**UNIVERSIDAD DE OVIEDO**
**MÁSTER UNIVERSITARIO DE ORTODONCIA Y ORTOPEDIA DENTOFACIAL**

# PHARMACOLOGICAL MANAGEMENT OF ANCHORAGE IN ORTHODONTICS BASED ON ZOLEDRONATE AND OSTEOPROTEGERIN

**Felipe J. Fernández González**

**Trabajo Fin de Máster**
**22-01-14**

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**Trabajo Fin de Máster**

**Felipe J. Fernández González**

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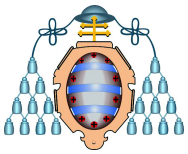
**Tutor**



**Juan M. Cobo Plana**, Catedrático de Ortodoncia adscrito al Departamento de Cirugía y Especialidades Médico Quirúrgicas de la Universidad de Oviedo

**CERTIFICO**:

Que el trabajo titulado "**Pharmacological management of anchorage in orthodontics based on zoledronate and osteoprotegerin**" presentado por **D. Felipe J. Fernández González** ha sido realizado bajo mi dirección y cumple los requisitos para ser presentado como Trabajo de Fin de Máster en Ortodoncia y Ortopedia Dento-Facial.

En Oviedo a 29 de Abril de 2014

*ÍNDICE*

# 1. INTRODUCCIÓN

El movimiento ortodóncico se basa en la reabsorción/aposición ósea que ocurre tras la aplicación de fuerzas mecánicas sobre los dientes. Por este motivo, cualquier fármaco que actúe sobre la función de los osteoclastos puede tener repercusiones en la efectividad del tratamiento ortodóncico[1].

Diversos modelos experimentales muestran los efectos de diferentes tipos de fármacos que modifican el ciclo de reabsorción ósea influyendo, por tanto, en el movimiento ortodóncico[2-5]. Fármacos como los bisfosfonatos (BF) o la osteoprotegerina (OPG) disminuyen la reabsorción ósea mediante la inhibición de la actividad osteoclástica. Éstos son comúnmente utilizados en el tratamiento de enfermedades óseas como la osteoporosis, enfermedad de Paget y las metástasis óseas[6,7].

Los BP están estructuralmente relacionados con el pirofosfato, formado por una estructura fosforo-carbon-fosforo característica, que es esencial para su unión a la hidroxiapatita. Actúan en áreas de gran actividad ósea y son incorporadas rápidamente a los osteoclastos involucrados en la reabsorción ósea. Se distinguen dos tipos de BF en cuanto al tipo de cadenas laterales unidas al carbón central: los BF amino (Alendronato, Acido Zoledrónico; Pamidronato, Ibandronato) y BF no amino (Etidronato, Tilodronato). Los BF tipo amino son de 10 a 10000 veces más potentes que los tipo no amino[8,9] . Su acción interrumpe la función citoesquelética y las comunicaciones intracelulares, lo que produce una disminución de la actividad osteoclástica y eventualmente la apoptosis de los osteoclastos. Por otro lado, los PF tipo no amino, tienen una menor potencia e inhiben la función osteoclástica por medio de metabolitos de adenosin trifosfato tóxico[10].

El movimiento de los dientes de anclaje es un efecto indeseable en ortodoncia, ya que se aumenta el tiempo de tratamiento obteniendo unos resultados clínicos menos predecibles. Los métodos tradicionales utilizados para conseguir anclaje

incluyen la utilización de aparatología extraoral, aparatología intraoral y elásticos. Sin embargo, todos ellos son dependientes de la colaboración del paciente[4].

Hoy en día, los microimplantes son el sistema de elección, ya que proveen de anclaje absoluto, son pequeños y relativamente fáciles de colocar. Sin embargo, estas unidades de anclaje temporal requieren de un procedimiento quirúrgico invasivo y traumático, añaden costes al tratamiento, requieren de una segunda cirugía para su retirada[6,11], tiene una elevada tasa variable de éxito y no siempre se mantienen estables durante el tratamiento[12,13].

Debido a los efectos de los BF sobre la pérdida ósea, se han desarrollado múltiples investigaciones en el campo de la odontología. Los BF se han utilizado en el tratamiento de la periodontitis en ratas[14,15] , para mejorar la osteointegración de los implantes en un modelo en conejos[16], para aumentar la fijación y el volumen óseo alrededor de implantes ortopédicos[17], y para disminuir la pérdida ósea en ratas tras extracciones dentales[4].

En el campo de la ortodoncia, los BF han sido utilizados en la prevención de recidivas posteriores a tratamientos de ortodoncia en ratas[18,19]. También han demostrados disminuir la reabsorción radicular tras el movimiento dentario[20] y prevenir la pérdida de anclaje en ortodoncia[3-6].

Recientes avances en investigación sugieren que los moduladores biológicos que inhiben la actividad osteoclástica, podrían ser utilizados para solventar dichos problemas y proveer de nuevas estrategias en el tratamiento ortodóncico. Varios de estos inhibidores han sido analizados, incluyendo los BF y la OPG, una proteína soluble que inhibe las interacciones receptor-activador del factor nuclear k-B ligand (RANKL), e interfiere en la diferenciación y activación osteoclástica[21,22].

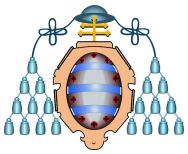
Ya que la OPG ha demostrado ser un factor clave en la inhibición de la diferenciación y activación osteoclastica, y está involucrada en la modulación mecánica de la remodelación ósea, su administración local adyacente a los dientes de anclaje podría dar lugar a una estrategia farmacológica novedosa en la prevención del movimiento dental innecesario, lo cual es muy deseable para el refuerzo del anclaje ortodóncico[23].

Si el movimiento indeseado puede ser prevenido mediante inhibidores de la reabsorción ósea, el tratamiento ortodóncico podría resultar menos complicado y más seguro.

El objetivo de este estudio ha sido evaluar y comparar en un modelo de experimentación en ratas, el efecto de la aplicación local de la OPG-Fc y el BF Zoledronato sobre el anclaje ortodóncico.

A continuación se presenta el artículo publicado y posteriormente una serie de consideraciones sobre las repercusiones que los resultados obtenidos en este estudio pueden tener en el ámbito ortodóncico.

# 2.ARTÍCULO



Elsevier Editorial System(tm) for Bone
Manuscript Draft

Manuscript Number:

Title: PHARMACOLOGICAL MANAGEMENT OF ANCHORAGE IN ORTHODONTI
ZOLEDRONATE AND OSTEOPROTEGERIN

Article Type: Original Full Length Article

Keywords: osteoprotegerin; zoledronate; RANKL inhibitor; tooth movement; o

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Abstract: ABSTRACT
Introduction - Some drugs commonly used for the treatment of bone diseases
Paget's disease or bone metastases, can alter the bone remodeling cycle thus i
movement. In this context, bisphosphonates (BPs) or osteoprotegerin (OPG-fc
inhibiting osteoclastic activity.
Material and Methods - The right first maxillary molars of male Sprague-Dawle
mesially using a calibrated nickel-titanium spring connected to a 6mm length
different drugs were used. A single dose of Zoledronate (16 µg) and a twice-w
recombinant fusion protein (OPG-Fc) (5.0 mg/kg) were injected in mesial and
molars. Tooth movement was measured using stone casts that were scanned a
structural and immunohistochemical studies were carried out to analyze the o
changes in bone and periodontal ligament. We assessed RAKN, Runx, type 1 co
metalloproteinases 2 and 9, S100 protein, vimentin, and the putative mechano
TRPV4.
Results - The 5.0 mg/kg OPG-Fc dose and the 16 µg Zoledronate showed a pote
molar movement. Animals administered a dose of 5.0 mg/kg OPG-Fc demonstr
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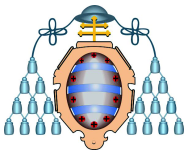
7,14, and 21, respectively ($p<0.001$). Rats receiving 16 µg Zoledronate had a st
52%, 46,2% and 30,3% of the molar movement relative that in control rats at
respectively ($p<0.001$). At days 14 and 21, the 5.0 mg/kg OPG-Fc group showe
($p<0.001$) percent molar movement compared to the Zoledronate group ($p<0.$
Conclusions - Local delivery of OPG-Fc or Zoledronate inhibits tooth movemen
provide maximum anchorage in orthodontics. According to the results of the p
immunohistochemical studies, OPG-Fc was more effective than Zoledronate in
osteoclasts in the orthodontic model of tooth movement analized.



Highlights

- We investigated the effect of OPG-Fc and zoledronate on anchorage.
- We study the effects of these drugs on bone density through micro-CT analysis.
- A locally applied dose of zoledronate provide maximum anchorage.
- A twice high dose a week of OPG-Fc provide maximum anchorage.
- The OPG-Fc proved to be a more effective drug in achieving anchorage.

