Bone Healing in Drill Hole Defects in Spontaneously Hypertensive Male and Female Rats' Femurs. A Histological and Histometric Study

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Objective: The aim of this study was to evaluate the bone healing in spontaneously hypertensive rats (SHR) and compare the results with normotensive rats, evaluating male and female animals.

Methods: A bone drill defect was created in the left femur of 24 SHR (12 males and 12 females) and 24 normotensive rats (12 males and 12 females). The animals were divided into two groups and sacrificed 7 and 21 days after the surgical procedure. After the routine laboratory processing, histological and histometric analysis were carried out and data were submitted to ANOVA and Tukey's test (5%).

Results: Males and females from the same group had similar histological characteristics. After seven days, all animals presented irregular bone trabeculae. The periosteal osteoblasts were flattened in SHR, and presented a cuboid shape in normotensive animals. After 21 days, the bone defects of all specimens showed a linear closure in all the superficial extension. In addition, SHR presented flattened osteoblasts surrounding the bone trabeculae, while normotensive ones showed cuboidal cells. Statistical analysis of the histometric data indicated similar means between the male and female groups, except for normotensive rats on day 7. In addition, a larger amount of new bone formation was observed in hypertensive when compared to normotensive rats on day 21, in males as well as females.

Conclusion: We conclude that bone healing in SHR was more significant than in normotensive ones, as shown by the histological and histometric evaluation 21 days after surgery.

Key words: Femur/abnormalities, hypertension, rats, inbred (SHR).

Systemic arterial hypertension is one of the most important world's health problems. Some bone pathologies may be aggravated in hypertensive individuals due to their pattern of calcium regulation, which induces bone loss in hypertensive human individuals[1-3] as well as in spontaneously hypertensive rats (SHR)[4-6]. Thus, bone mineral density (BMD) is reduced in hypertensive humans[1-3] and in SHR[4-6], when compared to normal normotensive controls.

Despite the possibility that this pathology can affect both sexes, most experimental studies were carried out in male rats. Liang et al[7] stated that more detailed investigations of bone behavior in female rats were necessary, due to the fact that, in humans, osteoporosis is more prevalent in women than in men.

The connection between hypertension and bone metabolism is evident; however, the process of bone repair in hypertensive individuals is little known. Hence, the aim of this study was to evaluate, histological and histometrically, the process of bone repair in SHR of both sexes, comparing the results to those obtained from normotensive animals.

Methods

This study was approved by the Ethics Committee of the Institution (Protocol # 051/2002 PA/CEP).

A monocortical bone defect (2mm) was created in the local of largest diameter in the proximal region of the left femur diaphysis of 48 rats (24 hypertensive and 24 normotensive rats). Seven days after the surgery, 12 normotensive rats and 12 SHR (six males and six females from each group) were sacrificed. The remaining animals were sacrificed 21 days after the surgery that created the bone defect.

After the sacrifice, the femurs were removed, fixed in a 10% formaldehyde solution and decalcified in a Plank-Rychlo's solution. After histological processing, 5 μm thick slices were obtained, which were hematoxylin-eosin (HE) stained.

The HE-stained slides were then examined under light microscopy and the histological aspects of the neoformed bone were evaluated, based on the remodeling of the immature bone trabeculae, the morphology of the osteoblasts and osteocytes and the periosteum characteristics.

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For the histometric analysis, five slices per animal were selected and photographed in their central area. The measurements were performed using a reticulum created by an image analysis program (Image-J 1.32 for Windows; National Institutes of Health, Bethesda, USA), containing 100 equidistant intersection points (total area: 3,000μm^2). The reticulum was then placed on the image and the points that overlapped the neoformed bone trabeculae were counted (Fig. 1). The data obtained from this measurement were tabulated and submitted to descriptive and inferential statistical analysis. At the first, the data were expressed as means and standard deviations and at second, the analysis of variance (ANOVA – 3 factors) was applied as well as Tukey's test (5%) in order to correlate the values of bone neoformation with the presence of hypertension, gender and the time of the sacrifice.

