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Carga mecânica na atividade funcional da anidrase carbônica II no periodonto

Mechanical loading on the functional activity of carbonic anhydrase II in the periodontium

Carga mecánica sobre la actividad funcional de la anhidrasa carbónica II en el periodonto

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Resumo

A anidrase carbônica II (AC II) participa da homeostase ácido-básica do tecido. Este estudo tem como objetivo avaliar o efeito da oclusão dentária traumática (ODT) através da expressão de AC II em osteoclastos e osteócitos (próximo à lâmina dura e no centro do septo do osso alveolar), no ligamento periodontal (LP) e nas células de revestimento. (periósteo). Para este estudo, 50 ratos Wistar machos com sete semanas de idade foram divididos em 2 grupos: um grupo com carga e um grupo sem carga. Os períodos de estudo foram de 2, 5, 7, 14 e 21 dias. O teste U de Mann-Whitney para análise quantitativa e o teste Qui-quadrado para análise semiquantitativa foram usados para a comparação dos grupos, juntamente com o teste post-hoc de Bonferroni. Diferenças estatisticamente significativas foram observadas entre os grupos no número de osteoclastos na lâmina dura (dias 5, 7 e 21); o septo do osso alveolar (dias 2 e 7); osteócitos próximos à lâmina dura (dias 2, 5, 7 e 14); e no centro do septo do osso alveolar (dias 2, 5, 7 e 14). Também houve diferenças de grupo na expressão de AC II em células de revestimento nos dias 7 e 14. ODT aumenta a expressão de AC II em osteoclastos, osteócitos, LP e células de revestimento periosteal. Relevância clínica: a oclusão dentária traumática estimula o aumento da atividade das células ósseas alveolares em distâncias curtas (lâmina dura) e longas (centro do osso alveolar e periósteo).

Palavras-chave: Anidrases carbônicas; Periodonto; Oclusão dentária traumática.

Abstract

Carbonic anhydrase II (CA II) is involved with the acid-base homeostasis of tissue. This study aims to evaluate the effect of traumatic dental occlusion (TDO) by means of CA II expression in osteoclasts and osteocytes (near the lamina dura and in the centre of alveolar bone septum), in the periodontal ligament (PDL) and in lining cells (periosteum). For this study, 50 male Wistar rats aged seven weeks were divided into 2 groups: Loaded and Unloaded group. The study periods were 2, 5, 7, 14 and 21 days. The Mann-Whitney U test for quantitative, and the Chi-square test for semi-quantitative analyses were used for group comparison, along with Bonferroni's post-hoc test. Statistically significant differences between the groups were observed in the number of osteoclasts in the lamina dura (days 5, 7 and 21); the alveolar bone septum (days 2 and 7); osteocytes near the lamina dura (days 2, 5, 7 and 14); and in the centre of the alveolar bone septum (days 2, 5, 7 and 14). There were also differences between-group in CA II expression in the lining cells on days 7 and 14. TDO increases CA II expression in osteoclasts, osteocytes, the PDL and lining cells of the periosteum. Clinical Relevance:

Traumatic dental occlusion stimulates higher cells activity of the alveolar bone at short (lamina dura) and long (centre of alveolar bone and periosteum) distances.

Keywords: Carbonic anhydrases; Periodontium; Traumatic dental occlusion.

Resumen

La anhidrasa carbónica II (AC II) participa en la homeostasis ácido-base del tejido. Este estudio tiene como objetivo evaluar el efecto de la oclusión dental traumática (ODT) mediante la expresión de CA II en osteoclastos y osteocitos (cerca de la lámina dura y en el centro del tabique del hueso alveolar), en el ligamento periodontal (LP) y en las células de revestimiento. (periostio). Para este estudio, 50 ratas Wistar macho de siete semanas se dividieron en 2 grupos: un grupo cargado y un grupo descargado. Los periodos de estudio fueron 2, 5, 7, 14 y 21 días. La prueba U de Mann-Whitney para análisis cuantitativos y la prueba de Chi-cuadrado para análisis semicuantitativos se utilizaron para la comparación de grupos, junto con la prueba post-hoc de Bonferroni. Se observaron diferencias estadísticamente significativas entre los grupos en el número de osteoclastos en la lámina dura (días 5, 7 y 21); el tabique del hueso alveolar (días 2 y 7); osteocitos cerca de la lámina dura (días 2, 5, 7 y 14); y en el centro del tabique del hueso alveolar (días 2, 5, 7 y 14). También hubo diferencias entre los grupos en la expresión de AC II en las células de revestimiento los días 7 y 14. La ODT aumenta la expresión de AC II en los osteoclastos, osteocitos, el LP y las células de revestimiento del periostio. Relevancia clínica: la oclusión dental traumática estimula una mayor actividad de las células del hueso alveolar a distancias cortas (lámina dura) y largas (centro del hueso alveolar y periostio).

