Simvastatin modulates gingival cytokine and MMP production in a rat model of ligature-induced periodontitis

的目的：本研究的目的是评估辛伐他汀对合成的细胞因子TNF-α和IL-10以及金属蛋白酶（MMP）2和9在大鼠模型中引起的龈炎的影响。

材料和方法：使用20只Wistar大鼠，使用棉线作为牙周袋位置，围绕左上第一磨牙的根颈。将动物随机分为两个实验组（每组n=10）：1）带有结扎+药物（生理盐水10 mL/kg；口服）和2）带有结扎+辛伐他汀（25 mg/kg；口服）。14天后，将动物以安乐死处死并去除并破碎龈组织。

结果：没有观察到TNF-α在治疗组和对照组之间的差异（p>0.05）。然而，IL-10在辛伐他汀治疗组中上调（64.3%），与药物治疗组相比（p<0.05）。辛伐他汀降低了龈组织中MMP-9的水平（8.1%），与药物治疗组相比（p<0.05）。

结论：口服辛伐他汀增加了IL-10的释放和MMP-9的抑制在结扎诱导的牙周炎模型中。

关键词：细胞因子，金属蛋白酶，牙周疾病，辛伐他汀

引言

牙周炎是一种影响支持牙齿的牙周组织的免疫性疾病，导致骨破坏和牙龈退缩。其发病率约为11%的成人世界范围内的（由Kassebaum et al3和Richards4）。现在已经知道，这种持续损伤是由牙周炎的免疫反应导致的。3不同炎症成分被建议参与这种反应，包括细胞因子和金属蛋白酶（MMPs）3,4。5

异常识别的细菌产物导致激活定居细胞，如成纤维细胞和抗原递呈细胞，以及中性粒细胞和单核细胞的移入牙龈，启动了持续的炎症过程。5内生免疫细胞因子（TNF-α, IL-1, 和IL-6）被产生，促进细胞通过根部的基质金属蛋白酶（MMPs）的激活，促进骨吸收。
and contributing to tooth loss.\(^7\) Also, as a result of cytokine generation, an increase in MMPs is noted. MMPs comprise a group of proteases that target the extracellular matrix and are associated with remodeling of the soft and mineralized periodontal tissues.\(^5\) As the disease progresses, another cytokine, IL-10, is released into the gum by activated T-cells and macrophages.\(^4\) IL-10 is produced in an attempt to counteract the inflammatory response and is known to trigger the expression of molecules that downregulate the transcription of proinflammatory cytokines and MMPs.\(^4,5\)

Current treatment of periodontitis includes surgical and nonsurgical (systemic or local antibiotics) procedures.\(^6\) Side effects, dysbiosis of gut microbiota, and increased bacterial resistance to antibiotic therapy have raised concerns regarding its use to treat periodontitis.\(^6\) On the other hand, the recent knowledge on the inflammatory pathways involved in this disease has unleashed novel venues for therapy, with increased attention to strategies that modulate the inflammatory response.\(^7\) Recently, an anti-inflammatory effect was suggested for antihypertensive drugs (angiotensin II receptor blockers, angiotensin-converting enzyme inhibitors, \(\beta\)-blockers) in periodontitis.\(^8,9\) More recently, the cholesterol-lowering drug simvastatin, used as adjuvant in hypertensive patients with dyslipidemia, was suggested to have a protective effect against experimental\(^10,11\) and human periodontitis.\(^9,12\) This effect was attributed to its capacity to reduce bone loss and modulate the expression/release of inflammatory markers in gingival crevicular fluid samples.

Herein, this study evaluated the effect of simvastatin on the synthesis of cytokines TNF-\(\alpha\) and IL-10 and the MMPs 2 and 9 in a rat model of ligature-induced periodontitis. We found that this drug is able to increase the levels of IL-10 while reducing MMP-9 expression in gingival tissue samples.

**Materials and methods**

**Animals**

Twenty nonfasted, outbred male Wistar rats (~250 g) were used. Rats were obtained from the Centro Multidisciplinar para Investigação Biológica na Área da Ciência em Animais de Laboratório (Campinas, Brazil), and experiments were performed in the biological service unit of the University of Campinas. Animals were kept (n=5/cage) in a climatically controlled environment (room temperature of 22\(^\circ\)C±2\(^\circ\)C and humidity of around 60%) under 12:12 hour light-dark cycle. This study and all procedures were approved by the Ethics Committee of the University of Campinas and carried out in accordance with the guidelines of the Brazilian Society for Animal Welfare (SBCAL).