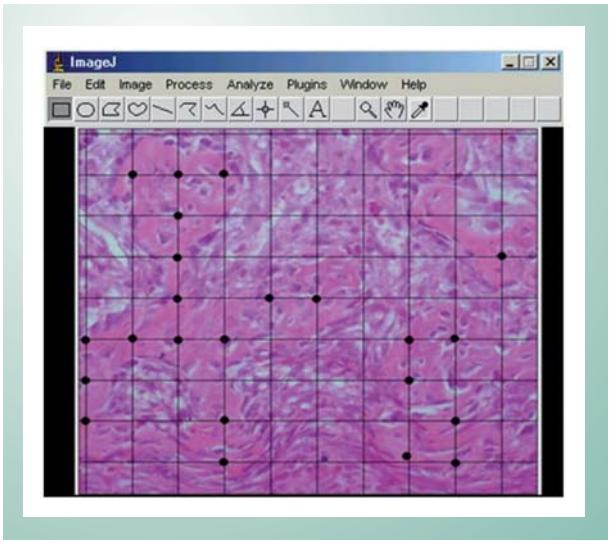

Fig. 1 - *Histometric analysis carried out with the help of a reticulum placed on the image, aiming at counting the points that overlapped the neoformed bone trabeculae.*

Results

Histological findings - The normotensive and hypertensive animals presented, after seven days, similar general histological characteristics of bone neoformation, with small variations in the cells close to the periosteum. On the 21st day post-surgery, normotensive and hypertensive animals presented distinct characteristics. It was also verified that males and females from the same group presented similar behaviors.

On the 7th day post-surgery, all animals presented the defect area filled by delicate and intertwined bone trabeculae (Fig. 2a), in addition to the intertrabecular space filled by conjunctive tissue. The neoformed trabeculae contained large osteocytes and were surrounded by cuboid osteoblasts in all specimens (Fig. 2b).

The normotensive animals presented, in their majority, red blood cells forming a clot on the surface of the fibrous conjunctive tissue (Fig. 2a) and cuboid osteoblasts in the periosteal region. However, these findings were not observed in SHR, considering that these animals had flattened osteoblasts on the periosteal surface (Fig. 2b).

Twenty-one days after the surgery, all specimens were characterized by a layer of neoformed bone joining the borders of the defect, as a bridge (Figs. 3a-b), and this showed to be thicker in SHR (Fig. 3b). The neoformed bone trabeculae were dense and the intertrabecular fibrous conjunctive tissue was substituted by hematopoietic tissue in all specimens, from normotensive as well as hypertensive animals. The bone trabeculae contained small osteocytes, reverse lines, and flattened osteoblasts in the periosteal region. At the surgical borders, there was union of the neoformed bone with the mature adjacent bone.

Still on day 21, the bone trabeculae of the normotensive animals showed to be recovered by cuboid osteoblasts while flattened cells recovered the bone trabeculae of SHR (Figs. 4a-b).

Histometric findings - The data obtained are summarized in Table I and are represented by a column chart in Figure 5.

The data were submitted to ANOVA (3 factors) to evaluate the association between the presence of hypertension, gender and the sacrifice period. The interaction effect among all variables was not significant (p=0.33).

Tukey's test (5%) was performed and the formation of homogeneous groups is represented in Figure 6.

The statistical analysis showed that the means of neoformed bone percentage were similar for males and females, except for the normotensive rats on day 7. It is possible to state that the condition in which there was a higher percentage of bone formation was that disclosed by hypertensive animals on day 21, in males as well as in females.

