# Comparison of the Effects of Local Injection and Oral Intake of Diclofenac and Atorvastatin in Alveolar Bone Density Assessed with CT in Experimental Periodontitis in Rat

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**Objectives** The first cause of tooth loss in developed countries is periodontitis. Chronic periodontitis is the most common form of periodontitis and it is characterized by loss of periodontal attachment, destruction of alveolar bone and eventual loss of teeth. Atorvastatin is a statin drug used for the treatment of high cholesterol. Statins can stop the inflammatory process by inhibiting the cholesterol pathway. Diclofenac is an NSAID with anti-inflammatory, anti-pyretic and analgesic effects. Its primary mechanism is through the inhibition of prostaglandins synthesis by the inhibition of the cyclooxygenase enzyme (COX). The purpose of this study is to compare the effects of local injected and oral intake of Diclofenac and atorvastatin on alveolar bone density measured in HUs with the use of a CT scan in a periodontitis-induced model in rats.

**Methods** Thirty rats were randomly divided into 6 groups of 5 rats each. Ligatures were placed around the left second maxillary molar to induce periodontitis for 10 days. Administration of 12.5 mg/kg of oral atorvastatin (group 1), 0.25 mg/kg of injectable atorvastatin (group 2), 7.5mg/kg of oral Diclofenac (group 3), 6.25mg/kg of injectable Diclofenac (group 4), the oral solvent without medicine as oral control (groups 5), and the injectable solvent without medicine as injectable control (group 6). In each group, the right side of maxilla was considered as control group (without ligature and drug interaction). At day eleven, the rats were sacrificed and the maxillary bone was separated from the soft tissue and fixed in 4% formalin. The prepared samples were then radiologically evaluated to determine the bone density with CT in fixed exposure conditions.

**Results** There was a statistically significant difference between the alveolar bone density of the oral atorvastatin group and the oral Diclofenac (P = 0.006). There was no statistical significant difference in alveolar bone density between the injectable atorvastatin and the injectable Diclofenac groups (P=0.228).

**Conclusion** Both atorvastatin and Diclofenac have shown better results when assessing bone density in a periodontitis rat model as compared to controls. Additionally, Diclofenac has been shown to be more effective at both oral and injectable administrations as compared with atorvastatin in the prevention of loss of bone density in a rat model with periodontitis.

**Keywords** Atorvastatin, Diclofenac, Alveolar Bone Loss, Bone Density, Rat

## Introduction

The first cause of tooth loss in developed countries is periodontitis.[1] Chronic periodontitis is the most common form of periodontitis. It is an inflammation of the periodontal tissues resulting in loss of periodontal attachment, destruction of alveolar bone, periodontal pocketing formation, and eventual loss of teeth. Chronic periodontitis is a multi-factorial disease initiated by microbial plaque. Its extension and severity within each individual is influenced by the genetic and environmental factors of each subject.[2] The process of inflammation starts from superficial tissues (sulcular and junctional epithelium) and spreads to deeper connective tissues.[3] The initial inflammatory response to the dental microbial plaque is headed by polymorphonuclear neutronphils (PMNs), which will detect the antigens of the microorganisms through their surface receptors. The activation of these receptors by the pathogen results in their phagocytosis and

the secretion of pro-inflammatory cytokines such as IL-8, IL-6, IL12 and TNF- α, which cause periodontal tissue destruction.[4] In the inflamed gingiva, high levels of the tissue- degrading enzymes such as matrix metalloproteinase (MMPs) are found. MMPs cause the destruction of the extracellular matrix; including collagen, gelatin, and elastin which will eventually result in periodontal tissue destruction and the loss of alveolar bone.[3] When the inflammatory products reach the bone surface bone destruction might start representing in this way the transition from gingivitis to periodontitis. The height and density of the alveolar bone is regulated by a balance between bone forming cells (osteoblasts) and bone-resorbing cells (osteoclasts), all affected by the systemic and local environment. If the bone resorption surpasses its formation rate, there will be a reduction in bone density, a reduction of alveolar bone height, or both.[4] Atorvastatin is a member of the drug class known as statins, which had a revolutionary impact on the treatment of high cholesterol.

