

Immunohistochemical and histomorphometric analysis of alveolar repair in spontaneously hypertensive rats (SHR) treated with losartan

Análise imunohistoquímica e histomorfométrica do reparo alveolar em ratos espontaneamente hipertensos (SHR) tratados com losartan

Análisis inmunohistoquímico e histomorfométrico de la reparación alveolar en ratas espontáneamente hipertensas (SHR) tratadas con losartan

Received: 11/26/2020 | Reviewed: 11/29/2020 | Accept: 12/01/2020 | Published: 12/05/2020

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Abstract

Hypertension is a multifactorial condition with high rates of complications such as cardiovascular and renal diseases, making it a worldwide public health concern. This disease alters calcium regulation by inducing bone loss, which is limited by anti-hypertensive drugs. One such drug, losartan, inhibits angiotensin II (Ang II) AT1 receptors. The aim of this study was to compare the process of alveolar repair in spontaneously hypertensive rats (SHR) and Wistar rats, and to assess the effect of losartan on bone dynamics. Treated and untreated rats underwent dental extraction of the upper right incisor and were euthanized 7, 14, or 28 days after surgery. Alveolar repair was then analyzed histomorphometrically and immunohistochemically by measuring proteins involved in bone metabolism. Data were analyzed using the nonparametric Kruskal-Wallis test, followed by the Mann Whitney test for comparison of samples at different times. Alveolar repair was slow in SHRs, while losartan increased bone formation and trabecular thickness in both SHRs and Wistars. Because the analyzed proteins are found in dynamic bone, it is suggested that losartan interferes with the actions of angiotensin II and the renin-angiotensin system and limits bone metabolism.

Keywords: Losartan; Angiotensin II; Bone.

Resumo

A hipertensão é uma condição multifatorial com altos índices de complicações, como doenças cardiovasculares e renais, o que a torna um problema de saúde pública mundial. Esta doença altera a regulação do cálcio induzindo a perda óssea, que é limitada por medicamentos anti-hipertensivos. Uma dessas drogas, o losartan, inibe os receptores AT1 da angiotensina II (Ang II). O objetivo deste estudo foi comparar o processo de reparo alveolar em ratos espontaneamente hipertensos (SHR) e ratos Wistar, e avaliar o efeito do losartan na dinâmica óssea. Ratos tratados e não tratados foram submetidos à extração dentária do incisivo superior

direito e eutanasiados em 7, 14 ou 28 dias após a cirurgia. O reparo alveolar foi então analisado histomorfometricamente e imunohistoquimicamente através da medição de proteínas envolvidas no metabolismo ósseo. Os dados foram analisados por meio do teste não paramétrico de Kruskal-Wallis, seguido do teste de Mann Whitney para comparação de amostras em momentos diferentes. O reparo alveolar foi lento em SHRs, enquanto o losartan aumentou a formação óssea e a espessura trabecular em SHRs e Wistars. Como as proteínas analisadas são encontradas no osso dinâmico, sugere-se que o losartan interfere nas ações da angiotensina II e do sistema renina-angiotensina e limita o metabolismo ósseo.

Palavras-chave: Losartan; Angiotensina II; Osso.

Resumen

La hipertensión es una enfermedad multifactorial con altas tasas de complicaciones, como enfermedades cardiovasculares y renales, lo que la convierte en un problema de salud pública mundial. Esta enfermedad altera la regulación del calcio induciendo la pérdida ósea, que está limitada por fármacos antihipertensivos. Uno de estos fármacos, losartán, inhibe los receptores AT1 de angiotensina II (Ang II). El objetivo de este estudio fue comparar el proceso de reparación alveolar en ratas espontáneamente hipertensas (SHR) y ratas Wistar, y evaluar el efecto del losartán sobre la dinámica ósea. Las ratas tratadas y no tratadas se sometieron a extracción dental del incisivo superior derecho y se sacrificaron 7, 14 o 28 días después de la cirugía. La reparación alveolar se analizó luego histomorfométricamente e inmunohistoquímicamente midiendo las proteínas implicadas en el metabolismo óseo. Los datos se analizaron mediante la prueba no paramétrica de Kruskal-Wallis, seguida de la prueba de Mann Whitney para comparar muestras en diferentes momentos. La reparación alveolar fue lenta en SHR, mientras que losartán aumentó la formación ósea y el grosor trabecular en SHR y Wistars. Como las proteínas analizadas se encuentran en hueso dinámico, se sugiere que losartán interfiere con las acciones de la angiotensina II y el sistema renina-angiotensina y limita el metabolismo óseo.