Discussion

The present study compared the healing of bone defects in the femurs of normotensive and hypertensive rats of both sexes, on days 7 and 21, post-surgically. At the shortest period, bone neoformation was similar between normotensive and hypertensive animals, with the defect being filled by immature bone trabeculae in all specimens. On day 21, there was a linear closure of both groups; however, the amount of neoformed bone in the defect region was higher for the hypertensive animals. Additionally, there were no differences between males and females from a same group.

The SHR presented an alteration in the process of bone turnover, when compared to the normotensive ones[5,8,9]. Despite the knowledge of these data, we did not find studies in literature that compared the process of bone healing between SHR and normotensive ones.

Due to the bone metabolic alterations, such as low calcium content and increased resorption rates, SHR could be characterized as osteopenic[5,8,9] or osteoporotic[9] animals. In osteoporotic normotensive animals, bone healing is considered deficient when compared to that of normal animals[10-13].

Nevertheless, in the present study we observed that, 21 days after the bone defect was created, bone healing in the SHR was more significant, histological and histometrically, than that observed in normotensive animals.

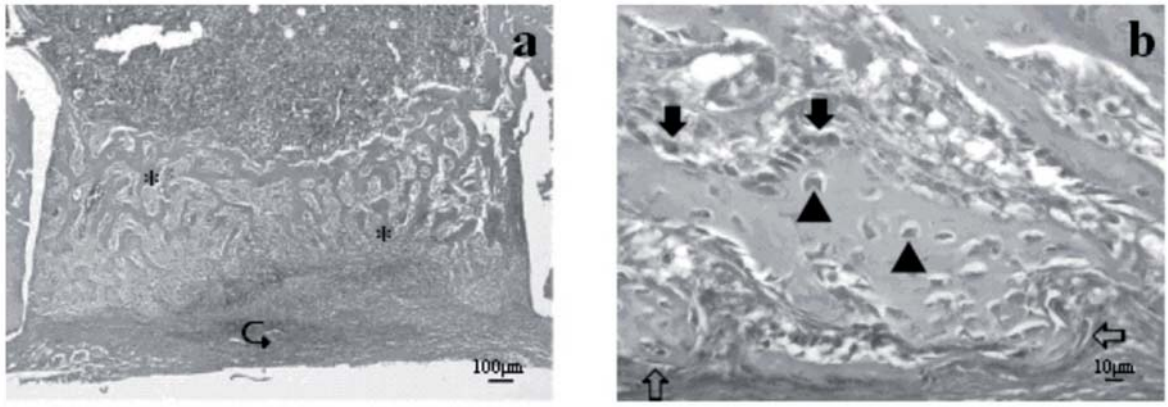

Fig. 2 - *Microscopic morphology of bone defects in the rats sacrificed 7 days after surgery: normotensive (a) and hypertensive (b). Delicate bone trabeculae (*) and red blood cells in the superficial region (↳). (b) Large osteocytes (▶), flattened osteoblasts (⇨) and cuboid osteoblasts (➡). HE.*