Palabras clave: Anhidrasas carbónicas; Periodoncio; Oclusión dental traumática.

1. Introduction

Mechanical stimulus is necessary for the maintenance of periodontal tissue homeostasis, whereas the absence or excess of occlusal load results in a disharmonic functioning of periodontal tissues (Glickman, 1971; Brandini, et al., 2018).

Primary occlusal trauma has been defined as an injury to the attachment apparatus as result of excessive occlusal force applied to a tooth or teeth with normal and healthy support tissues (Davies, et al., 2001). It can affect tooth mobility (Harrel & Nunn, 2001), fremitus, persistent discomfort or pain on eating (Wan, et al., 2012) and aseptic pulp necrosis. Discontinuity and thickening of the lamina dura, widening of the periodontal ligament (PDL)

space, radiolucency and condensation of alveolar bone, and/or root resorption (Davies, et al., 2001) can be observed using radiographic imaging.

In the first 24 hours of traumatic dental occlusion (TDO), osteogenesis in the rats, alveolar bone was inhibited, and osteoclastogenesis was not significant (Wan, et al., 2012). An increase in the pressure of the interstitial fluid of the PDL was observed after 48 hours (Palcanis, 1973; Brandini, et al., 2018). Until the 5th day the PDL space width decreased, and there was an increase in the number and a disorientation (Glickman, 1971; Palcanis, 1973; Brandini, et al., 2018) of fibroblasts, cell necrosis and venous thrombosis in the PDL (Palcanis, 1973), a decrease in collagenous fibres and elevated osteoclast activity (Glickman, 1971; Palcanis, 1973; Kaku, et al.,2005; Brandini, et al., 2018). There was also a significant increase in Ruffini endings and free nerve endings in the PDL. The PDL space returned to normality on day 7 (Kaku, et al.,2005), probably due to bone remodeling (Glickman, 1971; Brandini, et al 2016; Brandini, et al 2018).

CA II is involved with acid-base homeostasis. It provides the proton source for extracellular acidification by H+-ATPase and the HCO3- source for the HCO3-/Cl- exchanger (Teo, et al., 2012), and it catalyzes the reversible hydration of carbon dioxide into bicarbonate. It plays a critical role in the cells as a high neuronal activity in the periodontal Ruffini endings (Ochi, et al., 1998) and a decreased differentiation of ameloblasts (Wang, et al.,2010). Whereas some transporters are responsible for the bone resorption process, others are essential for the pH regulation in osteoclasts (Lehenkari, et al.,1998; Oksala et al., 2010).

CA II expression was found in the early stage of the osteoclast differentiation (Lehenkari, et al.,1998; Oksala et al., 2010). Bone resorption is stimulated by pH reduction in the osteoclast. Osteoclasts are multinucleated bone-reabsorbing cells that use multiple pH regulation mechanisms to create an acidic pH in the resorption lacuna (Riihonen, et al.,2007; Jansen, et al.,2009). Under conditions that favors bone resorption, osteoclasts reabsorb bone by attaching to the surface and then secreting protons into an extracellular compartment formed between osteoclasts and the bone surface. This secretion is necessary for bone mineral solubilization and the digestion of organic bone matrix by acid proteases (Jansen, et al.,2009).

The question now is to what extent traumatic dental occlusion can affect homeostasis of the periodontal tissues and maxillary bone.

This study aims to evaluate the effect of traumatic dental occlusion by means of CA II expression in osteoclasts and osteocytes the periodontal ligament and in lining cells of the periosteum.

2. Materials and Methods

First, approval was obtained from the Animal Care Committee of the Dentistry School of Araçatuba-UNESP (Process- 2012-00980).