Palabras clave: Losartán, Angiotensina II; Hueso.

1. Introduction

Systemic arterial hypertension (SAH) is characterized by an increase in blood, systolic and / or diastolic pressure beyond the limits currently defined by VII JNC (2003). It is frequently associated with functional and / or structural changes in the target organs (heart,

brain, kidneys and blood vessels) and metabolic changes (Williams, 2010). Abnormal calcium metabolism in hypertensive subjects results in lower concentrations of vitamin D and magnesium, and a higher concentration of parathyroid hormone (PTH), this causes gradual bone loss and decreased bone body mass index (Gealh et al., 2014; Afghani & Goran., 2007).

Formation of trabecular bone in the tibia after a critical size defect is significantly lower by mean percentage area in SHR than in normotensive rats (Bastos et al., 2010). Alveolar bone repair is delayed in SHR, which also exhibit a more severe response to periodontal disease than normotensive animals (Manrique et al., 2012). Blocking AT1 receptors with losartan prevents periodontal bone loss in rats with induced periodontitis (Santos et al. 2015). Since many hypertensive dental patients require rehabilitative treatments of bone quantity and quality, proper treatment is fundamental in oral rehabilitation with implant-supported prostheses requiring substantial bone healing (Afghani & Goran, 2007).

The renin-angiotensin system contributes to regulation of blood pressure and tissue remodeling. In bone, angiotensin II (Ang II) interferes with receptors on osteoblasts and osteoclasts and affects capillary blood flow in the marrow (Al-Majed et al., 2015; Whitebread et al., 1989; Donmez et al., 2012; Yamamoto et al., 2015). Among the drugs currently used are angiotensin II AT1 receptor blockers (BRA II), such as losartan, which is an antagonist of the action of angiotensin II through the specific blocking of its AT1 receptors in vascular smooth muscle and in the adrenal. In this way they inactivate the vasoconstrictor effects and the secretion of aldosterone from angiotensin II (See, 2001).

Losartan is most often used to treat hypertension because it is efficacious, with a low incidence of adverse effects while protecting target organs (Al-Majed et al., 2015; See., 2001).

This drug also increases bone mass by affecting expression of receptor activator nuclear kappa-B ligand (RANKL) and activating osteoclastogenesis (Bastos et al., 2010; Chen et al., 2015). The losartan also reduces endothelial cell adhesion, probably due to decreased blood pressure, thus limiting revascularization of bone grafts fixed to the mandible (Gealh et al, 2014).

The purpose of this study was to investigate losartan's effect on bone healing and dynamics in the alveolar bone tissue of SHR after tooth extraction.

2. Methodology

2.1 Study design and ethics

Thirty male SHRs and 30 male normotensive Wistar rats, weighing 180-230 g were obtained from the Animal Center of School of Dentistry of Araçatuba, UNESP, SP, Brazil. Rats were maintained at a temperature of 22 °C in a 12h light, 12h dark cycle with food and water ad libitum. The study was conducted according to ethical principles of laboratory animal care and national laws on animal use, and was approved by the Animal Research Ethics Committee of School of Dentistry of Araçatuba, UNESP, SP, Brazil (protocol #2008-005158).

2.2 Blood pressure monitoring

Systolic arterial blood pressure (SBP) was measured pre and postoperatively by indirect tail-cuff plethysmography using a Physiograph®MK-III-S (Narco Bio-systems, Houston, Texas, USA) adapted for measurements of rats.(Manrique et al., 2012; Manrique et al., 2015)

2.3 Groups

The rats were divided into the following groups: untreated Wistar, untreated SHR, Wistar treated with losartan, and SHR treated with losartan. Groups consisted of 15 animals, five each of which were evaluated at one of three different times (7, 14 and 28 days) after upper right incisor extraction.

2.4 Treatment

Treated rats were given losartan 30 mg/kg/day diluted in 50 ml water and taken orally over 24 h (Gealh et al., 2014). Treatment was initiated seven days prior to extraction and continued until euthanasia 7, 14, or 28 days afterward. Untreated controls received the same volume of water each day without the drug.