**Ligature-induced periodontitis**

Briefly, animals were anaesthetized with a single intramuscular injection of ketamine (90 mg/kg; Dopalen, Ceva, Brazil) and xylazine (10 mg/kg; Rompun, Bayer, Brazil). Periodontitis was then induced as previously described by Branco-de-Almeida et al.,\(^13\) with minor modifications. For this, a cotton ligature was placed in a subgingival position encircling the entire cervix of both sides of the first molar on the left (ipsilateral) side of the mandible. In order to immobilize the ligature, two knots were made at the mesial aspect of the first molar. The right (contralateral) side of the mandible had no ligature placed and was used as the control.

Disease was allowed to develop for 14 days, and then the rats were euthanized by anesthetic overdose. Gingival tissue samples were collected from the mandibular first molar regions, immediately frozen, and kept at \(-80^\circ\)C for further analysis. In order to confirm the establishment of periodontal disease, contralateral and ipsilateral mandibular alveolar bone specimens were collected at day 14 and submitted to macroscopic and histologic analysis.

For macroscopic analysis, mandibles were immersed in sodium hypochlorite 1% solution for 4 hours and the whole remaining bone soft tissue was mechanically removed. The pieces were stained with methylene blue (1 g/100 mL) for 1 minute to delimit the cement–enamel junction. For histological analysis, the mandibles were fixed in 10% neutral buffered formalin and then demineralized in 5% formic acid for 7 days. 7 \(\mu\)m sections from the decalcified bone were stained with hematoxylin and eosin staining and evaluated to confirm bone loss.

**Treatment with simvastatin**

After ligature placement, animals were randomly assigned to two experimental groups (n=10 animals/group): 1) rats with ligature + vehicle (saline; 10 mL/kg; orally) and 2) rats with ligature + simvastatin (25 mg/kg; orally). Simvastatin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline. Either saline or simvastatin were administered for 14 days following periodontitis induction.

**Histologic analysis of the mandible**

Ipsilateral mandible samples were immediately fixed in 10% neutral buffered formalin for 24 hours, washed 3\(\times\) in phosphate-buffered saline, and then stained with 1% methylene blue.\(^11\) Bone loss was evaluated in both contralateral and ipsilateral sides by comparison of digital images.
Sample preparation
Gingival tissue samples were homogenized in 300 µL of RIPA lysis buffer (Santa Cruz Biotechnology, Dallas, TX, USA) and then centrifuged for 10 minutes at 10,000 rpm at 4°C. The supernatants were collected and used for evaluation of cytokine (TNFα and IL-10) and MMP (MMP-2 and 9) levels.

Tissue cytokine and MMP levels
Gingival TNF-α, IL-10, MMP-2, and MMP-9 levels were evaluated by using commercially available kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Results are expressed as picogram per milliliter (pg/mL).

Data analysis
The results are presented as the mean ± standard deviation for the animals. The percentage of inhibition is reported as the mean ± standard deviation for each individual experiment. Statistical comparison was performed using unpaired Student’s t-test in GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). *p<0.05 was considered significant.

Results
Figure 1 shows the bone integrity of both the contralateral and ipsilateral sides of the mandible. The ipsilateral (ligature; Figure 1B), but not the contralateral (Figure 1A) side, presented loss of the alveolar crest (black arrow), indicating the successful induction of periodontitis in the tested rats. In addition, the histological analysis demonstrated an inflammatory cell infiltration with cementum and alveolar process destruction confirming the periodontitis (ligature, Figure 1D). In contrast, the contralateral side showed a normal periodontium (Figure 1C).

The levels of TNF-α and IL-10 were evaluated in gingival tissue samples with established periodontitis obtained from vehicle- and simvastatin-treated rats. No differences were observed for TNF-α production between the groups (Figure 2A). However, IL-10 was upregulated in simvastatin-treated animals (1.8-fold increase) in comparison with those of the vehicle-treated group (Figure 2B).

Gingival levels of MMP-2 and -9 were measured in samples obtained from vehicle- and simvastatin-treated rats. MMP-2 levels were similar in vehicle and simvastatin gingival samples (Figure 3A). Oral treatment with simvastatin reduced the gingival levels of MMP-9 (64.3%) in comparison with vehicle-treated samples (Figure 3B).