# Pharmacological management of anchorage in orthodontics based on zoledronate and osteoprotegerin

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**Running title**: Pharmacological anchorage in orthodontics

**Keywords**: osteoprotegerin, zoledronate , RANKL inhibitor, tooth movement, orthodontics.

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## INTRODUCTION

Orthodontic tooth movements are based on the bone remodeling that occurs after the application of mechanical forces, and therefore drugs influencing bone cells biology may affect the effectiveness of orthodontic treatment[1]. Different drugs are able to alter the bone remodeling cycle thus influencing tooth movement, as shown in different experimental models[2-7]. Interestingly, some drugs commonly used for the treatment of bone diseases such as osteoporosis, Paget's disease or bone metastases, like bisphosphonates (BPs) or osteoprotegerin (OPG) limit bone loss by inhibiting osteoclastic activity[8,9]. BPs are structurally related to pyrophosphate, but containing a characteristic phosphorus-carbon-phosphorus structure, which is essential for binding to hydroxyapatite[10,11]. They act in areas of increased bone turnover and are rapidly incorporated into osteoclasts involved in bone loss. Two different classes of BPs can be considered regarding the type of side chains attached to the central carbon: amino BPs (alendronate, zoledronic acid, pamidronate, ibandronate) and non-amino BPs (etidronate, tilodronate). The amino BPs are 10 to 10000 times more potent than the non-amino BPs inhibiting bone resorption processes. Its action disrupts cytoskeletal function and intracellular signaling, which leads to impaired osteolytic activity and eventually to osteoclasts apoptosis[12]. Because the effects of BPs on the bone loss, many researches have been developed in the field of dentistry. BPs have been used to treat periodontitis in rats[13,14], for improving osseointegration of the implants in a rabbit model[15], improved fixation and bone volume surrounding orthopedic screws[16] and to decrease bone loss in rats following tooth extraction[6]. In the field of orthodontics, BPs have been used to prevent post orthodontic treatment recurrences in rats[17,18]. BPs has also been

shown to decrease the root resorption after tooth movement[19] and prevent loss of anchorage in orthodontics[2,5-7,9]

Recent research advances suggest that biological modulators which inhibit osteoclasts could be used to address these problems and provide new adjunctive approaches to orthodontic treatment. Several such inhibitors have been examined, including bisphosphonates and OPG, a soluble protein that inhibits receptor-activator of nuclear factor κ-B ligand (RANKL) interactions with its cognate receptor, and prevents osteoclast differentiation and activation [20,21]. Since OPG is a key factor in the inhibition of osteoclast differentiation and activation, and it is involved in mechano-modulation of bone modeling, its local delivery adjacent to the anchorage teeth may provide a novel pharmacological approach in preventing unneeded tooth movement[22]. If undesirable tooth movement could be prevented with blockers of bone loss treatment could be less complicated and safer.

Here we have investigated the effect of the local application of OPG-Fc and BP zoledronate on orthodontic anchorage in an experimentation rat model, and was aimed to serve as a baseline for future studies in the field of the biopharmacology of tooth movement.

**MATERIAL AND METHODS**

***Animals.***- Male Sprague-Dawley rats with approximate weight 420-460 g (n = 36) were utilized in this study, and were obtained from the Animalarium of the Veterinary´s Faculty, (University of León, Spain). Twelve animals were randomly allocated in each group: experimental group 1 (Zoledronate), experimental group 2 (OPG-Fc) and the untreated control group. The number of animals was pre-established according to the results of a power analysis based on an estimate of effect sizes (f = 0.7 mm) of orthodontic tooth movement reported in previous studies evaluating bisphosphonates and OPG, 12 rats per

group were necessary in order to obtain a power of 95% with an alpha at 5%. The rats were acclimated for 5 days in a plastic shoebox in a room at 25°C with a 12-hour alternating light and dark schedule. They were fed with a powdered rodent chow (Harlan Laboratories, Indianapolis, Ind.) and distilled water *ad libitum*. The animals were evaluated at baseline and every 7 days (days 0, 7, 14 and 21) after appliance placement and they were sacrificed on day 21. The experimental period lasted 21 days based on previous studies on tooth movement in rats[2,6,23-26]. All experimental procedures were approved by the Animal Welfare Committee, University of León, Spain.

***Orthodontics treatment***.- Before baseline procedures, animals were weighed and deeply anesthetized with an IP injection of Ketamine (35mg/kg) and Medetomidine (0,25mg/kg). A 6mm length mini-screw (Tomas®-pin Ø 1,6mm, Dentaurum, Ispringen, Germany.) was placed on the anterior region of the maxilla, between the roots of the upper incisors. After that, small retention grooves were prepared along the distal-lingual line angles of the right first maxillary molar with a high-speed hand-piece and a carbide bur, so as to prevent the ligatures from loosening from the teeth due to their lingual curvature. Then a 0,010-in steel ligature was passed through the lumen of a super elastic closed coil nickel-titanium spring (Tomas®-coil spring, Dentaurum, 0.036-in lumen, 8 mm) at approximately the third coil to ensure optimal strength and then fixed around the right first maxillary molar, passing between the mesial contact of the second molar and twisted five times, to ensure the security of the appliance. The coil was activated by being connected to an anterior mini-screw. Coils´ force was measured with a push and pull gauge (YS-31D, YDM corporation Yamaura, Japan), applying a force around 50 g, maintaining the level of force between 40-60 g as recommended in previous studies for rat models[1,20,27,28]. It was a used a super elastic

nickel-titanium coil to guarantee the regular force along the experiment (Figs. 1a,d,g). After tied and cut the ligature, right first molar surface was etched using 37% ortophosphoric acid for 30 seconds and rinsed with water for 15. The tooth was dried with an air syringe and a thin coat of 3M Transbond XT primer (3M Unitek, Monrovia, CA, USA) was applied over the ligature and then, light cured in order to avoid its displacement and pulpar damage due to dentine exposure.

Following Ortega et al.[6] (2012) findings, a 50-µl solution of phosphate-buffered saline (PBS), containing 16 µg of zoledronate was injected in the experimental group 1. In the experimental group 2, PBS with 5mg/kg of human OPG-Fc (AMGEN, Inc., Thousand Oaks, CA USA) was delivered and an equal volume of PBS vehicle was injected into the control rats. All the injections were delivered into the palatal mucosa corresponding to the mesial and distal surface of the maxillary right first molar and the vestibule above the first molar, by using a 3/10mL syringe (BD Ultra-Fine™ 31G x 8mm). Because of the possible systemic effects of the drugs, no treatment was performed on the contralateral side.

At the 7, 14 and 21-day time-points, the animals were anesthetized as on the day of baseline procedures. Impressions of the diastema distal to the right maxillary first molar were made using a polyvinylsiloxane (Imprint II, 3M ESPE Dental Products, St. Paul, MN) impression material waiting the necessary time to allow the material polymerize, the impression was evaluated to be sure that the diastema was captured. The impressions were poured using an improved die stone (ade Stone, Whip Mix Corp., Louisville, KY). The stone models were separated from the impressions after 8 hours. The occlusal tooth surfaces were scanned (Epson Expression 1680, Epson America, Inc., Long Beach, CA) with a 100 mm ruler and then magnified 100x and diastema was measured using an imaging software (Adobe Photoshop

7.0.1, Adobe Systems, Inc., San Jose, CA).

At the end of the experiment, animals were deeply anesthetized as previously described and were sacrificed by decapitation. The jaws were dissected in order to have only the maxillae. Then photographs were taken.

***Microcomputed tomography***.- Two animals from both the control and experimental groups were randomly chosen for microcomputed tomography (micro-CT) and histologic analyses. The appliance was removed, and a section between mesial aspects of the first molar to the distal aspect of the third molar was excised with a thin diamond table saw. The 6 samples measured 12 x 5 x 5 mm, and were scanned with a micro-CT (Skyscan 1174, Kontich, Belgium), to quantify alveolar bone in the proximity of the first molar roots as previously described[24,30.] The furcation area and root apex was selected as they provide reproducible, morphological landmarks (Figs. 1b-c-, 1e-f-nad figs 1h-i).

***Histology and immunohistochemistry***.- The same 6 samples used for micro-CT were then decalcified in 0.5 mol/ L of EDTA in a microwave (24° -27°C) for 3 weeks. After decalcification, the samples were dehydrated, embedded in paraffin, and sectioned by using conventional methods at a thickness of 8 µm. The sections ere mounted on gelatin-coated glasses, deparaffinised, rehydrated and stained with Harris hematoxylin and eosin. For immunohistochemistry, deparaffinized and rehydrated sections were processed for imunohistochemistry using the EnVision antibody complex detection kit (Dako, Copenhagen, Denmark), following supplier's instructions. Briefly, in rehydrated sections the non-specific binding was blocked with 1% bovine serum albumin for 20 min. Sections were then incubated overnight at 4°C with primary antibodies whose characteristics are described in table 1. Subsequently, sections were incubated with the anti-rabbit EnVision system-labelled

polymer (Dako-Cytomation) for 30 min, washed in buffer solution, and treated with peroxidase blocking buffer (Dako-Cytomation, Copenhagen, Denmark). Finally, the slides were washed with buffer solution and the immunoreaction was visualized with diaminobenzidine as a chromogen. To ascertain structural details sections were counterstained with Mayer's haematoxylin, dehydrated, and mounted with Entellan® (Merk, Dramstadt, Germany).

***Statistical analysis***.- Descriptive statistics (mean, standard error) for each parameter were calculated for all groups. Each animal was used as the experimental unit. Comparisons of variables by groups were made using non-parametric Krukal-Wallis for non-normal variables and analysis of variance (ANOVA) for those variables that presented normality according to the results of the Anderson-Darling test.

**RESULTS**

Regarding the animal status, there were no significant differences in weight gain among the groups. Appliance placement and injections did not appear to impair the animals ability to thrive. The mean + SD starting weight of all animals at baseline was 440,45 ± 5,37 g. At 21 days, the animals weighed 495.54 ± 15,16 g, 492,88 ± 10,95 g and 496,23 ± 12,56 g in the vehicle treated, 16 µg Zoledronate treated and 5.0 mg/kg OPG-Fc treated groups, respectively. The appliance success rate was 97,22% over the 3-week experimental period as only one appliance broke and was immediately replaced.