2.5 Surgical procedure

Rats were anesthetized with intramuscular administration of 50 mg/kg ketamine hydrochloride (Vetaset, Fort Dodge Animal Health Ltd., Campinas São Paulo, Brazil) and 10mg/kg xylazine hydrochloride (Dopaser, Calier Laboratory of Brazil Ltda. - Osasco, São Paulo, Brazil). Upper right incisors were extracted utilizing standard techniques (Okamoto and Russo, 1973), and surgical wounds were sutured with sterile absorbable surgical thread (Vicryl 5-0, polyglactin 910, Ethicon®). Each animal received 0.2 mL of the veterinary pentabiotic Small Arms (1.7g/3mL Fort Dodge®) intramuscularly.

2.6 Sample processing

The right maxilla was removed after euthanasia and fixed in formalin solution and 10% 0.1M phosphate buffer (pH 7.4), washed for 24 h in running water, demineralized in 4.13% EDTA (Merck, Darmstadt, Germany), dehydrated in ascending grades of alcohol, and embedded in paraffin. Serial sections 5µm thick were obtained along the long axis of the middle third of the alveolus and stained with hematoxylin and eosin (HE).

2.7 Histomorphometric analysis

Histomorphometric analysis of bone mass and trabecular thickness was performed on two HE-stained slices using light microscopy at a magnification of 200x in a blind setup. Images were captured with a digital camera (JVC TK-1270 Color Video Camera) mounted on the microscope and analyzed with Leica Qwin Color/RGB software. Mean values were statistically analyzed by a two-way ANOVA with Tukey's post-hoc multiple comparison tests at statistical significance $p < 0.05$.

2.8 Immunohistochemical analysis

Immunohistochemical reactions were performed using antibodies on slices incubated in citrate buffer (pH 6.0 at 55 °C) and then incubated for 18 h at 4 °C with anti-OPG and anti-RANKL, (OPG, SC21038; RANKL, SC7627) Santa Cruz Biotechnology, CA, USA) polyclonal goat antibodies at 1:100 dilution. Slices were then triple-rinsed with PBS and incubated with a 1:200 secondary biotinylated antibody (anti-goat IgG-HRP, Pierce, CA,

USA) for 18 h after 1 h at room temperature. After another rinse with PBS, the slices were incubated with streptavidin-biotin complex (StreptABCComplex/HRP; Dako Corp., Carpinteria, CA, USA). Finally, slices were rinsed with 3, 3-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO, USA) and counterstained with Harris HE45 min prior to detection.

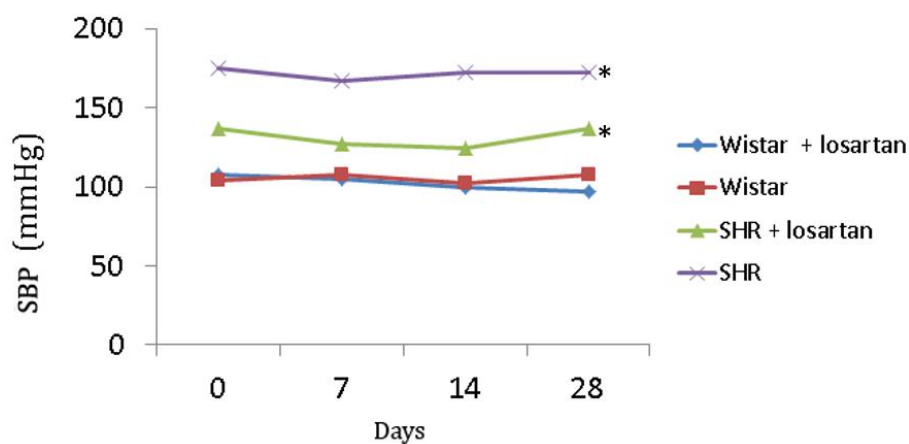
Expression of osteoprotegerin (OPG) and RANKL, proteins were each evaluated semi-quantitatively using goat isotype IgG for the primary antibody, which was suppressed in negative controls. Immunolabeled cells present at each stage of the alveolar repair process were scored by a blind examiner using a conventional optical microscope as described above for immunohistochemistry (light labeling: 1; moderate labeling: 2; intense labeling: 3).

3. Results

3.1 Systolic arterial blood pressure (SBP)

SBP was measured before surgery and in the postoperative periods. The SBP of SHR was higher ($p < 0.05$) than that of Wistar in all periods analyzed. At the beginning of treatment with losartan, a significant reduction in SBP was observed in SHR and this was maintained throughout the treatment period, so SHR treated with losartan showed lower SBP ($p < 0.05$) when compared to untreated SHR (Graphic 1)

Graphic 1 - Wistar rat PAS determined by different colors, in which the points represent the average of the experiments performed ($n = 5$). In addition, $p < 0.05$ was observed between SHR and other groups and $p < 0.05$ between treated SHR and other groups. * $p < 0.05$.