Discussion
Gingival inflammation triggered by bacteria is a hallmark of periodontitis and progressively leads to bone resorption and tooth loss. A plethora of cells and inflammatory mediators are involved in this process, leading to the higher production of proinflammatory molecules such as TNF and MMPs, accounting for disease progression. Nonsurgical treatment of periodontitis is currently used in the clinics and is primarily based on administration of either systemic or local antibiotics. Increased bacterial resistance to the therapy, in addition to its side effects, has lead to the search for novel therapeutic approaches. The increased knowledge on the inflammatory pathways involved in periodontitis has prompted the investigation of the effects of anti-inflammatory drugs as strategies to reduce gingival inflammation. Recent studies have suggested that cholesterol-lowering drugs such as simvastatin are protective against experimental and human periodontitis. These evidences have suggested that simvastatin is able to reduce bone loss and modulate the expression/release of inflammatory markers in gingival crevicular fluid samples. However, little is known of simvastatin effects on the gingival tissue itself. Herein, we provided data that this drug modulated inflammation in the gingival tissue of animals with experimental periodontitis induced by ligature placement.
This study initially investigated the levels of cytokines in gingival tissue samples obtained from animals with established periodontitis. TNF-α is well known for its involvement in inflammatory disorders, contributing to inflammation and pain, and plays a leading role in bone resorption in periodontitis.20 Indeed, this inflammatory mediator is one of the detrimental factors for disease progression as it stimulates osteoclast activity by inducing RANKL production, in addition to MMP production.17,21 Simvastatin was previously suggested to inhibit TNF-α.12 This study found that simvastatin does not affect TNF-α production at 14 days after disease induction, as animals treated with this drug exhibited similar levels of this mediator in their gingival samples to those of the vehicle-treated group. However, Fentoglu et al22 demonstrated that simvastatin inhibits TNFα release in to the gum when administered for 3 months in vivo; this suggests that in order to modulate this cytokine, simvastatin may have to be taken for longer periods than assessed in our study. Also, we cannot overrule the possible modulatory effects of this drug on the release of other proinflammatory cytokines in our model. Indeed, recent studies showed that simvastatin anti-inflammatory effects are also related to its ability to reduce IL-1β and IL-6 levels and to increase the anti-inflammatory cytokine IL-10.4 Studies in IL-10 knockout mice showed that these mice present with higher osteoclast activity and bone loss,23 and this has been linked to increased RANKL expression.24 Here, simvastatin increased the gingival release of IL-10, reinforcing its protective effects in periodontitis.

MMPs, proteases that target the extracellular matrix, are released by infiltrating inflammatory cells such as macrophages and fibroblasts residing in the gum, and they...
are associated with remodeling of the soft and mineralized periodontal tissues.1 Their expression is known to be regulated by cytokine production in periodontitis and other chronic diseases in which tissue destruction occurs.1 MMP gene polymorphisms have been recently linked to periodontitis susceptibility.25 Our data show that simvastatin reduced gingival MMP-9 but did not affect MMP-2 levels in samples with established periodontitis. An inhibitory effect on MMP expression was previously shown for simvastatin. This drug was found to decrease MMP-9 mRNA expression in gingival tissue samples from rats with periododontis induced by Aggregatibacter actinomycetemcomitans LPS, and a similar effect was observed when simvastatin was incubated in vitro with human leukemia cells.26 Also, patients treated with simvastatin present with lowered levels of circulating MMP-9 and reduced expression of this molecule in aortic wall samples.27 The effects of simvastatin on MMP-2 have been shown to be rather controversial, with some reports suggesting inhibition of this molecule by simvastatin and others suggesting a lack of effect for this drug on this pathway.26,30 It is possible though that MMP regulation by simvastatin differs according to the disease model investigated. In the case of periodontitis, MMP-2 has been linked with asymptomatic apical disease, while MMP-9 is associated with chronic gingival inflammation.31

**Conclusion**

Overall, our data showed that simvastatin may be useful for treating gingival inflammatory conditions, and more specifically, periodontal disease, as repeated treatment reduces MMP-9 levels while increasing IL-10 release into the inflamed gum. We suggest simvastatin be used as an aid therapy for chronic inflammatory diseases in which bone resorption occurs.

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**Disclosure**

The authors report no conflicts of interest in this work.

**References**


