 300 ms integration and image were reconstructed with 16 μ m isotropic voxels. Analyses were performed using the manufacturer's software. A three-dimensional (3D) volume of interest was standardized and defined by the following points: apex of the mesial root of the first molar (apical limit); first molar furcation area (coronary limit); mesial of the mesial root of the second molar (posterior limit); and mesial of the mesial root of the first molar (anterior limit) (Fig. 2). For consistency between measurements, the cortex and roots were excluded. Within the volume of interest, a threshold of 548 mg HA/cm³, sigma value of 0.3, and support of one were applied to segment bone from soft tissue, and the bone volume fraction (BV/TV), trabecular number (Tb.N) and thickness (Tb.Th) and tissue mineral density (TMD) were calculated using standard methods.

Histomorphometry

After decalcification, the left hemi-mandibles were embedded in paraffin. The hemi-mandibles were then sectioned (5 μ m) in a mesial–distal direction and stained with a modified hematoxylin and eosin–phloxine–orange G stain and picosirius red.

Histomorphometric analysis was performed using image analysis software (ImageJ 1.31p; National Institutes of Health, USA). Three semi-serial slices, separated from one another by at least 20 μ m, for each sample, were digitalized at $\times 50$ and $\times 100$ original magnification. The area of alveolar bone loss was evaluated in the furcation of the first molar at $\times 50$ magnification. The distance between the distal interproximal alveolar crest and the cementum–enamel junction (distal bone loss), and the distance between the cementum–enamel junction and the most apical level of the epithelial cells (distal attachment loss), were evaluated, at $\times 100$ magnification (19).

Three semi-serial slices stained with picosirius red were digitalized at $\times 100$ magnification using polarization microscopy to analyze the area of collagen in the distal gingival region. The collagen fibers displaying a red hue inside a predetermined rectangle (0.14 mm height $\times$ 0.30 mm width) over the distal bone crest were selected using image processing software (Adobe Photoshop 7.0.1 Adobe Systems Incorporated, San Jose, CA, USA). The image was then binarized, and the area of collagen within the rectangle was calculated using the software ImageJ 1.31p.

One blinded and calibrated observer performed the data analysis.

Statistical analysis

Data normality was tested (Kolmogorov–Smirnov test) and statistical analysis was performed using one-way analysis of variance with Tukey's *post-hoc* test (GraphPad Prism 5.0, GraphPad Software, San Diego, CA, USA). $p < 5\%$ was considered statistically significant. Data are presented as the mean $\pm$ SD. Owing to technical difficulties during sectioning, the number of samples for histological analysis in the control group was seven.

Results

X-ray and microcomputed tomography

The ligature model was effective in reducing the percentage of alveolar bone support (X-ray), since the L group showed less support (mean $\pm$ SD; $43.99 \pm 2.02\%$) than the control group ($60.14 \pm 3.55\%$) ($p < 0.001$). Using microcomputed tomography (micro-CT) analyses, the L group also displayed a statistically lower BV/TV (L group: $0.50 \pm 0.05\%$; C group: $0.65 \pm 0.04\%$, $p < 0.001$), Tb.Th (L group: 0.19 ± 0.03 mm; C group: 0.25 ± 0.03 mm, $p < 0.001$) and TMD (L group: 1011 ± 11.32 mg HA/cm³; C group: 1036 ± 18.53 mg HA/cm³, $p < 0.05$) than control group (Fig. 3). When comparing the L versus the T group, no significant difference was observed in bone support percentage (X-ray) ($p > 0.05$; T group: $41.47 \pm 4.78\%$; and L group: $43.99 \pm 2.02\%$, $p > 0.05$), or on the micro-CT analysis, BV/TV (T group: $0.50 \pm 0.07\%$; and L group: $0.50 \pm 0.05\%$; $p > 0.05$), TMD (T group: 996.5 ± 15.24 mg HA/cm³; and L group: 1011 ± 11.32 mg HA/cm³; $p > 0.05$), trabecular number

(T group: 4.52 ± 1.47 ; and L group: 3.96 ± 0.76 ; $p > 0.05$) or trabecular thickness (T group: 0.18 ± 0.03 mm; and L group: 0.19 ± 0.03 mm; $p > 0.05$) between the two groups (Fig. 3).