Local delivery of OPG-Fc and Zoledronate resulted in a substantial decrease in mesial movement of the first molar tooth when compared to PBS control animals (Fig. 2). At day 7, there was a significant decrease in molar movement in both the 16 µg Zoledronate group

(0.13 ± 0,01 mm, $p < 0.01$) and 5.0 mg/kg OPG-Fc group (0.13 ± 0.01 mm, $p < 0.01$) when compared to controls (0.25 ± 0,03 mm). However, at this time-point there was no significant difference in amount of molar movement between groups receiving the two different drugs. By day 14, not only was there a significant decrease in mesial molar movement in the zoledronate group (0.25 ± 0,01 mm, $p < 0.001$) and OPG-Fc group (0.17 ± 0.01 mm, $p <0$ .001) compared to controls (0.54±0.01 mm) but there was also a significant difference between the experimental groups ($p < 0.001$). Both the zoledronate group (0,30 ± 0,01 mm) as the OPG group (0,21 ± 0,01 ($p< 0.001$) showed a significant decrease in molar movement at day 21 compared to controls (0.99 ± 0,03).

Using the data reported to determine percentages of tooth movement, it was noted that rats receiving a dose of 5.0 mg/kg OPG-Fc demonstrated only 52%, 31,4%, and 21,22% of the total mesial molar movement compared to that observed in control rats at days 7, 14, and 21, respectively. These differences were all statistically significant. Rats receiving 16 µg Zoledronate had a statistically significant 52%, 46,2% and 30,3% of the molar movement relative that in control rats at days 7 and 14 and 21 respectively. At days 14 and 21, the 5.0 mg/kg OPG-Fc group showed significantly less ($p < 0.001$) percent molar movement compared to the Zoledronate group.

The structural changes induced by orthodontic movement in the differential experimental conditions were then analyzed focusing attention on the epithelium of the diastema, the bone subjacent to diastema and the periodontal ligament (compression and tension zones). In the epithelium there were string inflammatory signs in vehicle treated animals as well as large bone spaces (Figs. 3a and d), i.e. bone remodeling compartment (BRC). In relation to the periodontal ligament, osteoclasts were found in bony lacunae of the compression zone

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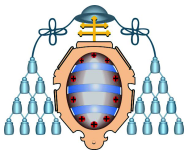
whereas they were absent from the tension zone[31]. In the experimental groups the inflammatory signs in the diastema epithelium were reduced after Zoledronate treatment (Figs. 3b and e) and showed a normal structure in the OPG treated animals (Figs. 3c and f) although in this groups large vascular spaces were observed. Interestingly, the density of BRC progressively was reduced in Zoledronate (Fig. 3g) and OPG (Fig. 3h) treated animals. Regarding the periodontal ligament, the number of bone lacunae and ostoclasts present in the bone of the compression zone were reduced in Zoledronate treated animals and more clearly in the OPG treated group (data not shown).

To identify bony cells immunohistochemistry for RANK and Runx was performed. Both RANK and Runx positive cells were detected in the lacunae showing an intense immunostaining. The positive cells were irregular and were attached to the walls of the bony cavities. Conversely RANK or Runx positive cells were rarely found in the compression zone of the periodontal ligament or in the BRC (Fig. 4). In any case, the number of RANK and Runx immunoreactive cells was reduced in Zoledronate treated animals and still more in OPG treated animals (Fig. 4). Interestingly, The mature superficial osteoclasts of the compression zone were identified by theirs expression of vimentin (see Harre et al., 2012), and followed an progressive reduction in number in Zoledronate and OPG treated animals respectively (Fig. 5).

Another marker of osteolitic activity investigated was the changes of MMPs 2 and 9 in the experimental conditions we created. MMP9 was primarily detected in cells placed in the bony lacunae (Figs. 6a-c) and in the periodontal ligament (data not shown). The morphology of these MMP9 positive cells resembled that of the RANK positive ones but their density was lower. Moreover the density of MMP9 positive cells was found reduced in Zoledronate



treated animals and were still more infrequent in OPG treated ones (Fig. 5) with respect to the controls. MMP2 was primarily detected in the periodontal ligament (Figs. 5d-f) and no differences in the pattern or intensity of immunostaining were observed among experimental groups. The only remarkable finding was the occurrence of this protease in superficial bony cells, presumably osteoclasts, in the vehicle treated animals which were not detected in the other two groups.

Fibroblasts are the predominant cell type in the periodontal ligament, and type I collagen is the predominant in the extracellular matrix of this structure. Vimentin, used as a marker of fibroblasts, showed a large distribution in the periodontal ligament. In the vehicle treated animals all most all cells displayed vimentin immunoreactivity (Figs. 7a,d,g) being more intense in zones of ligament-bone attachment (Fig. 7g). With treatment of Zoledonate (Figs. 7b,e,h) the number of cells vimentin positive decreased, and in the OPG treated animals vimentin was restricted to some zones of the periodontal ligament, especially in the vicinity of the bone (Figs. 7c,f,i). Regarding the expression of type I collagen no changes were observed among the three experimental groups (Figs. 7j-l).

Finally, we investigated whether or not Zoledronate and OPG treatment alters the expression of TRPV4 and ASIC2, two putative mechanoproteins (see del Valle et al., 2012). TRPV4 was detected in the periodontal ligament, bony lacunae and cells of the boy surface identifies as osteoclats (Fgs. 8a,d). As a rule the expression of TRPV4 remains unchanged in Zoledronate treated animals (Figs. 8b,e) and was reduced in the OPG treated ones (Figs. 8c,f). On the other hand, ASIC2 immunostaining was present in the periodontal ligament and osteoclast, and the treatments reduced its expression in the osteoclasts whereas remained unchanged in the ligament (Figs. 8g-l).

As a summary, and based on the results of the present structural and immunohistochemical studies, OPG was more effective than Zoledronate in blocking the action of osteoclasts in the orthodontic model of tooth movement we created. In these effects are involved MMPs and mechanoproteins. Furthermore, the treatment also alters the biology of the fibroblast in periodontal ligament.

**DISCUSSION**

The movement of anchored teeth is an undesired complication in orthodontics that results in prolonged treatment time and not optimal clinical outcomes. Traditional methods for achieving anchorage include headgears, intraoral appliances and elastics, but the results obtained are dependent on the patient's compliance[6]. Nowadays miniscrew implants are the preferred system because they provide maximum anchorage, are small and relatively easy to place[32]. However, this temporary anchorage units require an invasive and traumatic surgical procedure, add costs to the orthodontic treatment, require a second surgery to remove the implants[9,33] have highly variable success rates, and do not always remain stable during the treatment [34,35].

In the last decade several researches have been developed trying to get an alternative strategy for maintaining anchorage by means of acting directly on the bone resorption, and thus prevent undesirable tooth movement avoiding the drawbacks associated to the traditional anchorage methods[2-6]. As the tooth movement is a result of the interaction between bone apposition (osteoblast) and bone resorption (osteoclast), interfering in the osteoclast function and therefore in the ostelysis, should reduce these orthodontic movement or even avoid it. For that purpose, a variety of bioactive molecules have been

studied and related to decreased tooth movement. Most of these studies have been focused on the influence of different bisphosphonates on orthodontic movement, obtaining a significant decrease of bone resorption through the inhibition of osteoclasts activity. However, due to the long permanency of these agents in the bone, their irreversible effects and specially the possible osteonecrosis associated[20], its use for this purpose is still under debate.

Otherwise, recent studies have shown significant results in the use of OPG-Fc as pharmacological anchorage agent. Considering that RANKL to RANK binding is critical for osteoclastogenesis, acting at this stage by the use of a competitive inhibitor such as OPG, would hinder osteoclast formation, activation and survival[36]. Moreover, OPG has shown to be more effective as pharmacological anchorage agent than bisphosphonates, showing a higher potency even when they are administrated at high doses[8]. This could be explained by the fact that bisphosphonates require to be first incorporated into the bone in order to inhibit osteoclast activity efficiently[37].

The concentration of OPG used in the present study is according to the results obtained by Dunn et al in 2007. In their experimental rat model they found a significant greater decreased tooth movement (78,7%) with a 5.0mg/kg twice weekly local injection in comparison with that obtained with a lower concentration of 0,5mg/kg, although there was still a significative reduction ($p<0.05$). Nevertheless is important to remember that in order to overcome the animal´s immune response to foreign human OPG-Fc, the dosage used in rats is higher and more frequent than would likely be used in humans[20].

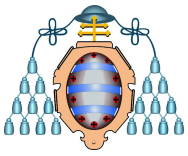
Regarding the Zoledronic effect on tooth movement, Ortega et al.[6] found similar results in a study also made in a rat model. They concluded that a single low dose application of the

bisphosphonate Zolendronate, which was 16 µg as used in the present study, provides maximum anchorage by preventing bone resorption. Moreover, they found that Zolendronate also prevented severe periodontal bone loss at the extraction site and around the second and third molars. As in our study, none of their rats developed osteonecrosis.