Statins reduce the production of mevalonate, geranyl pyrophosphate, and farnesyl pyrophosphate and the other production in the way of cholesterol creation. Statins can stop the process of inflammation by inhibiting the cholesterol pathway and by interfering with the function of the RAS family of proteins.[5] Additionally, atorvastatin has shown to suppress MMPs.[6] Statins are also strong stimulants of bone morphogenetic protein-2 (BMP-2).[7] Diclofenac is an NSAID derived from phenyl-acetic acid and has a structure similar to Flurbiprofen. It has anti-inflammatory, anti-pyretic and analgesic effects. Its primary mechanism is through the inhibition of prostaglandin synthesis by the inhibition of the cyclooxygenase enzyme (COX). This drug is used in the treatment of osteoarthritis and rheumatoid arthritis.[8] Computed-Tomography (CT) uses computer- processed combinations of X-ray slices of a structure to obtain cross-sectional images. CT scanners use the emitted x-ray from the body of a patient to produce sectional images. The X-ray taken out of the body of the patient is sensed by a detector. The detector measures the output x-ray severity of the patient and sends it as digital data to a computer for analysis. The computer converts this digital data to numerical information that is expressed as the number of CT (CT number) or Hounsfield Units (HUs). Numerical data is represented in images taken from the patient as a gray shade to indicate the different tissue densities that in the darkest (radiolucent) case may represent the air density and in the clearest (opaque) case represents very dense tissues such as bone. Hounsfield Unit (HU) scale is a standard para-clinical densitometry method that is used to check the density of the body bones, including maxilla and mandible. As of today, many studies indicate that the use of HU scale is a very useful method to evaluate the bone density of maxilla and mandible.[9]

Numerous laboratory animal species including non-human primates, dogs, and rodents have been used to study the pathophysiology, the radiographic changes, or the outcomes of preventive treatments in periodontitis.[10]

Several studies showing different results have been conducted on the effect of atorvastatin on bone formation and bone loss.

Balli et al. (2014) performed a study in various phases of periodontitis on 100 rats, where the effect of topical atorvastatin was examined for the first time. They concluded that atorvastatin was useful in the treatment of periodontal disease.[11] In another study by Araújo Jr et al. (2013) on the anti-inflammatory properties of atorvastatin, it was concluded that there was a decrease of the gingival inflammation and a decrease in bone resorption.[5] In a study performed by Goes et al. (2010) to assess the effect of atorvastatin on bone density with the help of digital radiography, high doses of atorvastatin were capable of preventing bone loss in a ligature-induced periodontitis model.[12] On the other hand, Chang et al. (2011) found that atorvastatin did not affect the bone density even at high doses[13] by DEXA method (The method in which is used x-ray to measure the bone density). Several studies have also

been performed to evaluate the effects of Diclofenac and bone loss.

Kurunj Kumarun et al. (2012) showed that the Diclofenac sodium even at low concentration diminishes the number of osteoclasts hence reducing bone loss.[14] Additionally; Ghalyani et al. (2014) assessed the effect of Diclofenac and celecoxib in relation to the osteoclastogenesis during the healing period after tooth extraction. Both Diclofenac and celecoxib caused a decrease in osteoclast production, reducing in this way the bone loss after tooth extraction.[15]

The cited studies have not examined deeply the topical injection and oral effects of Diclofenac and atorvastatin on the alveolar bone density in periodontal disease. To our knowledge, there is not a study comparing the effects of these two drugs together. Moreover, very few studies have been performed using HUs to measure the bone density despite it is considered the standard method for densitometry.

The aim of this study is to compare the effects of the topical injection and oral administration of either Diclofenac or atorvastatin in the alveolar bone density in areas with ligature–induced periodontal disease assessed in HU with the use of CT in rat.