For this study, 50 male Wistar rats (*Rattus Norvegicus, albinus*) aged seven weeks were used. They were originating from the central bioterium of the Dentistry School of Araçatuba, the animals were transferred to the bioterium of the Department of Surgery and Integrated Clinic 5 days prior to the experiment. They were kept in cages with five animals each and given granulated food and water *ad libitum*. The environment was kept at a constant temperature of 22 °C (±2 ºC) and 50% (±10%) humidity, with light/dark cycles of 12/12 hours.

To assess the influence of mechanical loading on the alveolar bone of the right first upper molar following traumatic dental occlusion, the animals were divided into a Loaded (L) and Unloaded (U) group.

The periods of study were 2, 5, 7, 14 and 21 days for both groups. Group U group consisted of 25 same-aged rats – animals with the same age as those in the experimental group, in order to enable comparison with normal conditions. Group L was comprised of 25 rats, submitted to mechanical loading on the occlusal surface of the teeth by placing a composite filling in the right inferior first molar.

Prior to the experiment, the animals were intramuscularly anaesthetized with a solution of ketamine hydrochloride (25 mg/kg, Vetanarcol, Laboratórios König, Argentina) and xylazine (10 mg/kg, Coopazine, Coopers Brasil, Brazil). All animals received a single intramuscular 24,000 IU antibiotic dose (benzathine benzylpenicillin - 12,000 IU, procaine benzylpenicillin - 6,000 IU, potassium benzylpenicillin - 6,000 IU, dihydrostreptomycin sulfate - 5 mg, streptomycin sulfate - 5 mg; Fort Dodge, Animal Health Ltda., Campinas, SP, Brazil).

In group L, the right first inferior molar was raised with a direct filling using 37% phosphoric acid etchant for enamel and dentin (FGM, Brazil), microbrush (Microbrush® International, Grafton, USA); scotchbond multi-purpose light adhesive (3M ESPE, Saint Paul, USA), Estelite composite resin (Tokuyama Dental Corp, Japan) and light-cured (Dabi Atlante, Ribeirão Preto, Brazil), as indicated by the manufacturer's manual; creating a flat high occlusal table on the highest occlusal cusp. A piece of ligature wire 0.20mm (.008´´) (Morelli, Sorocaba, São Paulo, Brazil) was attached to the surface of the composite filling. Prior to the restoration, superficial micro retentions were made with Carbide burs FG ¼

(Beavers Dental, Canada) attached to a high-speed handpiece with water and without invasion to the pulp chamber.

Animals were excluded in case they died of natural causes or if they lost the occlusal composite filling.

Following anesthesia, transcardial perfusion was performed. An intraventricular injection of heparin (0.1 ml/5,000 U.I/ml) was administered. After 1 minute, 100 ml saline was perfused via the aorta, followed by a mixed solution of 200 ml of paraformaldehyde fixation solution at 4% (Sigma Chemical Co., St. Louis, MO, USA) and 200 ml phosphate buffered saline (PBS) at 0.1M, pH 7.4, 4ºC (Sigma Chemical Co., St. Louis, MO, USA).

After dissection, the specimens were washed in PBS and kept in a paraformaldehyde fixation solution at 4% (Sigma Chemical Co., St. Louis, MO, USA) for 24 hours, before starting the decalcification with ethylene diamine tetra acetic acid disodium (EDTA) at 10% for 20 days.

The specimens were processed with progressive dehydration in ethyl alcohol, diaphanized in xylol, impregnated with paraffin at a low fusion temperature (56-58 ºC) for 3 hours and embedded according to standard protocols.

Transversal 4 µm wide histological sections at the interradicular alveolar bone between the roots of the right upper first molar and surrounding tissue were obtained with an automated rotary microtome (Leica SMR 2000), transferred to a bain-marie (40-50 ºC) and then collected using silanised slides.

The slides were deparaffinized and rehydrated. Next, they were subjected to the following washing and incubation stages: a) 3 min. rinsing in 3% H_2O_2; b) 5 min. washing in distilled water for inactive endogenous peroxidase; c) 10 min. washing in PBS (pH 7.4, 0.1M, room temperature); d) incubation for 30 min. in 2% BSA (albumin bovine serum) (Sigma); e) gently tap off the blocking buffer (2% BSA); f) incubation for 24 hours at room temperature, using rabbit primary antibodies anti-carbonic anhydrase II (ab6621) (1:100); g) three 10 min. washings in PBS at room temperature; h) exposure with an incubation solution (1 drop DAB plus 1 ml DAB substrate buffer (Dako kit, Dako North America, Inc - USA); i) blocking of the reaction through washing phosphate buffered saline (PBS); and j) counterstaining with hematoxylin (Maier).