Another interesting result found at the present study is that the major total molar movement in all groups occurred within the first 7 days. Dunn et al[20] in their study also found the most important tooth movement at first time check, which in their case was done at 3th day. This initial movement is associated with the physical compression of the viscoelastic periodontal ligament, and no tissue remodeling is still noticed. At the 3 control stages of this study, there were statistically significant differences between the 3 groups regarding the molar movement, being in all cases higher in the control group. The comparison of the experimental groups showed that OPG significantly decrease molar movement in a major extent than Zoledronic.

The study of Kaipatur et al[28] highlighted the long durability of the effect of BP on bone, specifically alendronate. They referred the impact of BP bone burden in retarding dental movement of 77% and 86% in the 4-week and 8-week controls respectively, significantly greater to those related to concurrent dosage of BP with orthodontic treatment where the reduction of movement was 56% and 65% in the two time controls. It is important to bear in mind this characteristic of BP, as the difficulty to easily reverse their effect may become a complication when these agents are used as pharmacological anchorage system.

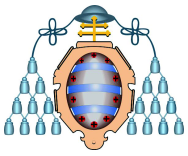
On the other hand, OPG has as short-term persistence in bone, which means an advantage when comparing to the long lasting effects of bisphosphonates. Accordingly, a human study has showed that a single subcutaneous dose (3.0mg/kg) of OPG-Fc has a half-life of 6-7 days



remaining effective for at least 30 days. Moreover, the same single subcutaneous dose of a specific fully human monoclonal antibody to RANKL based on OPG (AMG 162) has demonstrated an 81% of suppression in bone turn over after six months of its administration[38]. Nevertheless, this human antibody cannot be analyzed in a rat model for directly comparing its efficacy to humans. Therefore, further investigation should be done to achieve a better understanding of these human monoclonal antibodies.

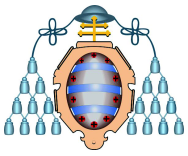
The results of this study indicate that inhibition of osteoclastogenesis and osteoclast activity by local delivery of recombinant OPG protein could serve as a rational pharmacological approach for achieving orthodontic anchorage, avoiding the need of patients cooperation and discomfort of traditional anchorage methods and invasive and traumatic surgical procedures and variability of success rates of the actual preferred anchorage system, the mini screws. In addition, its use over bisphosphonates provides a more effective and predictive action.

How mechanical forces stimulate bone remodeling remains a mystery but some key facts are known. First, intermittent forces stimulate more bone remodeling than continuous forces. It is likely that during orthodontic tooth movement intermittent forces are generated because of 'jiggling' effects as teeth come into occlusal contact. Second, the key regulatory cell in bone metabolism is the osteoblast. It is therefore relevant to examine what effects mechanical forces have on these cells. The application of a force to a cell membrane, triggers off a number of responses inside the cell and this is usually mediated by second messengers. It is known that cyclic AMP, inositol phosphates and intracellular calcium are all elevated by mechanical forces. Indeed the entry of calcium to the cell may come from G-protein controlled ion channels or release of calcium from internal cellular stores. These messengers



will evoke a nuclear response, which will either result in production of factors responsible for osteoclast recruitment and activation, or bone forming growth factors. An indirect pathway of activation also exists whereby membrane enzymes (phospholipase A2) make substrate (arachidonic acid) available for the generation of prostaglandins and leukotrienes. These compounds have both been implicated in tooth movement.

The structural changes observed in control animals are in total agreement con previous studies using similar models of tooth movements[31]. Moreover, several cellular and molecular mechanism to explain the functional effects o Z and OPG were analyzed, centering the research in the bone and periodontal ligament. During orthodontic treatment, the early response of periodontal tissues to mechanical stress involves several cellular changes that allow tooth movement. These changes include activation of osteoclasts, release and/or activation of MMPs. Bone remodeling proceeds in a specialized vascular structure (the bone remodeling compartment, BRC). Reduced bone turnover leads to a decrease in the number of BRCs, while increased turnover causes an increase in the number of BRCs. The outer lining of this compartment is made up of flattened cells, displaying all the characteristics of lining cells in bone including expression of OPG and RANKL. The process is regulated by a wide variety of factors including calcitropic hormones (PTH, thyroid hormone, sex steroids etc.). The secretion of regulatory factors inside a confined space separated from the bone marrow would facilitate local regulation of the remodeling process without interference from growth factors secreted by blood cells in the marrow space. The dominant pathway regulating osteoclast recruitment is the RANKL/OPG system, while many different factors (RUNX) are involved in osteoblast differentiation[39]. On the other hand in vivo experiments using transgenic mouse models have demonstrate that also osteocytes are involvement in



osteoclastic bone resorption during orthodontic tooth movement[40]. OPG is used in maintaining anchorage may be the use of biological inhibitors of osteoclastic bone resorption. A recent research on the role of OPG in inhibiting bone resorption and tooth movement, using an orthodontic model in mice in which maxillary molars are moved for prolonged periods by near-constant, clinically relevant forces. Tooth movement paralleled osteoclast numbers. Minimal osteoclast apoptosis was observed, suggesting that recruitment, rather than programmed cell death, is a critical regulatory mechanism under conditions of constant force. In agreement with previous studies by Keles et al[9] osteoclasts were reduced at compression sites by both Zelodronate and OPG treatments, thus suggesting these cells are the targets of both molecules. We have also observed that the used molecules reduce de expression of both MMP9 and MMP2. The moedification of MMPs activity by orthodontic forces has been reported earlier[41] but is modification by molecules used to anchorage the teeth is reported here for the first time.

The periodontal ligament lies between the hard tissues of alveolar bone and cementum of teeth and serves to anchor the tooth to the alveolus and functions as a cushion between these hard tissues in physiological conditions i.e. mastication, and also in orthodontic movements. In this study we have demonstrated that its architecture, cell arrangement and immunohistochemical patter for vimentin, type I collagen and the mechanoproteins TRPV4 and ASIC2 is altered by tooth movement[42] and all these parameters altered by the applied treatments.

As a summary, and based on the results of the present structural and immunohistochemical studies, OPG was more effective than Zoledronate in blocking the action of osteoclasts in the orthodontic model of tooth movement we created. In these effects are involved MMPs and

mechanoproteins. Furthermore, the treatment also alters the biology of the fibroblast in periodontal ligament.

**CONCLUDING REMARKS**

A single, small, locally applied dose of zoledronate or a twice high dose a week of OPG-Fc was enough to provide maximum anchorage after an orthodontic force.

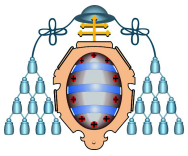
These drugs effectively inhibits mechanically induced osteoclastogenesis resulting in improved bone quantity, orthodontic anchorage, and would likely lead to enhanced treatment efficacy.

According to our results, OPg-Fc proved to be a more effective drug in achieving anchorage in orthodontics, with the advantage of its reversibility in time.

There were no signs of osteonecrosis associated with bisphosphonates of the jaws in any rat.

**Acknowledgements**

The OPG-Fc was kindly donated by (AMGEN, Inc., Thousand Oaks, CA).The mini-screw and coil springs were provided by Dentaurum Company. The authors appreciate Dr. José A. Vega for his histological expertise, Eva Pascual and Vanessa Loredo for their assistance with micro-CT. We gratefully acknowledge Dr. Marta Regueiro for her assistance with all the experiment.



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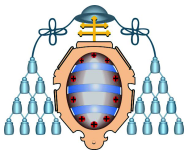

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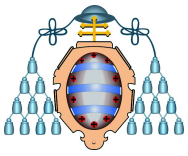

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**Legends for figures**

**Figure 1.-** Intraoral photographs of orthodontic appliances (a,d,g) in place at 21 days, and three-dimensional sagittal and coronal microcomputed tomographes of the same animals. The size of the diastema was reduced in Zoledronate (b,c) and OPG (h,i) treated animals with respect to those receiving the vehicle. Arrows indicate the diastema.

**Figure 2.-** Evolution of the intermolar distance (expressed in mm) throughout the time period observed. The differences between vehicle treated animals, and those treated with Zoledronate and OPG were always significant, whereas between Zoledronate and OPG treated animals were significant at 14 and 21 days.

**Figure 3.-** Structural analysis of the diastema in animals treated with the vehicle alone (a,d), Zoledronate (b,e) and OPG (c,f). The inflammatory signs observed in the control groups were almost absent in the experimental groups. In the bone subject to the diastema the number and size of the bone remodeling compartments was reduced in the experimental groups

especially in animals treated with OPG. Figures a-f: hematoxylin-eosin; figures g-i-: trichromic staining. Scale bar: 300 µm for a-c; 40 µm for (d-f); 80 µm for g-i.

**Figure 4**.- Immunohistochemical detection of RANK (a-f) and Runx (g-i) in vehicle, Zoledronate and OPG treated animals. Scale bar: 15 µm.

**Figure 5.-** Immunohistochemical detection of vimentin in vehicle, Zoledronate and OPG treated animals. Mature osteoclasts were immunolabelled by antibodies against this cytoskeletal intermediate filament. b: bone. Scale bar: 10 µm.

**Figure 6.-** Immunohistochemical detection of MMP9 (a-c) and MMP2 (d-f) in periodontal ligament of vehicle, Zoledronate and OPG treated animals. Scale bar: 15 µm.

**Figure 7.-** Immunohistochemical detection of vimentin (a-i) and type 1 collagen (j-l) in the periodontal ligament of vehicle, Zoledronate and OPG treated animals. Scale bar: 15 µm; 25 µm for b,e,h.