## Materials and Methods

Based on previous studies [5, 11, 12, 14, 15] and by the use of SPSS, 30 male Sprague-Dawley rats weighting of 180 to 200g and with the age of 8 to 12 weeks were used.

After adequate anesthesia with sub cutaneous injection of animal ketamine hydrochloride combination as 10% at a dose of 70 mg/ kg and 2% xylazine at a dose of 10 mg/ kg, periodontal disease was induced by placing a ligature thread (3-0 ETHIBOND EXCEL polyster green coated braided non absorbable) on the gingival sulcus around the left second maxillary molar and tied in the palatal area.[16]

In the study, 30 rats were randomly divided into 6 groups of 5 rats each. The ligature thread was tied around the left second maxillary molar for 10 days to induce periodontitis. The animals were daily administered with 12.5 mg / kg of oral atorvastatin by Gavage method (group 1), they were injected 0.25 mg / kg of injectable atorvastatin in the alveolar crest of the second left maxillary molar (group 2), 7.5mg/ kg of oral Diclofenac by Gavage method was administered (group 3), injectable Diclofenac as 6 .25mg / kg was injected in the alveolar crest of the second left maxillary teeth (group4), administration of solvent without drug as xsoral control (group 5) and the injectable solvent without drug as injectable control (group 6). In each group, the right side of maxilla was considered as control group (without ligature and drug interaction). The selection of drug doses have been done based on previous studies that have shown to be effective in reducing inflammation in rats.[17]

The animals in all the groups were kept in a standard condition (12 hours of day and 12 hours of night and at a temperature of 22 ° C with access to food and water).[18]

The sagittal profile of the prepared samples was radiologically evaluated to determine the bone density by Hounsfield Unit technique with CT in the exposure conditions of 1 mAs and 120 kvp. In the images that were taken from samples, gray shades indicate the different tissue densities (in the darkest case it may represent the air density and in the clearest case it represents very dense tissues such as bone). Then the bone density of the studied area (alveolar bone of second maxillary molar) was measured by using the existing software on CT scan device (GE: Milawaukee, USA) as numerical information that is expressed as CT values or HUs. These CT values or HUs were compared between the samples with periodontal defects without drug administration versus the samples having the periodontal defects with drug administration both as topical and as oral injection. That is how the considered bone tissues include the lower Hounsfield unit or in other words the lower number of CT, tissue density is lower and the higher Hounsfield unit or CT number is, the greater tissue density is.

Data analysis was performed by SPSS versoin 18. Data was described by using the mean (±Standard deviation). Analysis of one-way variance (One-Way ANOVA) was used as well as Tukey HSD test to compare the bone density at control sides. Also, the analysis of covariance (ANCOVA) was used to compare the amounts of bone density between the ligature- induced periodontitis groups when their values of bone density at control sides were considered as the independent variable (Covariate). Throughout the paper a $p<0.05$ statistically significant.

## Results

No statistical significant difference was found between the bone density at control sides (p=0.071) (Table 1).

Table 1- Comparison of mean bone density at the control sides between the groups

| Normal Group | Mean | p-value |
|---|---|---|
| ATV/ORAL | 1163.60±60.65[AB] | |
| ATV/INJECT | 1435.20±111.67[AB] | |
| DICLO/OAL | 1366.00±283.65[AB] | 0.071 |
| DICLO/INJECT | 1215.00±157.55[AB] | |
| GC /ORAL | 1450.20±253.09[AB] | |
| GC/INJECT | 1161.40±241.40[AB] | |

One- way ANOVA F TEST
Mean values with at least a common letter in superscript ([AB]) were not statistically different (Post-Hoc Turkey HSD test)
The mean difference is significant at the 0.05 level.