Each specimen consisted of three sections of the superior right first molar and surrounding tissues of each animal, with a distance of 40 µm between them. The samples were submitted to histomorphological analysis at the set times throughout the experimental period to detect time-related changes and enable group comparison.

For quantitative analysis, the images were processed using ImageJ. The number of osteoclasts was counted in the lamina dura around the distal root and in the centre of the alveolar bone septum in a selected area. The percentage of osteocytes was calculated in a limited area near the lamina dura and in the centre of the alveolar bone septum.

The semi-quantitative analysis of the entire PDL and lining cells of the periosteum used scores from 1 to 4, based on the color intensity of the CA II expression. The CA II expression in the lining cells of the periosteum was present on the vestibular side of the upper jaw in the correspondent region of the right first upper molar; and in the PDL of the right first upper molar's distal root.

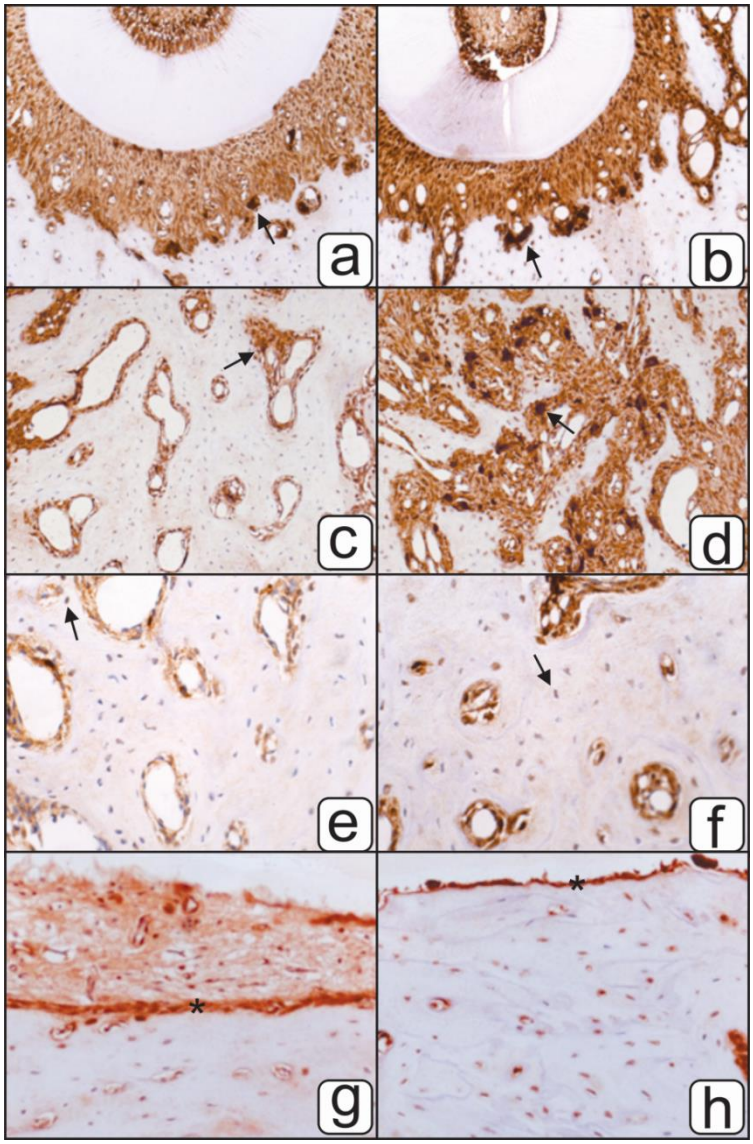
The examiner was not informed to which group the images belonged, in order to avoid bias during the analysis.

The data were analysed with SPSS 20.0 (IBM, Armonk, NY, USA) at α=0.05. The Mann-Whitney U test was used for group comparison on the number of osteoclasts and osteocytes, and data were expressed as mean ± SD and percentage, respectively. The Chi-square test was used in combination with Bonferroni's post-hoc test for semi-quantitative analyses of the PDL and the lining cells, with data expressed in percentages of the score prevalence.