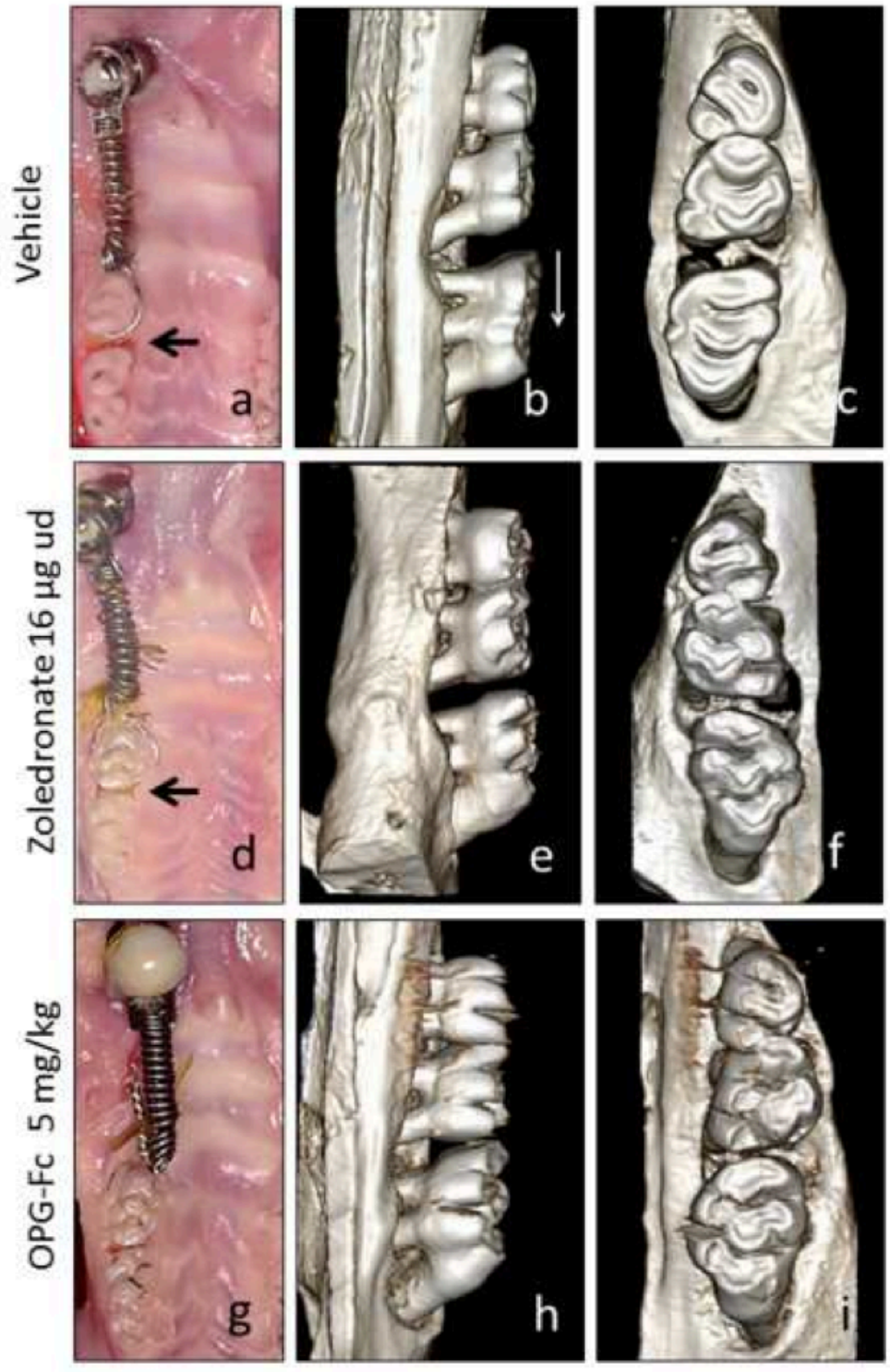
**Figure 8.-** Immunohistochemical detection of TRPV4 (a-f) and ASIC2 (g-l) in the periodontal ligament and cells of vehicle, Zoledronate and OPG treated animals. Scale bar: 25 µm; 10 µm for j-l.

**Table 1.- Primary antibodies used in the study**

| Antigen (clone) | Origin | Dilution | Supplier |
|---|---|---|---|
| ASIC2 | rabbit | 1:200 | Lifespan BioSciences[a] |
| Col 1 | rabbit | 1µg/ml | Chemicon[b] |
| MMP-2 | rabbit | 1:100 | Santa Cruz Biotechnology[c] |
| MMP-9 | rabbit | 1:100 | Santa Cruz Biotechnology[c] |
| RANK | rabbit | 1:100 | Abcamb[d] |
| Runx2 | rabbit | 1:100 | Santa Cruz Biotechnology[c] |
| S100P | rabbit | 1:1500 | Dako[e] |
| TRPV4 | rabbit | 1:200 | Abcamb[d] |
| Vimentin (V-9) | mouse | 1:200 | BioGenex[f] |

ASIC2: acid-sensing ion channel
Col 1: rat type 1 collagen
MMP-2 and MMP-9: matrix metalloproteinases 2 and 9
RANK: Receptor Activator of Nuclear Factor kappa B
RUNX2: Run-related transcription factor 2
S100: S100 protein
TRPV4: transient receptor potential vainilloid 4
VIM: vimentin