Fonte: Authors.

3.2 Histomorphometric analysis

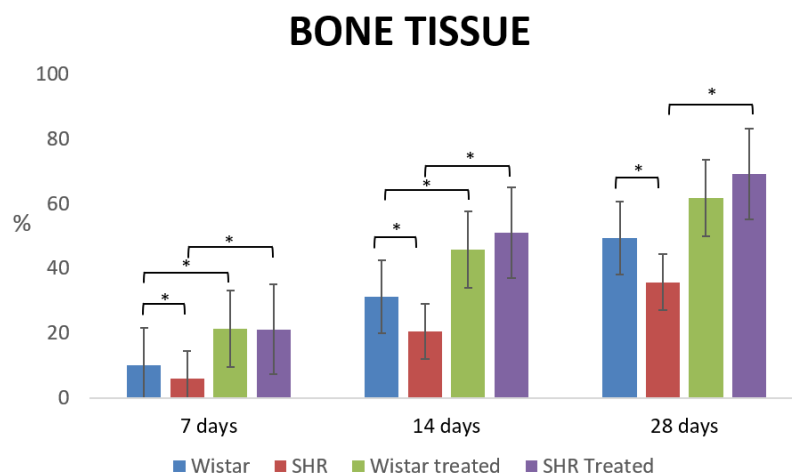
Bone formation gradually increased by percentage in all untreated rats from 7 to 28 postoperative days, and was statistically significant at 28 days. Overall, growth was lower by percentage in SHRs than in Wistar rats. Total bone percentage increased in both Wistar and SHR sockets over the same time, but was lower in SHRs at 7, 14, and 28 postoperative days.

In Wistar rats treated with losartan, bone percentage in the middle third of the alveolus increased between days 14 and 28. A higher percentage of newly formed bone was observed when compared to the untreated Wistar group and both SHR groups ($p < 0.05$ in all periods).

The bone percentage in treated SHRs gradually increased from days 14 to 28, with greater bone formation compared to untreated SHRs and Wistars ($p < 0.05$ for both).

In comparing the Wistar and SHR groups treated with losartan, bone growth percentage was lower in the Wistars at 14 and 28 days, but without statistical significance ($p > 0.05$). (Graphic 2)

Graphic 2 – Comparative percentage of bone tissue in the middle third of the alveolus of Wistar, treated Wistar, SHR and treated SHR rats. The bars represent the average of the values obtained in the different alveolus for each period analyzed. In the multiple comparison of the samples there was statistical difference ($p < 0.05$), between the Wistar and SHR groups 28 days; Wistar and Wistar treated in 7 and 14 days; Wistar and SHR, SHR and Wistar and SHR and SHR treated in all periods (7th, 14th and 28th), except for the treated Wistar group when compared to the treated SHR in which there was no statistically significant difference ($p > 0.05$). * $p < 0.05$.