3. Results

In the group L on day 2, the CA II expression was significantly higher in the osteoclasts (Figure 3) and osteocytes (Figures 1e, 1f, and 5) when compared with group U, located in the alveolar bone septum.

Figure 1. Transversal histological sections of the right upper first molar and surrounding tissue were stained with CA II. CA II positive osteoclasts (arrows) on day 5, the bone resorption and number of osteoclast (arrow) and osteocytes (arrows) can be seen in the group U (1a, 1c and 1e) and L (1b, 1d and 1f) (Magnification 200x). The presence of active osteoclasts (arrow) and the expression of CA II in the lamina dura can be compared between group U (1a) and L (1b) group. Detection of CA II positive osteocytes (arrows) on 2 day of the experiment in group L (1e) is more evident than in group U (1f). The lining cells show higher CA II expression (star) in group L (1h) than in group U on 14 day. (Magnification 400x).

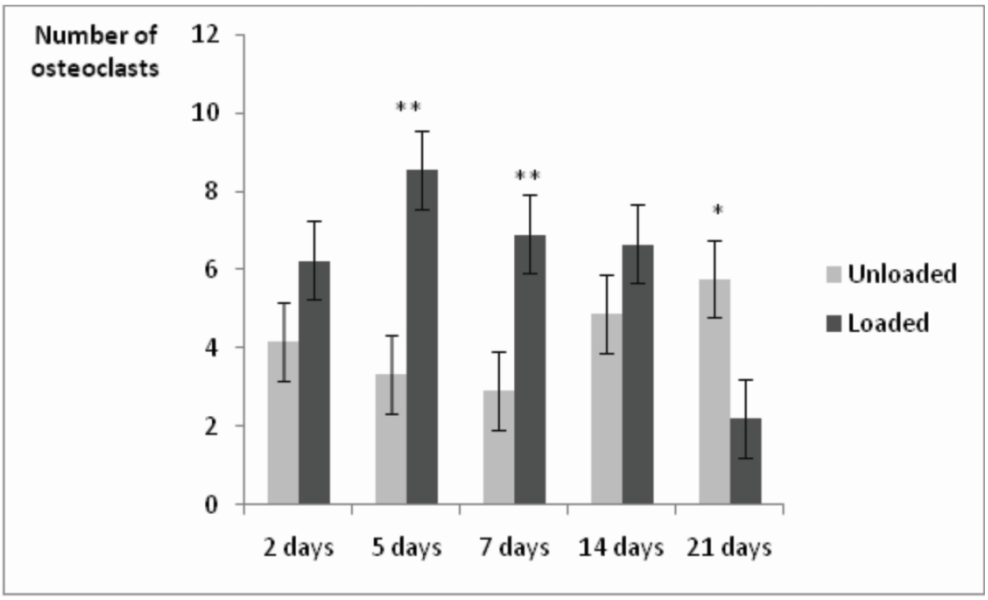


Source: Authors.

On day 5, the CA II expression was more evident in all cells: in osteoclasts (Figure 2), osteocytes (Figures 4 and 5) and the PDL (Table 1) in group L. At this time point, the stronger staining of the PDL on the distal side of the root (Figures 1a and 1b) indicated a higher

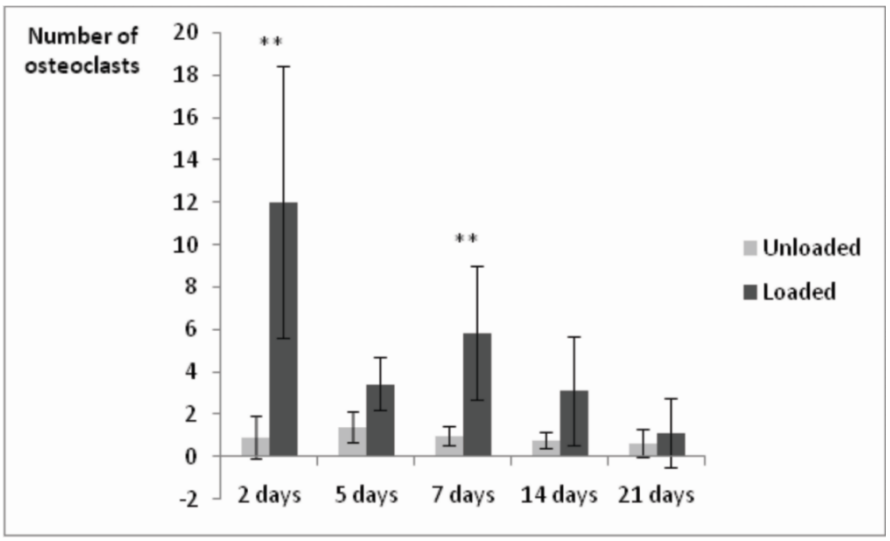
number of osteoclasts than on the day 2 – in the alveolar bone (Figures 1c and 1d), as well as in the lamina dura of group L (Figures 1a and 1b).