Histology

Regarding the histomorphometric results, the L group had higher alveolar bone loss in the furcation (L group: 0.46 ± 0.17 mm²; C group: 0.08 ± 0.04 mm², $p < 0.001$) (Fig. 4) and distal region (L group: 0.71 ± 0.11 mm; C group: 0.38 ± 0.1 mm, $p < 0.001$), as well as less attachment than the C group (L group: 0.52 ± 0.09 mm; C group: 0.007 ± 0.01 mm, $p < 0.001$) (Fig. 5). Further, histological analyses revealed that the area of gingival collagen (Picrosirius red) was lower in the L group (0.02 ± 0.005 mm²) than the control group (0.03 ± 0.005 mm²) ($p < 0.05$) (Fig. 6).

In addition, the bone loss in the furcation (T group: 0.56 ± 0.16 mm²; L group: 0.46 ± 0.17 mm², $p > 0.05$) (Fig. 4), distal bone loss (T group: 0.75 ± 0.10 mm; L group: 0.71 ± 0.11 mm, $p > 0.05$) and distal attachment loss (T group: 0.52 ± 0.12 mm; L group: 0.52 ± 0.09 mm, $p > 0.05$) did not differ between the T and L groups (Fig. 5). The collagen area (Picrosirius red) was also similar between the T (0.025 ± 0.004 mm²) and L groups (0.02 ± 0.005 mm², $p > 0.05$) (Fig. 6).

Discussion

In recent years, evidence was published supporting that serotonin regulates bone homeostasis and that inhibition of serotonin had anabolic and antiresorptive effects in bone (1,16,17). Based on these studies, we sought to evaluate the effect of gut serotonin inhibition, via Tph1 inhibition by using LP533401, on ligature-induced periodontal disease in rats, with the hypothesis that pharmacologic inhibition of Tph1 could prevent alveolar bone loss. Our results indicate that the Tph1 inhibitor LP533401 at a dose and regimen shown to revert OVX-induced bone loss in rats (17) does not prevent alveolar bone loss in rats after a 28 d treatment.

Current therapies for periodontal disease involve mechanical and surgical procedures that do not allow for the recovery of lost tissue. Drugs used and tested to treat osteoporosis, such as bisphosphonates (20), statins (21), (β-blockers (22) and anti-receptor activator of nuclear factor kappa-B ligand and sclerostin antibodies (23), as well as anabolic agents such as parathyroid hormone (24,25) have been studied in periodontal disease with good results. Previous studies showed that LP533401 was effective in preventing systemic bone loss, and enhanced bone mass in mice and rats (16,17). According to data from Yadav *et al.* (17), LP533401 indeed reversed the deleterious effect of OVX on bone mass of female rats at a dose of 25 mg/kg/d, even though it only decreased serum serotonin levels by 35–40%. There was no alteration in bone size or width. Further, no gastrointestinal side effects, platelet number changes or changes in coagulation time were observed, even at doses of 250 mg/kg/d, suggesting the drug is safe. Fu *et al.* (26), testing an inhibitor of Tph1 similar to LP533401 verified recovery of OVX-induced bone loss using a smaller dose of the tested drug (10 mg/kg/d) than the dose of LP533401 we used in our study (25 mg/kg/d). Moreover, with only 1 mg/kg/d Tph1 inhibitor, they could find significant reduction on the circulating serotonin levels though no bone parameter was changed. Although these results suggest that

LP533401 could be beneficial for bone mass conservation in the setting of osteoporosis, this drug showed no effect on alveolar bone loss induced by periodontal disease in rats in our study. It is worth noting that all in vivo studies regarding the bone protective effect of Tph1 inhibition were performed in OVX-induced osteoporosis models (16,17,27), whereas periodontal disease is a locally developed inflammatory disease. Thus, it is possible that the bone anabolic effect of gut serotonin inhibition could be blunted by the periodontal inflammatory microenvironment, where inflammatory factors could outweigh the beneficial effect of Tph1 inhibition on bone.

Some studies have shown an association between periodontal disease and serotonin (12–14,28), but the possible effect of gut serotonin inhibition on alveolar bone loss associated with periodontal disease has not been studied. The existing studies involve brain serotonin through selective serotonin reuptake inhibitor therapy (13,14,28), using varying doses to evaluate the anti-inflammatory effect of these drugs in periodontal disease. Two groups (13,14) found that treatment of periodontitis with 20 mg/kg/d fluoxetine reduced alveolar bone loss and disease progression. Further, Branco-de-Almeida *et al.* (13) found that fluoxetine decrease cyclo-oxygenase-2 and interleukin-1 β mRNA expression in the gingival tissue. These results suggest that serotonin reuptake has a differential effect on alveolar bone independently of bone regulation by gut serotonin.