[a]Seattle, WA, USA; [b]Temecula, CA, USA; [c]Santa Cruz, CA; USA; [d]Cambridge, UK; [e]Glostrup, Denmark; [f]San Ramon, CA, USA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Zoledronate 16 µg ud
OPG-Fc 5 mg/kg
a
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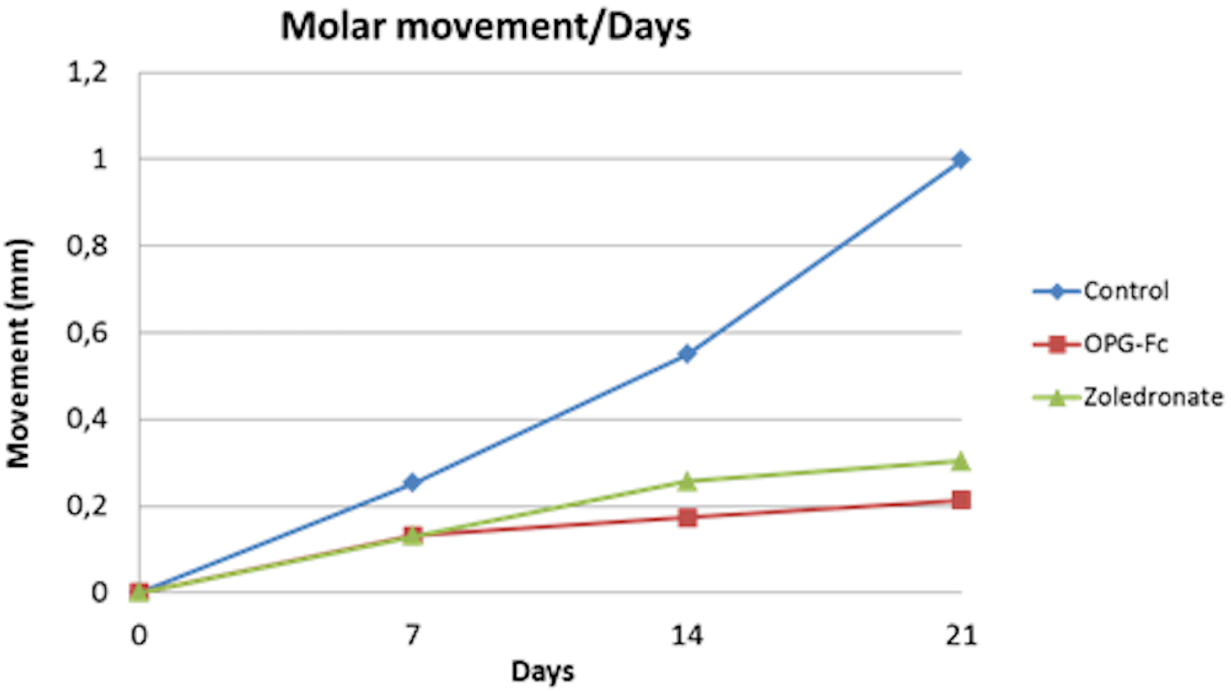
Molar movement/Days
Movement (mm)
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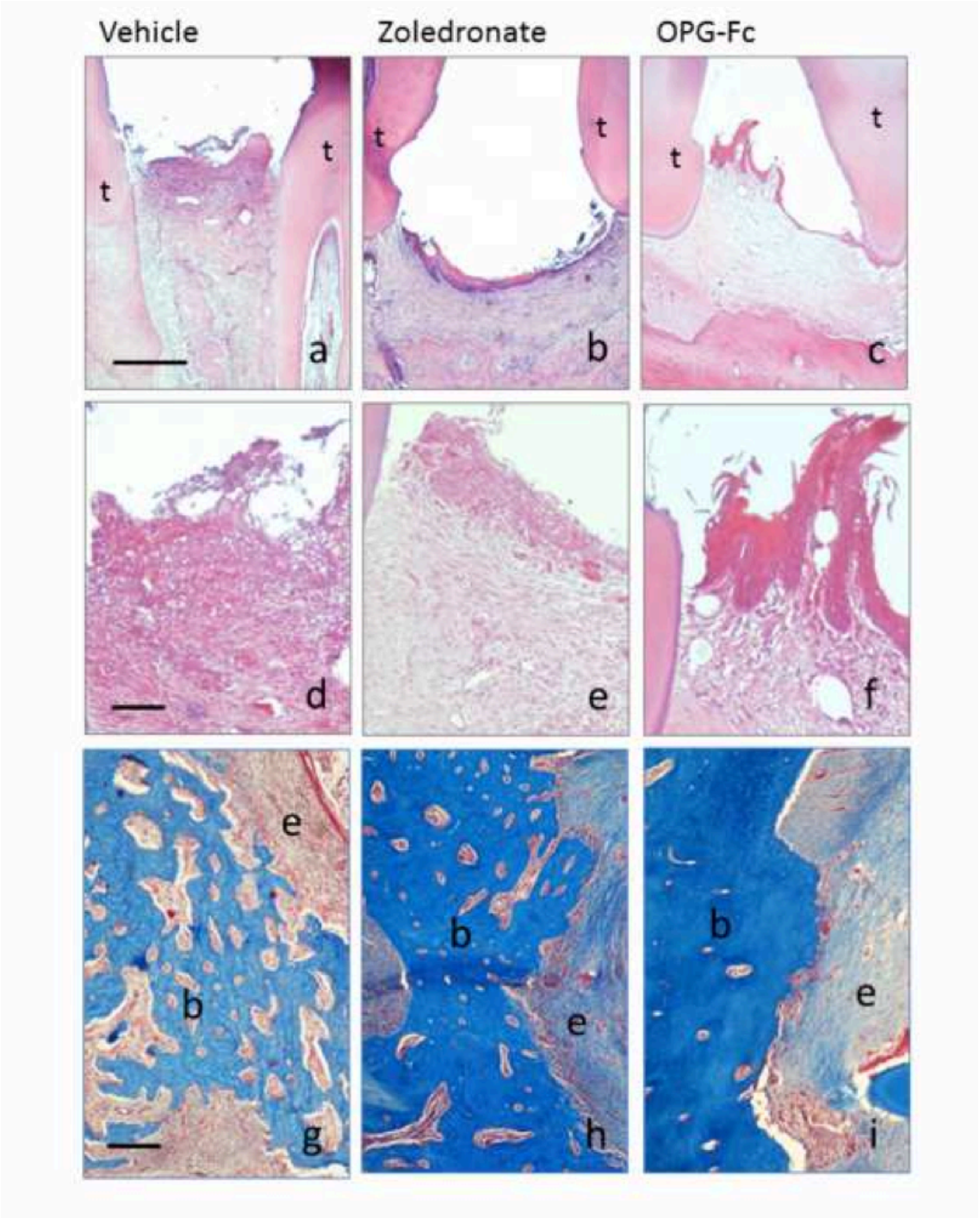
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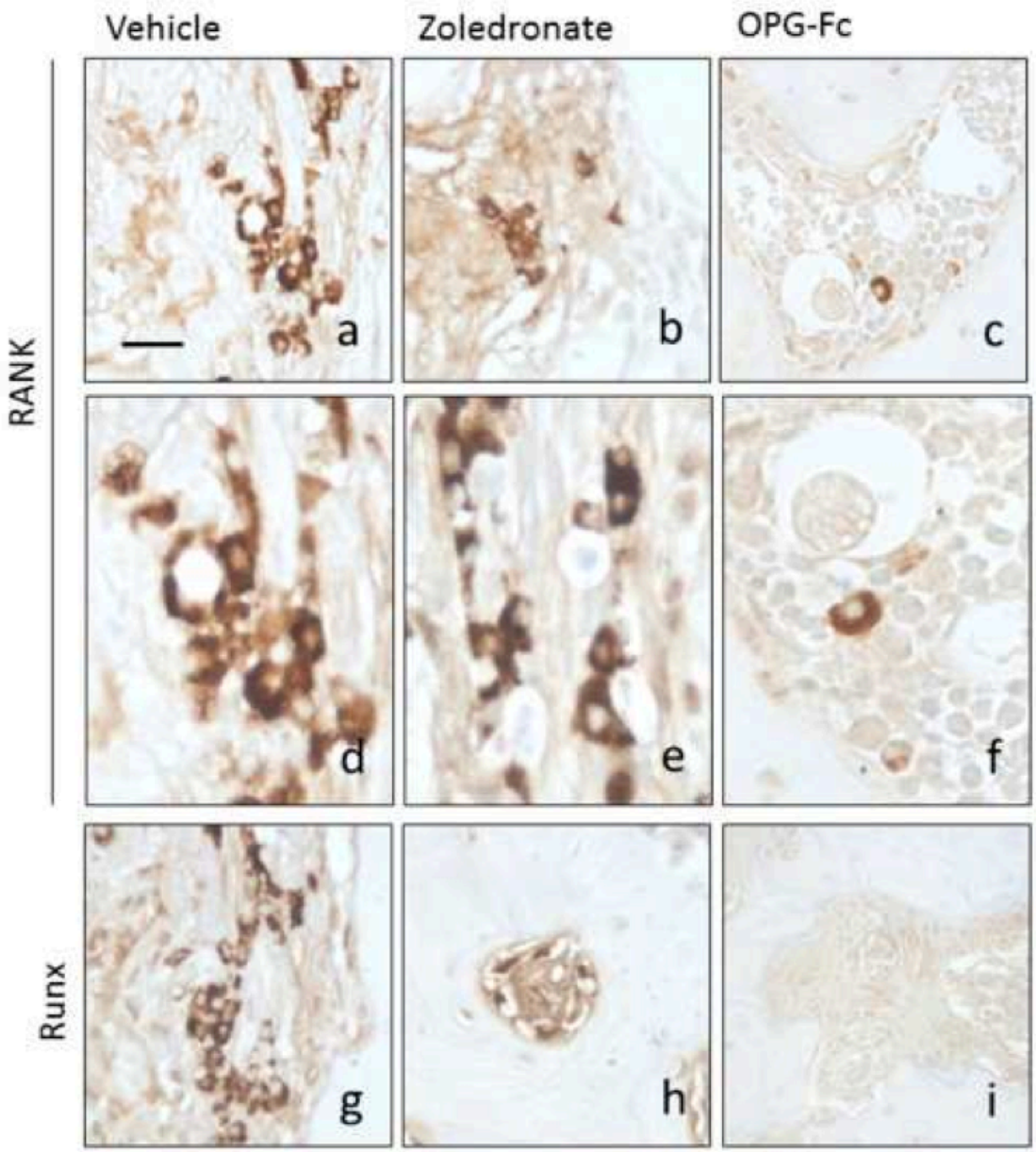
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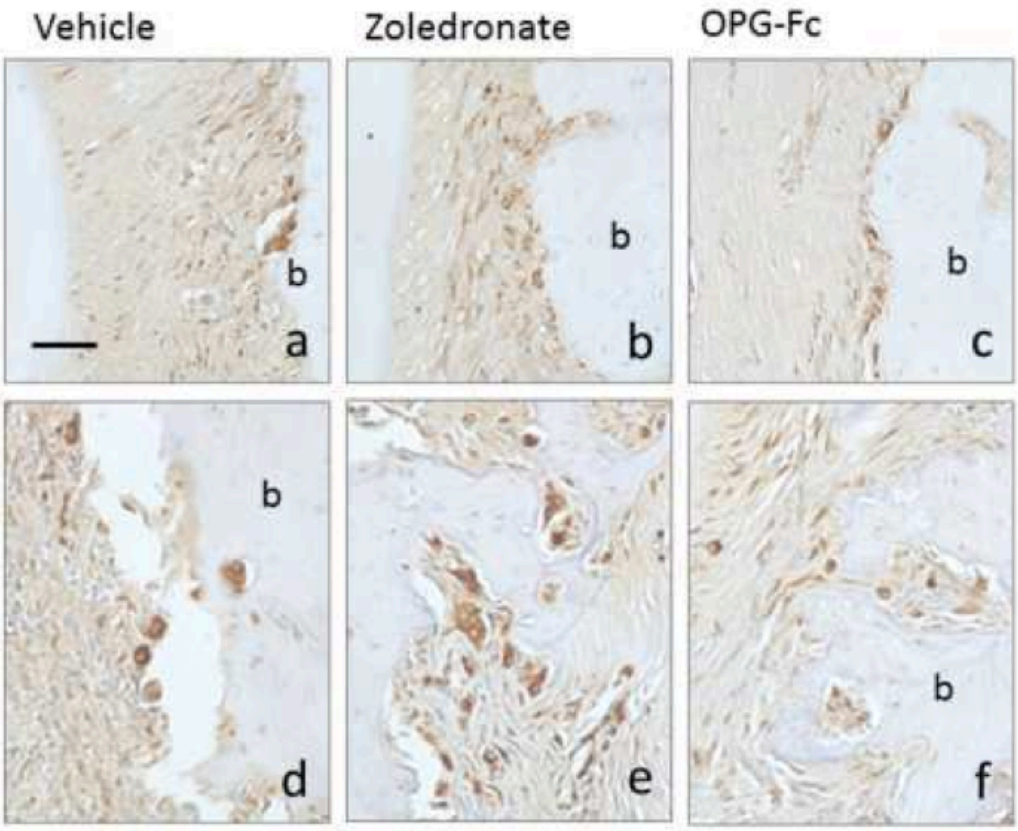
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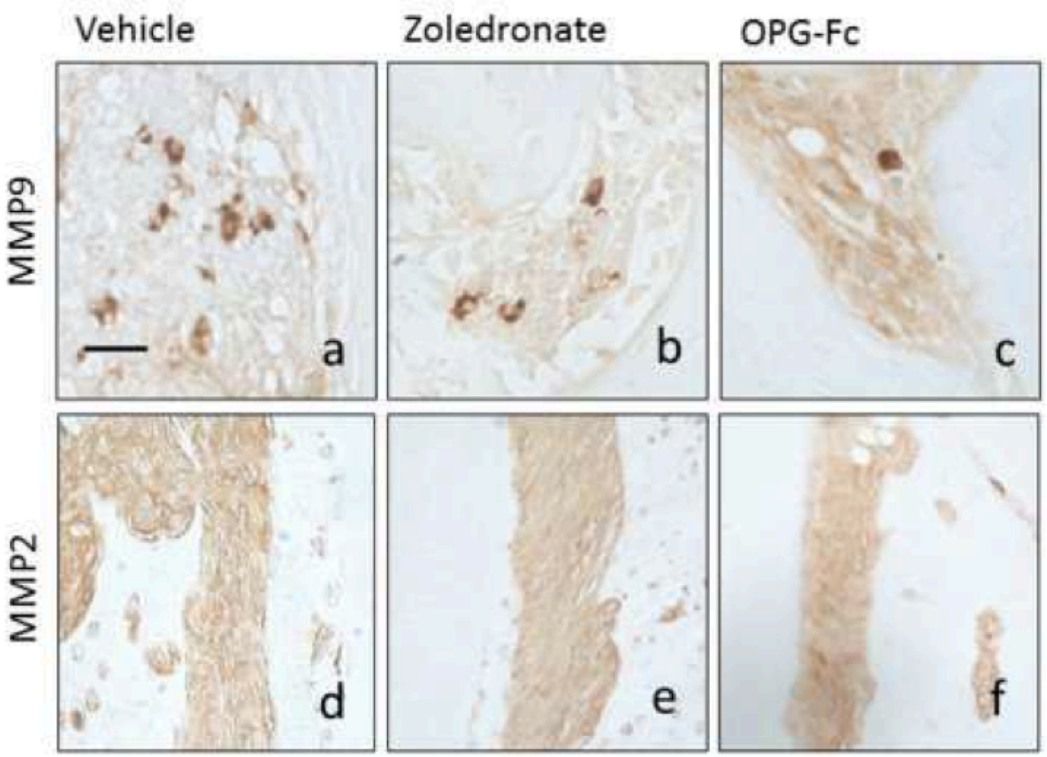
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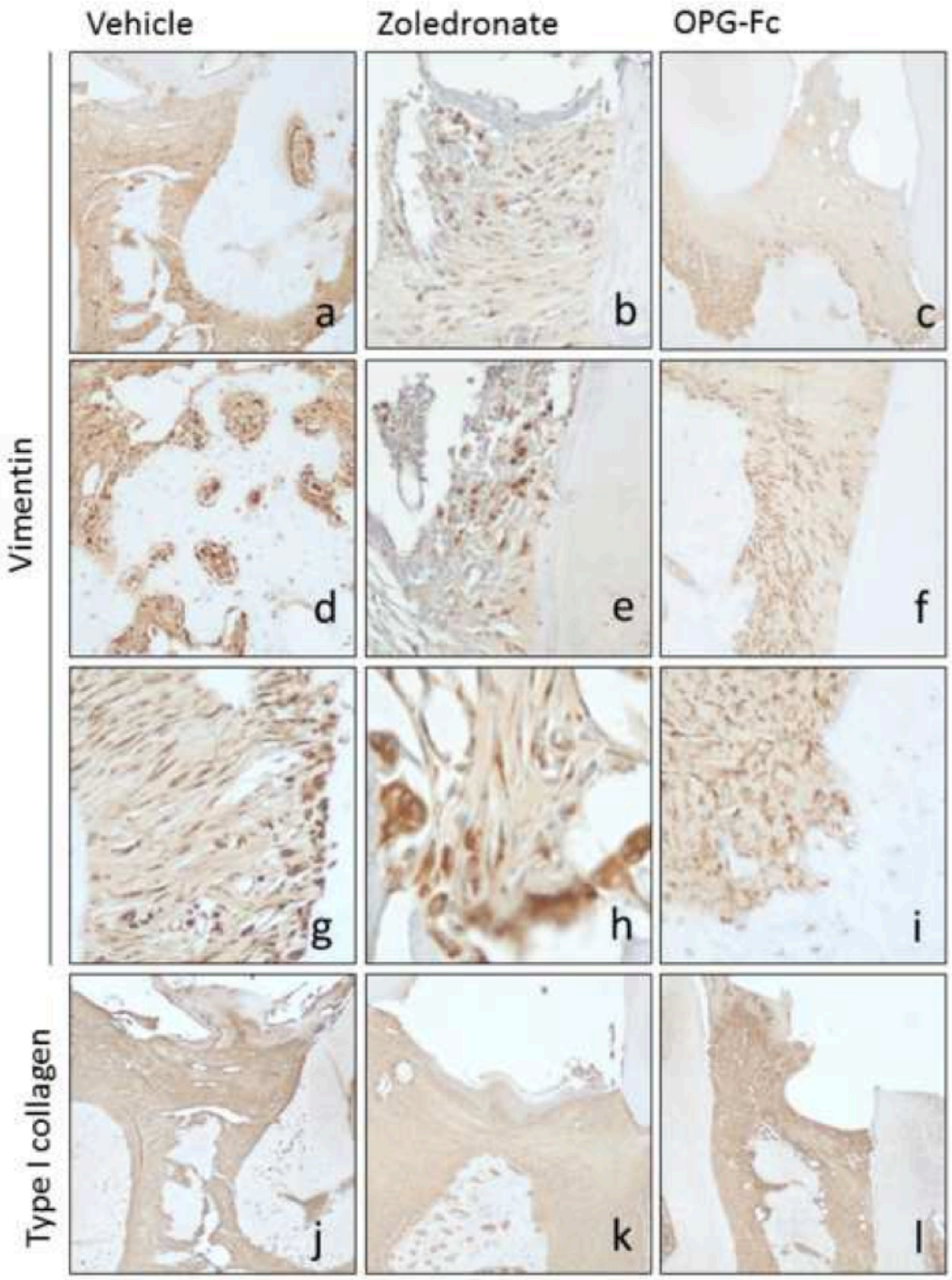
Zoledronate
OPG-Fc
MMP9
MMP2
a
b
c
d
e
f