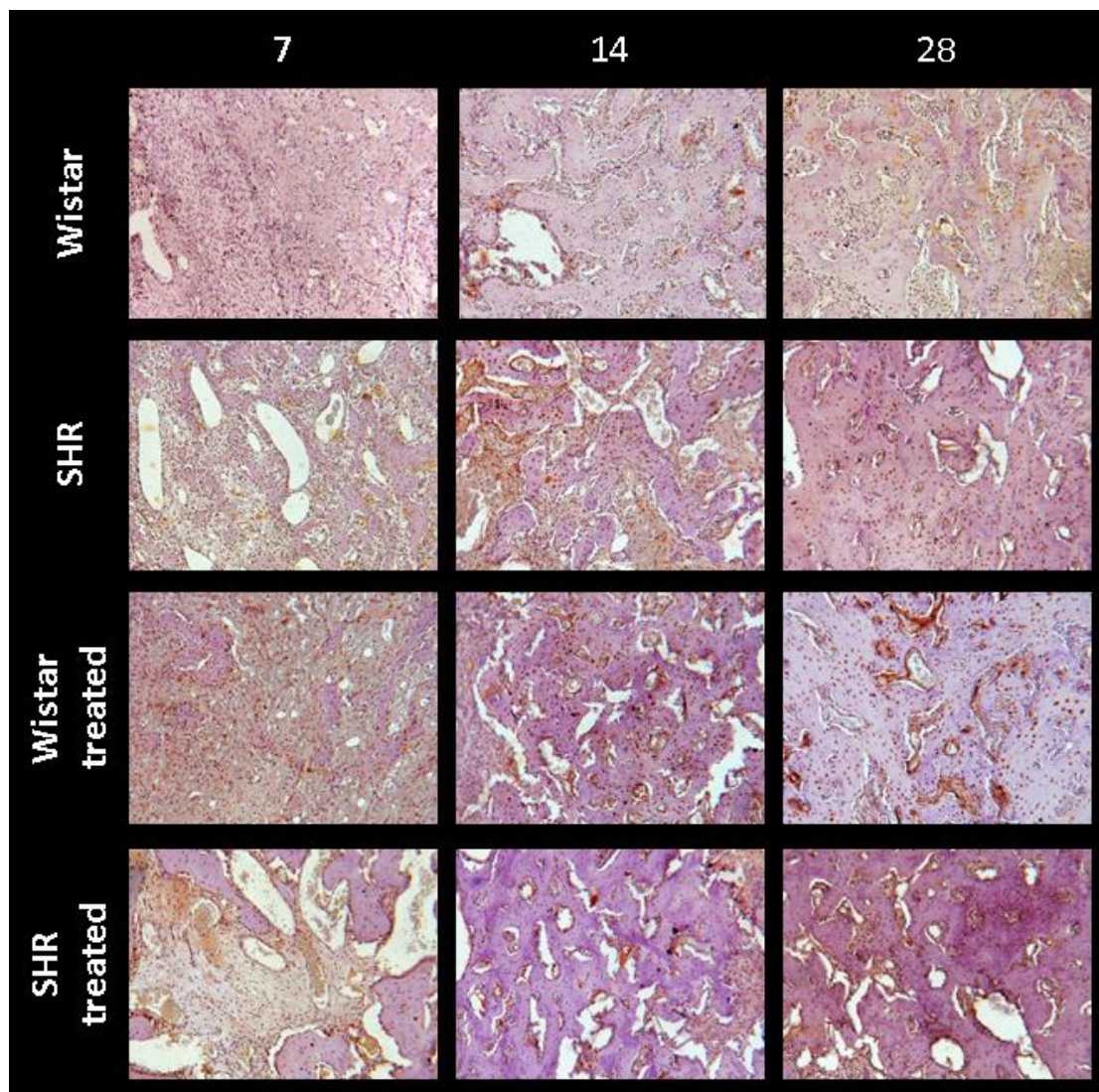


Fonte: Authors.

3.3 Immunohistochemical analysis

Immunohistochemical analysis revealed OPG, RANKL, and osteoblasts in the extracellular matrix of bone-forming tissue after 7, 14, and 28 postoperative days. At all three measurements, OPG appeared with intense markings in the treated Wistar group, while appearing light in treated SHR. Untreated Wistars showed light marking at 7 days, increasing to moderate 14 and 28 days after tooth extraction. In untreated SHR, intense OPG immunostaining was observed 14 days after extraction, but was moderate at 28 days (Figure 1).

Figure 1 – Immunolabeling of OPG for wistar, SHR, wistar treated and SHR treated groups at 7, 14 and 28 days.



Fonte: Authors.