Controversial results have been reported concerning the potential link between gut serotonin and bone mineral density. Cui *et al.* (11) inhibited gut serotonin in ovariectomized and sham-operated rats using an active enantiomer of the compound LP533401 and found a whole blood serotonin decrease but no change in femur and vertebral trabecular bone mass. In addition, van Dijk *et al.* (29) and Walsh *et al.* (30) demonstrated no correlation with serotonin levels and bone parameters in patients with carcinoid syndrome who present with excessive levels of circulating serotonin. Moreover, no difference on proliferation and mineralization was found when stimulating pre-osteoblasts with different doses of serotonin *in vitro* (30).

Our study has some limitations: (i) only one dose of LP533401 was tested, which limit the generalizability of the obtained results; (ii) despite the use of the same dose of LP533401 shown to protect from OVX-induced bone loss, and to decrease serotonin levels by Yadav *et al.* (17), we were not able to measure serotonin levels in our study; and (iii) data regarding RNA expression for inflammatory cytokines would be useful to determine if Tph1 modulation through LP533401 affects the inflammatory background involved in periodontal disease-induced alveolar bone loss, although not clinically relevant, as alveolar bone loss and connective tissue attachment were not influenced by the drug. Based on our data, we conclude that 25 mg/kg/d LP533401 is not sufficient to inhibit periodontal disease and prevent alveolar bone loss in a rat model of periodontal disease.

Acknowledgments

This study was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES) – process number 1913-13-1, the UNESP Research Pro-Rectory (PROPe) and the São Paulo Research Foundation (FAPESP) – Process number 2012/16547-6. MicroCT imaging was supported, in part, by NIH grant 1S10RR027631. DSP was supported by Career Development Award IK2BX001634 from the Department of Veterans Affairs, BLRD.

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Fig. 1.

Schematic drawing and lines used to measure the percentage of alveolar bone support, where AC is the distance from the top of the bone crest to the distal root apex, AB is the distance between the distal cusp top and the distal root apex, and $\hat{CAB}$ is the angle between these two segments. Measurements were performed using the image analysis software ImageJ 1.31p.

Fig. 2.

Micro-CT images: (A) 3D reconstruction of the mandibular scanned area, where the symbol (()) represents the analyzed periodontium area, from the first molar furcation roof to the apex of the mesial root (schematic drawing); (B) micro-CT image of the analyzed area after chosen rotation in a transverse plane (contours illustrate the determined area excluding the cortex and the roots that were excluded using the micro-CT software; Scanco Medical AG); (C) 3D reconstruction of the volume of interest analyzed. Micro-CT, microcomputed tomography.

Fig. 3.

Microcomputed tomography: Graphs of plotted values for the BV/TV (A), tissue mineral density (B), trabecular number (C) and trabecular thickness (D) of the studied groups: C group ($n = 8$), L group ($n = 8$) and T group ($n = 8$). (E) 3D reconstruction images of the total scanned area of each group illustrating their volumetric bone loss. *Significant difference ($p < 0.001$), and #significant difference ($p < 0.05$), related to the C group. The p value corresponds to the Tukey's test between the groups. BV/TV, bone volume fraction; C, control group; L, ligature group; T, treatment group.

Fig. 4.

Photomicrography and graph of plotted values, of the area of alveolar bone loss in the furcation region (hematoxylin and eosin stain; $\times 50$ original magnification) illustrating the area of bone loss for each group: C, $n = 7$; L, $n = 8$; T, $n = 8$. *Significant difference related to the C group ($p < 0.001$). The p value corresponds to the Tukey's test between the groups. C, control group; L, ligature group; T, treatment group.

Fig. 5.

(A) Graph of plotted values, and photomicrography of distal alveolar bone (hematoxylin and eosin stain, $\times 100$ original magnification) illustrating each group: C, $n = 7$; L, $n = 8$; T, $n = 8$. (B) Graph of plotted values, and photomicrography of distal attachment loss (hematoxylin and eosin stain, $\times 100$ original magnification) illustrating each group: C, $n = 7$; L, $n = 8$; T, $n = 8$. *Significant difference related to the C group ($p < 0.001$). The p value corresponds to the Tukey's test between the groups. BC, bone crest; C, control group; CEJ, cement-enamel junction; L, ligature group; JE, junctional epithelium; T, treatment group.

Fig. 6.

(A) Picrosirius red stain illustrating the distal area of collagen for each group: C, $n = 7$; L, $n = 8$; T, $n = 8$. (B) Graph of plotted values of the distal area of collagen. *Significant difference related to the C group ($p < 0.05$). The p value corresponds to the Tukey's test between the groups. C, control group; L, ligature group; T, treatment group.