Vehicle
Zoledronate
OPG-Fc
Vimentin
Type I collagen
a
b
c
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k
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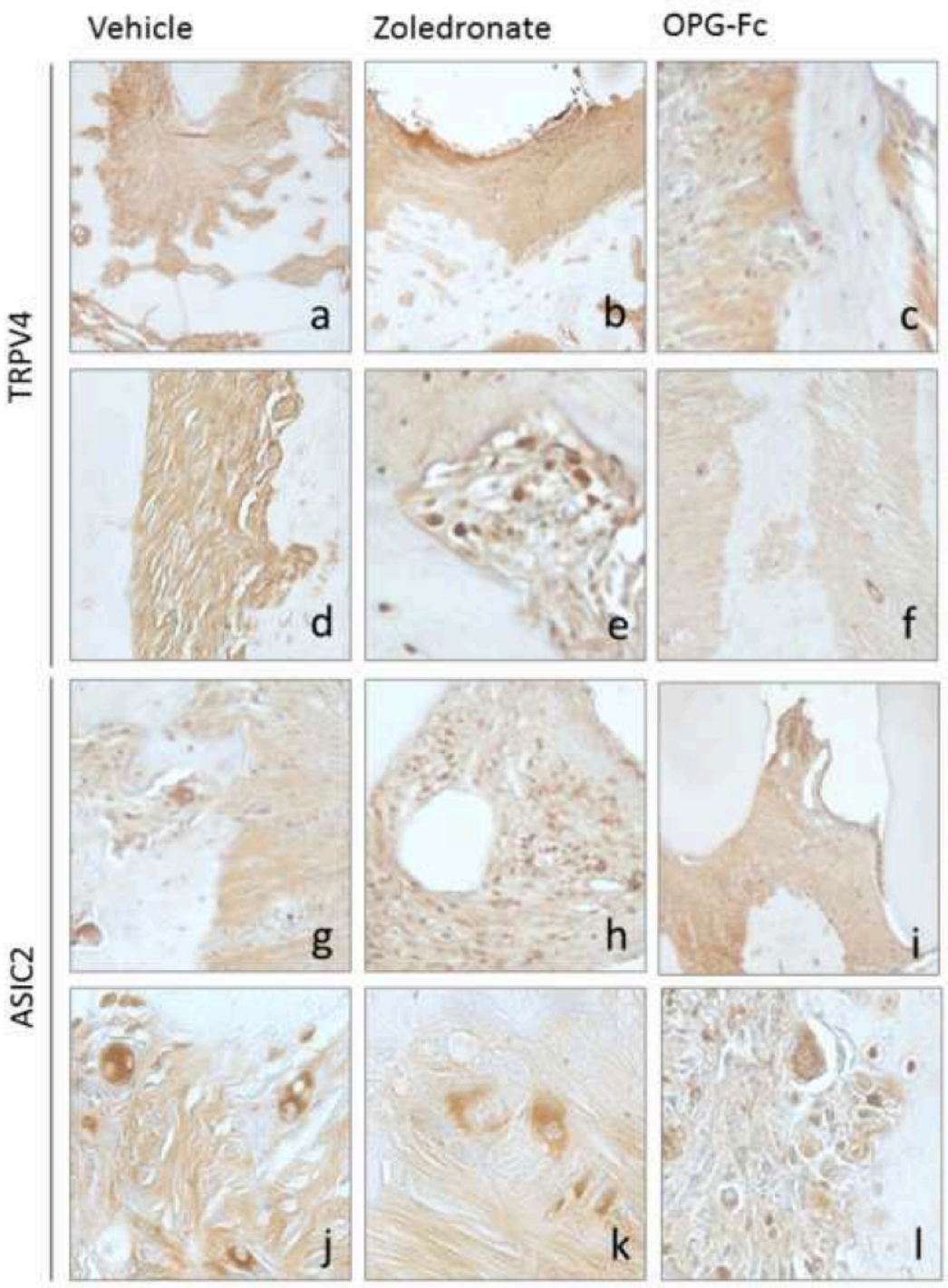
Vehicle
Zoledronate
OPG-Fc
TRPV4
ASIC2
a
b
c
d
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g
h
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# 3.CONSIDERACIONES EN EL ÁMBITO ORTODÓNCICO