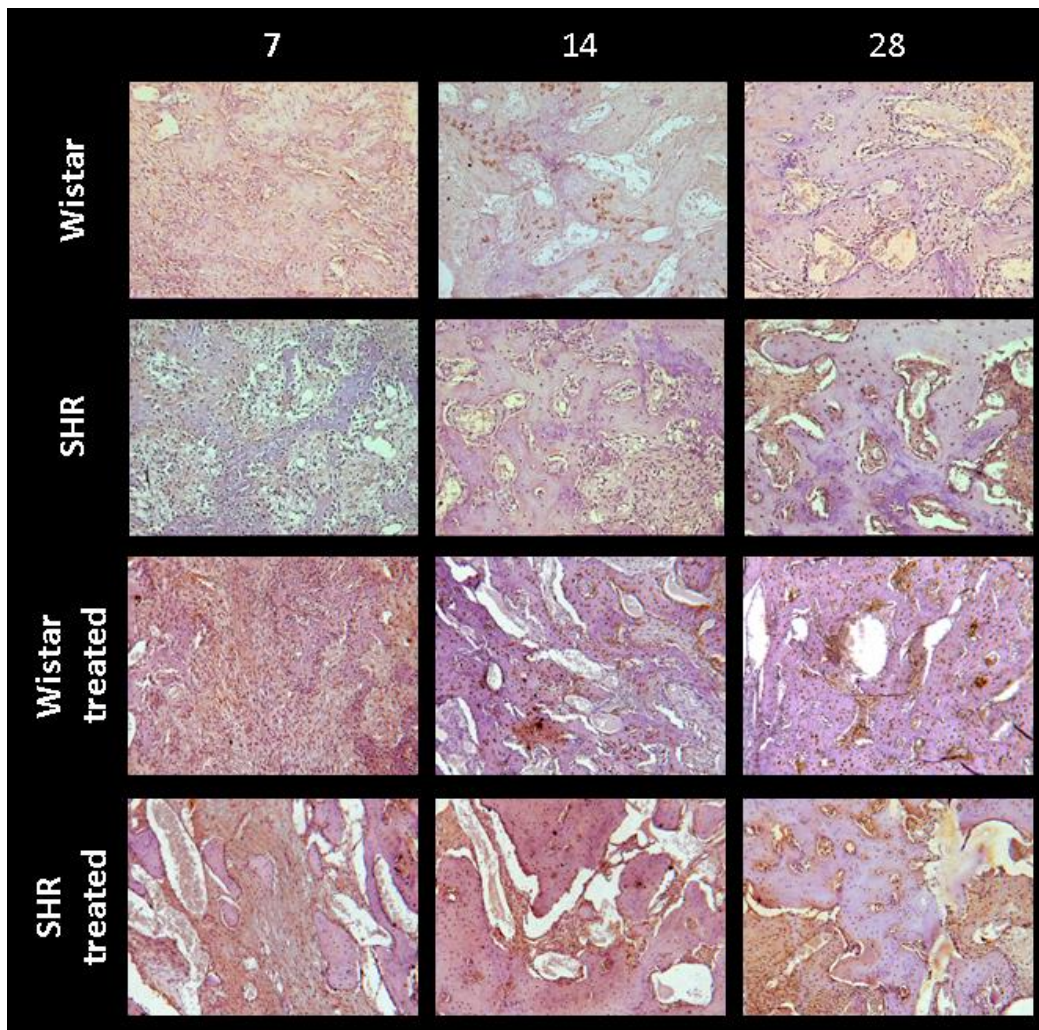
Figure 2 - OPG immunostaining. Score 1 (light), score 2 (moderate), score 3 (intense) in all groups and periods evaluated. (N = 5).

	7 days	14 days	28 days
Wistar	1	2	2
SHR	1	3	2
Wistar Treated	3	3	3
SHR Treated	1	1	1

Fonte: Authors.

Staining for RANKL was intense at 14 and 28 days in treated Wistars. Untreated Wistars and treated SHRs exhibited moderate staining. In the untreated SHR group, RANKL immunostaining was weak at 7 days, moderate at 14 days, and strong at 28 days (Figure 3).

Figure 3 - Immunolabeling of RANKL protein for wistar, SHR, wistar treated and SHR treated groups at 7, 14 and 28 days.



Fonte: Authors.

Figure 4 - RANKL immunostaining. Score 1 (light), score 2 (moderate), score 3 (intense) in all groups and periods evaluated. (N = 5).

	7 days	14 days	28 days
Wistar	2	2	2
SHR	1	2	3
Wistar Treated	2	3	3
SHR Treated	1	2	1

Fonte: Authors.

4. Discussion

This study evaluated the impact of hypertension on post-extraction alveolar repair using SHR, which are hemodynamically similar to humans with essential hypertension (Trippodo & Frohlich, 1981). Angiotensin I (Ang I) and Ang II are potent stimulators of osteoclast activity, suggesting that the renin-angiotensin system plays a role in bone resorption. Ang II is active in regulating hypertension, and can control the growth of bone cells alone or in combination with other regulatory factors by stimulating AT1 receptors and increasing DNA synthesis in cultured osteoblasts (Hatton et al. 1997; Soltis et al. 1993).