There was statistically significant difference between mean bone density at intervention sides of the groups when their values of control sides were controlled. (P=0.383)
There was not statistical significant difference between the alveolar bone density of the oral and injectable atorvastatin groups. (P=0.615)
There was a statistically significant difference between the alveolar bone density of the oral atorvastatin group as compared with the oral control group (P=0.31) (Table 2).
Although the value of the oral Diclofenac group was 1,011.51 HU, and the value of the oral control group was

909.42 HU, this difference was not statistically significant (P=0.505) (Table 2).
Also no statistically significant changes in the alveolar bone density were found when comparing the injectable control group with the injectable Diclofenac group (P=0.075) (Table 2). The mean alveolar bone density of the oral Diclofenac group was 797.91HU which was higher than the mean alveolar bone density of the injectable control group 759.76 HU.
There was a statistically significant difference between the alveolar bone density of the oral atorvastatin group and the oral Diclofenac. (P=0.006)
The difference between the alveolar bone density of the injectable atorvastatin group and the injectable Diclofenac did not reach statistical significance (P=0.228) (Table 2). The mean alveolar bone density of the injectable Diclofenac group had a value of 797.91HU which was more than the value of the injectable atorvastatin which was 601.59HU.
There was statistically significant difference between the alveolar bone density of the injectable atorvastatin and the oral Diclofenac (P=0.012) (Table 2).

Table 2- Comparison of mean bone density at the ligature-induced periodontits sides between groups

| Diseased group | Mean±SD | Adjusted mean | p-value |
|---|---|---|---|
| Oral Atorvastatin | 458.80±247.11 | 515.19[A] | |
| Injected Atorvastatin | 656.00±76.94 | 601.59[A] | |
| Oral Diclofenac | 1043.00±319.48 | 1011.51[B] | 0.038 |
| Injected Diclofenac | 779.40±250.56 | 797.91[AB] | |
| Oral Control group | 968.80±322.46 | 909.428[B] | |
| Injected Control group | 686.400±103.74 | 756.76[AB] | |

ANCOVA F TEST
Mean values with at least a common letter in superscript (AB ) were not statistically different (post – hoc sidac test)
The mean difference is significant at the 0.05 level

## Discussion

Periodontitis is related to several inflammatory mediators, which contribute not only to bone homeostasis, but also to tissue destruction. Considering that local bone loss is an immunoinflammatory exacerbated reaction and localized osteoclastogenesis combination, ligature-induced alveolar bone loss occurs due to abnormal activation of host immunological system with consequent uncontrolled inflammatory response.[16] In order to evaluate periodontal condition several assays have used conventional radiographic images, due to the obvious bone alterations seen However, when bone anabolic events are still subtle, conventional biochemical and radiographic markers are not always sensitive enough to reveal such changes. This way, images of Hounsfield units present an addition advantage, as they demonstrate capacity to reveal larger number of early sites with bone loss.[19] In this study, Ligatures were placed around the left second maxillary molar to induce periodontitis for 10 days. Administration of 12.5 mg/kg of oral atorvastatin (group 1), 0.25 mg/kg of injectable atorvastatin (group 2), 7.5mg/kg of oral Diclofenac (group 3), 6.25mg/kg of injectable Diclofenac (group 4), the oral

solvent without medicine as oral control (groups 5), and the injectable solvent without medicine as injectable control (group 6). In each group, the right side of maxilla was considered as control group a t eleven day, the rats were sacrificed and the maxillary bone was separated from the soft tissue. The prepared samples were then radiological evaluated to determine the bone density with CT. The results of the present study show that both Diclofenac and atorvastatin are effective in preventing bone density loss. Nevertheless, Diclofenac is more effective both orally and injectable as compared with atorvastatin in the prevention of bone density loss in rats with ligature-induced periodontitis.

Numerous studies have been performed on the effects of atorvastatin on bone formation and its resorption with different results.

Chang et al. (2011) performed a study by DEXA method they stated that atorvastatin did not have any effect on the bone density even at high doses.[14] The results of this study have been consistent with the present study results, making in that way questionable the effectiveness of atorvastatin on the bone density.