Figure 2. Quantification of osteoclasts in the lamina dura around the distal root of the first maxillary tooth labelled immunohistochemically for CA II expression. Data are presented in means ± with standard deviation. *P<.05; **P<.001.



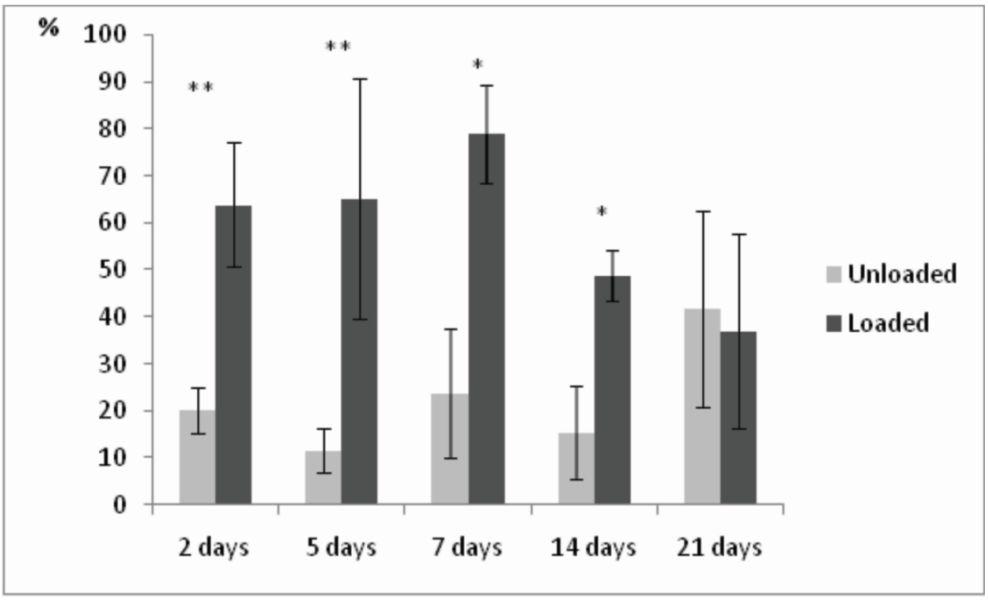
Source: Authors.

Figure 3. Quantification of osteoclasts in the alveolar bone septum of the first maxillary tooth labelled immunohistochemically for CA II expression. Data are presented in means ± with standard deviation. *P<.05; **P<.001.



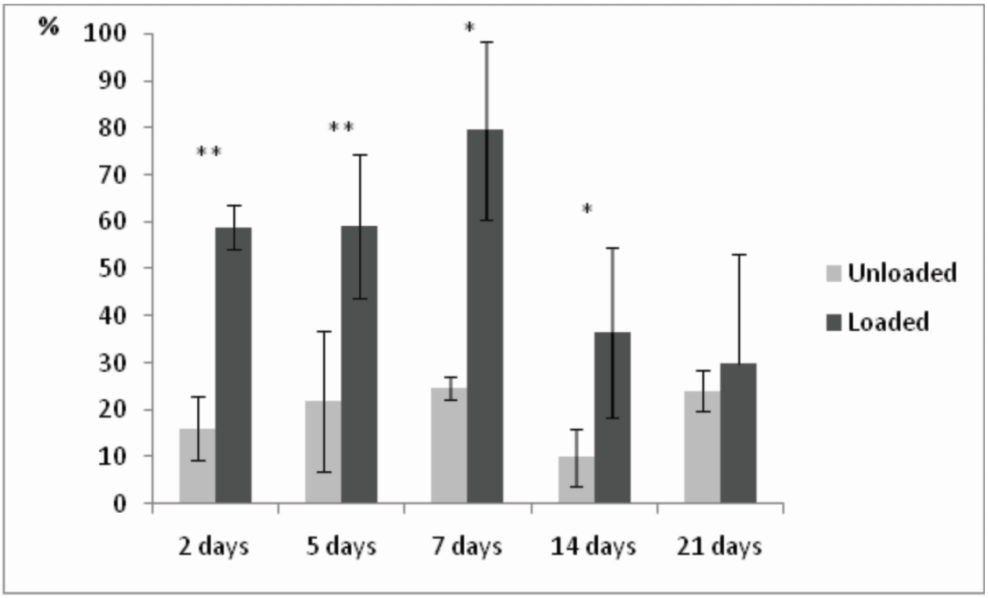
Source: Authors.