The effect of losartan on alveolar repair in SHR was also evaluated in this study. The dosage of 30 mg/kg/day used here confirms some studies where it was observed that treatment of hypertension with losartan also improved the healing process despite lack of statistical differences (Gealh et al. 2014). Analysis of the alveolar repair process revealed a significant difference in bone formed during alveolar repair between SHR and Wistar rats with therapeutic use of losartan; smaller prolonged doses up to 10 mg/kg/day may be employed using other routes of administration to provide similar benefits (Soltis et al. 1993).

The renin-angiotensin system is present in the periodontal tissues of humans and rats (Santos et al. 2015), and osteoblast differentiation is suppressed by Ang II (Hiruma et al. 1997; Santos et al. 2015), supported by the observation in this study of increased bone formation in normotensive Wistar rats. The delayed repair response observed in SHR at 7 and 14 days corroborates previous study, which detected formation of immature alveolar bone tissue in renal hypertensive rats between 9 and 21 post-operative days (kidney-1clip 1) (Carvalho et al. 1983).

Treatment of both Wistar and SHR groups with losartan increased the percentage of bone formation over untreated groups, supporting evidence that losartan increases bone mass by affecting RANKL and osteoclastogenesis (Queiroz-Junior et al. 2015). Similarly, other work reported reduced bone loss in ovariectomized SHR with the use of the Ang II receptor antagonist telmisartan (Ma et al. 2010). Increased trabecular thickness in the treated groups reaffirms the observation of increased bone formation with grafts in SHR treated with losartan (Gealh et al. 2014). As suggested, it is believed that losartan decreased osteoclast differentiation by acting on AT1 receptors and inhibiting Ang II (Shimizu et al. 2008).

Increased expression of RANKL in SHR, which have higher concentrations of Ang II and increased osteoclastia, confirms the role of hypertension in bone metabolism. Samples from Wistars treated with losartan also exhibited intense immunostaining of RANKL at 14

and 28 days, but the presence OPG at all three post-operative measurements indicates accelerated bone turnover in both groups. This turnover was slower in treated SHR's due to decreased osteoclast activity, as indicated by light staining for OPG and RANKL. These results suggest that inhibitors of Ang II AT1 receptors interfere with bone metabolism. Previous studies found that Ang II induces RANKL expression in osteoblasts, increasing the number of TRAP cells and leading to osteoclast activation (Shimizu et al. 2008, Moura et al. 2016). These observations are supported here by increased turnover, indicated by progressively intensifying TRAP staining from untreated SHR's through Wistar to SHR's treated with losartan. Hypertension is a major risk factor for cardiovascular disease characterized by endothelial dysfunction and altered control of vascular cell growth (Tea et al. 1999). Studies have shown that hypertension impairs post-ischemic neovascularization in cardiac tissue due to decreased angiogenic potential progenitor such as VEGF (You et al. 2008; Emanuelli et al. 2001). Losartan treatment could be acting in the angiotensin II vasoconstricting response, once the improvement of vascularization of bone tissue, could be an important factor related to the better bone formation response.

Skeletal muscle capillary density in SHR's can be increased using antihypertensive drugs such as losartan and enalapril (Rizzoni et al. 2008). Losartan blocks the renin-angiotensin system, preventing or reversing the microvascular rarefaction in rat hearts and brains (Munzenmaier et al. 2007). This study suggests that vasodilation caused by antihypertensive treatment resulted in higher-scoring vascular markers. The combined use of antihypertensive ACE inhibitors and diuretics was demonstrated to increase the number of circulating progenitor cells, restoring the angiogenic potential of SHR's (You et al. 2008). According to others works the use of antihypertensive drugs in hypertensive models leads to recovery of angiogenesis; probably related to peripheral vasodilatation afforded by the drug (Munzenmaier et al. 2007; Rizzoni et al. 2008; You et al. 2008).

Owing to the complexity of bone metabolism and the range of mediators involved in maintaining mineral homeostasis, many further studies are needed to understand the phenomena involved in bone dynamics, particularly regarding associated systemic changes.

5. Final Consideration

This study demonstrates that alveolar repair begins later in hypertensive subjects. Losartan influences this process in both hypertensive and normotensive subjects, resulting in greater bone formation and trabecular thickness in treated groups. The renin-angiotensin-

aldosterone system interferes with bone metabolism, while losartan affects the dynamic expressions of OPG protein and RANKL in bone healing.

Acknowledgments

This study was financially supported by the São Paulo Research Foundation – FAPESP with process numbers 2009/11227-0 and 2010/00173-4.

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