En la última década, se han llevado a cabo estudios con el objetivo de encontrar un sistema de anclaje que actúe directamente sobre los mecanismos de reabsorción ósea, y evite, por tanto, los movimientos indeseados y los inconvenientes que conllevan los sistemas de anclaje tradicionales. [2-7] Como sabemos, el movimiento dentario es el resultado de la interacción entre aposición ósea (osteoblastos) y la reabsorción ósea (osteoclastos), por lo que actuando sobre la función osteoclástica, es decir directamente sobre la reabsorción ósea, se debería poder reducir este tipo de movimientos dentarios incluso llegar a evitarlos. Con éste propósito se han estudiado distintas moléculas bioactivas que han sido relacionadas con el descenso del movimiento dentario. La mayoría de estos estudios se han centrado en la influencia de los distintos *bisfosfonatos* sobre el movimiento ortodóncico, obteniendo un marcado descenso en la reabsorción ósea mediado por la disminución de la actividad osteoclástica. No obstante, debido a la prolongada permanencia de estos fármacos en el hueso, sus efectos irreversibles y especialmente por la posible osteonecrosis asociada, [20] su uso con este propósito es controvertido.

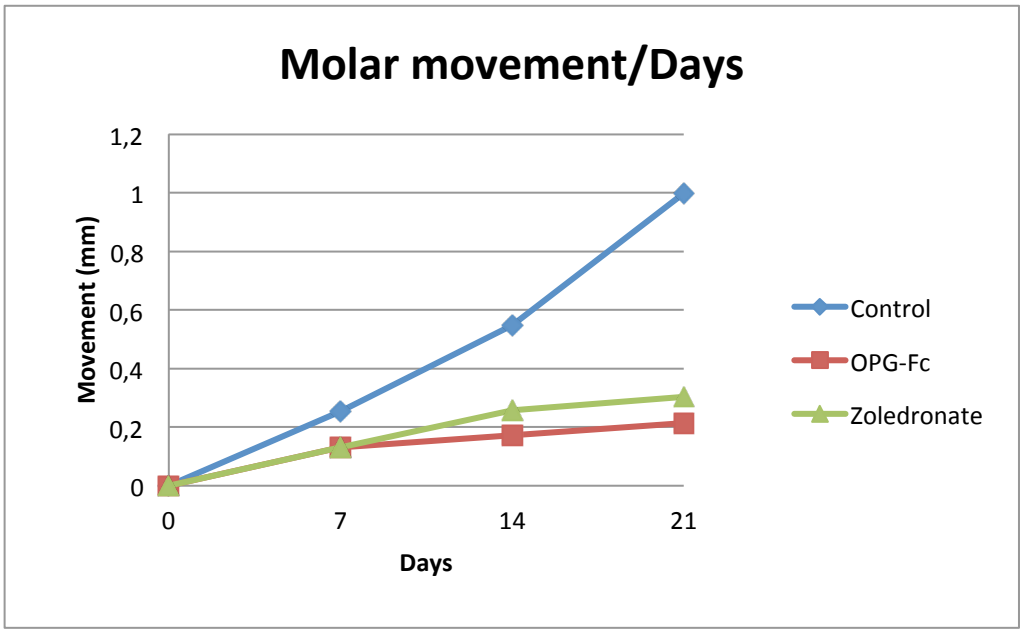
Por este motivo recientemente se han desarrollado estudios en los que se han obtenido resultados significativos en el uso de OPG-Fc como agente de anclaje farmacológico. Teniendo en cuenta que la unión del RANKL con el RANK es necesaria para la osteoclastogénesis , actuando sobre esta etapa mediante el uso de un inhibidor competitivo como la OPG, impediría tanto la formación de osteoclastos como su activación y supervivencia.[35] Asimismo , la OPG ha demostrado ser más eficaz como agente de anclaje farmacológico que los bisfosfonatos ,mostrando un efecto mayor incluso cuando éstos son administrados a alta dosis.[8] Esto podría explicarse por el hecho de que los bisfosfonatos tienen que ser incorporados primero en el hueso con el fin de inhibir la actividad de los osteoclastos de forma eficiente.[36]

En nuestro estudio hemos podido observar que el movimiento

dental se puede reducir considerablemente mediante el uso de fármacos que actúan directamente en el hueso inhibiendo la reabsorción ósea, actuando por tanto como agentes de anclaje farmacológico. En nuestro caso, el uso de Zoledronato ha supuesto una disminución del

70% del movimiento dental, mientras que la OPG ha resultado ser aún más potente, con una reducción del movimiento de 79%.

Nuestros resultados concuerdan con los obtenidos en otros estudios como el de Keles et al. en 2007[8] , en el cual la OPG demostró ser un mayor inhibidor del reclutamiento y de la actividad de los osteoclastos que el pamidronato, obteniendo en su modelo de ratones una reducción en el número de osteoclastos en los sitios de compresión del 95 % y 70 % , respectivamente, y el movimiento dental fue menor en el grupo de OPG ( 77 % vs 34 % ) .




Asimismo, para facilitar la reproducibilidad y la comparación con los diferentes estudios publicados anteriormente, la concentración de OPG utilizada en el presente

estudio ha sido similar a la empleada en diferentes investigaciones en roedores como estudios de Dunn et al. en 2007.[22] En su modelo en rata se encontraron con una disminución más significativa del movimiento dentario (78,7%) con un 5.0mg/kg inyección local dos veces por semana en comparación con la obtenida con una

concentración más baja de 0,5 mg / kg, aunque todavía había un reducción significativa ($p < 0,05$). Sin embargo es importante recordar que con el fin de evitar la respuesta inmunitaria del animal a una molécula (OPG-Fc) humana, la dosis utilizada en ratas es mayor y más frecuente de lo que sería si se utilizara en humanos.[20]

En cuanto al efecto del zoledronato sobre el movimiento dentario, Ortega et al. [4] encontraron resultados similares en su modelo en rata. Llegaron a la conclusión de que una única aplicación de 16µg del bisfosfonato zoledronato, como se usa en el presente estudio, proporciona máximo anclaje mediante la prevención de la reabsorción ósea. Por otra parte, encontraron que el zoledronato también previene la pérdida de hueso periodontal en la zona de extracción así como alrededor de los segundos y terceros molares. Al igual que en nuestro estudio, ninguno de los animales desarrolló osteonecrosis.

Otro resultado interesante del presente estudio es que la mayoría del movimiento molar total en todos los grupos se produjo dentro de los primeros 7 días. Dunn et al.[20] en su estudio también encontraron el movimiento dentario más importante en el primer control, que en su caso se llevó a cabo en el día 3. Este movimiento inicial se asocia a la viscoelasticidad del ligamento periodontal, aunque la remodelación de tejidos todavía es inexistente. En las etapas de control 3 (día 21) del presente estudio, se encontraron diferencias estadísticamente significativas entre los 3 grupos en cuanto al movimiento molar, siendo en todos los casos superior en el grupo control. En cuanto a los resultados de este estudio, en los días 14 y 21 los grupos experimentales mostraron que la OPG disminuye el movimiento molar de una forma mas eficaz que el zoledronato.

Los resultados de este estudio indican que la inhibición de la osteoclastogénesis así como de la inhibición de los osteoclastos por la administración local de la proteína OPG-Fc podría servir como un enfoque farmacológico racional para la consecución de anclaje ortodóncico, evitando la necesidad de cooperación por parte de los pacientes, el malestar de métodos tradicionales de anclaje y procedimientos quirúrgicos invasivos

como es el caso de los microtornillos. Además, su uso proporciona una acción más eficaz y predecible que los bisfosfonatos.

Por lo tanto en el presente estudio se concluye:

1. Una única aplicación de zoledronato o de OPG-Fc en alta concentración dos veces por semana es suficiente para proporcionar un anclaje máximo en ortodoncia.
2. Estos fármacos inhiben eficazmente la osteoclastogénesis dando lugar a una menor pérdida ósea, proporcionando anclaje máximo y por tanto una mayor eficacia en los tratamientos ortodóncicos.
3. De acuerdo a los resultados, la OPG-Fc resultó ser un fármaco más eficaz en la consecución de anclaje, con la ventaja de su reversibilidad en un corto periodo de tiempo.
4. No se encontraron signos de osteonecrosis asociada con bisfosfonatos en ninguno de los casos.

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