Figure 4. Quantification of osteocytes in the lamina dura around the distal root of the first maxillary tooth labelled immunohistochemically for CA II expression. Data are presented in means ± with standard deviation. *P<.05; **P<.001.



Source: Authors.

Figure 5. Quantification of osteocytes in the centre of the alveolar bone septum of the first maxillary tooth labelled immunohistochemically for CA II expression. Data are presented in means ± with standard deviation. *P<.05; **P<.001.



Source: Authors.

Table 1. Distribution of the percentage of CA II expression scores in the PDL

	Groups							
Periods	Unloaded	Loaded	Unloaded	Loaded	Unloaded	Loaded	Unloaded	Loaded
	Score 1 (%)		Score 2 (%)		Score 3 (%)		Score 4 (%)	
2 days	0 [a]	0 [a]	33,3 [a]	50 [a]	66,7 [a]	50 [a]	0 [a]	0 [a]
5 days	0 [a]	0 [a]	100 [a]	50 [a]	0 [a]	25 [a]	0 [a]	25 [a]
7 days	25 [a]	0 [a]	75 [a]	50 [a]	0 [a]	25 [a]	0 [a]	25 [a]
14 days	0 [a]	0 [a]	75 [a]	25 [a]	0 [a]	50 [a]	25 [a]	25 [a]
21 days	0 [a]	0 [a]	33,3 [a]	66,7 [a]	66,7 [a]	33,3 [a]	0 [a]	0 [a]

P-values are used for group comparison, and χ^2 or Fisher's exact test for appropriate categorical variables adjusted by Bonferroni's post-hoc test. Data are presented in percentages. Different letters indicate a statistically significant difference between groups.
Source: Authors.

After 7 days, the CA II expression became less intense, although the quantitative difference between groups L and U was still evident in the osteoclasts (Figures 2 and 3), osteocytes (Figures 4 and 5) and lining cells (Table 2).

Table 2. Distribution of the percentage of CA II expression scores in the lining cells of the periosteum.

	Groups							
Periods	Unloaded	Loaded	Unloaded	Loaded	Unloaded	Loaded	Unloaded	Loaded
	Score 1 (%)		Score 2 (%)		Score 3 (%)		Score 4 (%)	
2 days	0 [a]	25 [a]	66.7 [a]	75 [a]	33,3 [a]	0 [a]	0 [a]	0 [a]
5 days	25 [a]	25 [a]	25 [a]	25 [a]	50 [a]	50 [a]	0 [a]	0 [a]
7 days	25 [a]	0 [a]	75 [a]	25 [a]	0 [a]	75 [b]	0 [a]	0 [a]
14 days	75 [a]	0 [b]	25 [a]	0 [a]	0 [a]	100 [b]	0 [a]	0 [a]
21 days	0 [a]	33.3 [a]	66.7 [a]	0 [a]	33.3 [a]	66.7 [a]	0 [a]	0 [a]

P-values are used for group comparison, and χ^2 or Fisher's exact test for appropriate categorical variables adjusted by Bonferroni's post-hoc test. Data are presented in percentages. Different letters indicate a statistically significant difference between groups.
Source: Authors.

On day 14 of the experiment the between-group difference in CA II expression had decreased, but could still been seen in the osteocytes (Figures 4 and 5) and lining cells (Figures 1g, 1h and Table 2).

The group difference had further reduced on day 21. The CA II expression showed a slight increase in osteoclasts (Figure 3) and osteocytes (Figure 5) in the centre of the alveolar bone septum. However, the number of osteoclasts in the lamina dura was higher in the group U (Figure 2).