Other studies have concluded that periodontal disease causes an increase in lipid peroxidation.[19] In a study conducted by de Araújo et al. (2013) it was shown that oral atorvastatin at doses of 10 mg/kg in ligature-induced periodontitis model in rats reduced the lipid peroxidation, which ultimately led to a reduction on inflammation and bone loss.[5] In another study performed by Bali et al. (2014) on 100 rats, it was compared for the first time the effect of local versus systemic administration of atorvastatin. They reported a positive effect of both administration paths on the periodontium by histomorphometry and imonohistochemistry tests.[11]

Goes et al. (2010) performed a study to assess the effect of atorvastatin on bone density with the help of digital radiography. They concluded that atorvastatin can prevent bone resorption by reducing pro- inflammatory cytokines such as I L-8, IL-6, IL-12 and TNF-α.[12]

The results of the aforementioned studies show different findings as compared to the present study. The reasons for this differences could be related to the use of oral and injectable drug doses, as well as the examination method used to determine the bone changes in the present study. The doses used of atorvastatin have been higher than the ones used in the previous studies. The drug dose selection is based on previous studies, showing an effectiveness in reduction of inflammation.[17]

This study also differs in the examination method of the bone changes. HU scale which is considered to be the standard densitometry method of assessing bone density was used in this study, which differs from the previous studies.

HU scale assessment is one of the most accurate para-clinical methods to evaluate the bone density, and it is used in numerous studies.

Turkyilmaz et al. (2007) performed a study to assess the bone density in areas scheduled for dental implants. They analyzed their results with a standardized HU scale.[20] In another study conducted by Aksoy et al. (2009), it was stated that assessment with HUs is a suitable method to determine the bone quality to predict the implant stability.[21] Diclofenac is a NSAID which has been suggested in several studies to inhibit the bone resorption Additionally, evidence has concluded that the use of NSAIDs, such as Diclofenac can reduce the bone loss.[14, 15, 22]

Kurunj Kumarun et al. (2012) showed that Diclofenac sodium even at low concentration levels diminishes the number of osteoclasts probably by inhibiting the secretion of prostaglandins and in that way reducing the orthodontic tooth movement as a result of reducing the bone loss.[14] Ghalyani et al. (2014) performed a study to assess the effect of Diclofenac and celecoxib on the osteoclastogenesis during the healing process of the alveolar bone after tooth extraction. They concluded that both Diclofenac and celecoxib reduced osteoclast production by decreasing expressions ratio of RANKL / OPG genes, and resulting in the decrease of the bone loss after tooth extraction.[16]

In the present study, oral Diclofenac has been more effective in preventing bone density reduction as compared to the injectable Diclofenac in the ligature-induced periodontitis rat model. One of the reasons that oral Diclofenac performed better than its injectable form might be related to the trauma caused after the drug injection in the periodontal tissues. This might exacerbate the inflammation and destruction of the periodontium.

In this study, Diclofenac was more effective administered both orally or as an injection when compared to atorvastatin in the prevention of bone density loss in periodontal disease. The role of inflammation in the development of chronic periodontitis has been recognized.[3] Diclofenac is a potent anti-inflammatory drug due to its relation with the cyclooxygenase pathway and blocking both COX1 and COX2 metabolites.[8] As a result, it can be more effective in minimizing bone density reduction. The balance between the numbers of osteoblasts is the main determinant for bone density. Diclofenac can reduce the production of osteoclasts by decreasing expressions ratio of RANKL / OPG genes. As a result, it can also be more effective on the decrease of the bone density.[16]

## Conclusion

Finally, according to the present study, Diclofenac has been shown to be more effective at both oral and injectable administrations as compared with atorvastatin in the prevention of loss of bone density in a rat model with periodontitis. Hence, according to the present study that for the first time has been conducted using Hounsfield units (HU) that is the method of standard Para-clinic densitometry that is performable in all the bones in the body as well as for the first time, it has been compared the effects of these drugs on the bone density with each other, it

is required more studies in this field.,

## Acknowledgement

## Conflict of Interests

None Declared ∎

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