4. Discussion

Traumatic dental occlusion increases CA II expression in the PDL cells, osteoclasts, osteocytes and lining cells of the periosteum. The communication between these cells may be the network that preserves the homeostasis of the alveolar bone and periodontal connective tissue in the case of excessive mechanical loading.

Although the literature indicates that the PDL space returns to normal 7 days (6) after the incidence of traumatic occlusion, this study shows that a decrease in the number of

osteoclasts in the lamina dura only occurs after 21 days, and that a higher number of osteoclasts still remains at the centre of the alveolar bone, maybe because the adaptation of the PDL width needs a longer time to adapt until a point that it does not receive a excessive mechanical loading.

The osteoclastic bone resorption cycle begins with the dissolution of inorganic minerals. In the first step towards mineral dissolution, a large number of intracellular acidic vesicles fuse to the bone-facing plasma membrane and release acid into the space formed between the bone surface and cell membrane. Simultaneously, the vacuolar proton ATPase (V-ATPase) continues pumping protons across the cell membrane in order to maintain the intralacunar pH levels at 4.5–4.8.(Väänänen & Parvinen, 1983) Protons for the action of V-ATPases are continuously formed in osteoclasts in a cytoplasmic reaction facilitated by CA II, where carbon dioxide and water are converted into bicarbonate and protons (Sundquist, et al., 1987).

The induction of CA II in osteoclast progenitors requires their physical communication with stromal cells (i.e. fibroblasts) and is inseparable from the osteoclast differentiation process (Biskobing, et al., 1997).

The pH alteration may be a result of pH changes in the PDL. Fibroblasts in the PDL suffer damage from overloading, which causes an interruption in the pH regulation. PDL cells are exposed to a slightly acid pH. This acidity can demineralize the periodontal bone and activate osteoclasts. When inflammation prevents these cells from recovering, the osteoclasts disappear and the bone can acquire new minerals through physicochemical exchange with blood. The expectation is that these PDL cells respond to potential pH drops by producing more carbonic anhydrase involved in the buffering of acidity in cells and extracellular fluid.

The extensive osteocyte canalicular network passes through the bone matrix and contains gap junctions allowing continuous cell to cell communication (Gu, et al., 2006). There is evidence that osteocytes act as primary monitors for bone remodeling in cases of microfracture or in the presence of higher mechanical stress (Hazenberg, et al., 2009). Recent studies have revealed the function of osteocytes as mechanosensors in the early stage of bone remodeling (Nomura & Takano-Yamamoto, 2000). Mechanical stimulation is capable of maintaining osteocyte viability in human bone. However, osteocyte death by apoptosis has been associated with a range of conditions including under and overloaded bone (Mann, et al.,2006). The results corroborate with this concept, because initially the number of osteocytes that expressed CA II increased near the lamina dura as well as in the centre of the alveolar bone, while after 21 days their numbers showed a sharp decline near the lamina dura.

Bone lining cells are members of the osteoblast lineage; when they are not involved in bone remodeling they function as a membranous covering layer for bone tissue. Apparently, these cells can be induced to proliferate and differentiate into osteogenic cells. Moreover, some studies have suggested that bone lining cells may interact with osteocytes via gap junctions (Islam, et al., 1990), and are even able to stimulate osteoclast precursors through RANKL expression (Tanaka, et al., 2000). In addition, they can act as bone surface cleaners through matrix metalloproteinase (MMP) activity prior to osteoclastic bone resorption (Riihonen, 2010). This might explain the relation between bone exostosis and occlusal forces (Yoshinaka, et al., 2014).

As a limitation of this study, can be considered the sample size, 5 animals per group in each period; and the semi-quantitative statistical analysis of some variables.

These observations expand the knowledge about the effects of traumatic dental occlusion. The intensity and frequency of traumatic dental occlusion affect the network between the cells, and may lead to temporary or permanent changes in the periodontium caused by the mechanical stimuli applied to cells. Long term studies are needed to identify all the alterations that can be caused by this type of trauma. CA II affects the resting intracellular pH, but the effect differs per cell type; meaning that this effector depends on cell action, not on CA II itself.

5. Conclusion

So, traumatic dental occlusion causes a significant increase in the CA II expression in osteoclast, osteocytes and in lining cells of the periosteum.

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