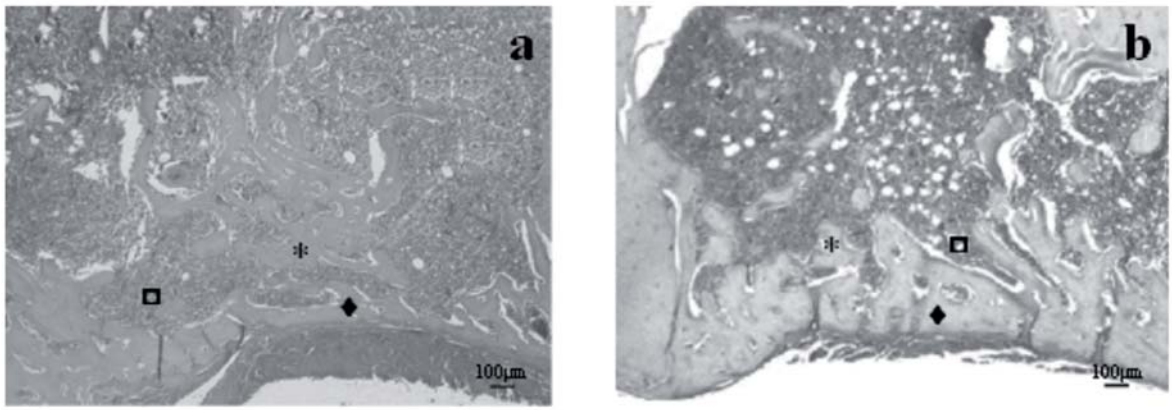

Fig. 3 - *Microscopic morphology of bone defects in the rats sacrificed 21 days after surgery: normotensive (a) and hypertensive (b). Bone bridge joining the defect borders (♦) and bone trabeculae (*) intertwined with hematopoietic (◘). HE.*

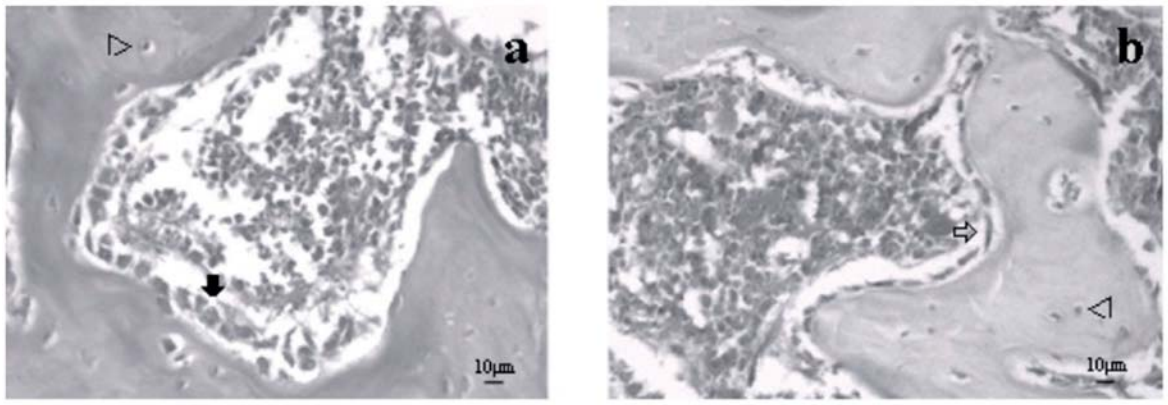

Fig. 4 - *Microscopic morphology of bone defects in the rats sacrificed 21 days after surgery: normotensive (a) and hypertensive (b). Small osteocytes (▷). (a) Cuboid osteoblasts (➡). (b) Flattened osteoblasts (⇨) in the periphery of the trabeculae. HE.*

	Normotensive		Hypertensive	
	7 days	21 days	7 days	21 days
Males	15.94±5.78	42.75±6.79	27.44±8.95	69.78±16.24
Females	37.05±6.26	37.03±12.40	27.05±17.16	55.33±10.09

Table 1 -Percentage data of bone neoformation (mean ± standard deviation) regarding the hypertensive and normotensive groups, males and females, on day 7 and day 21 (number of animals=6)

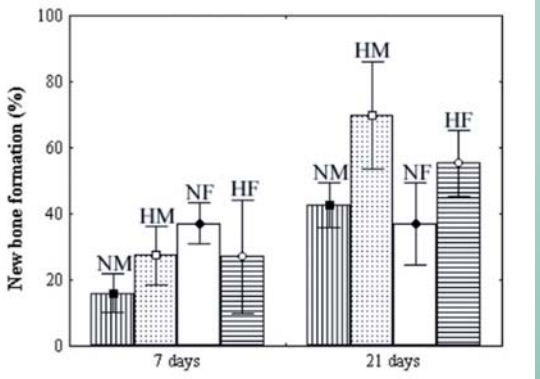

Fig. 5 - *Column chart (mean±SD) for the neoformed bone percentage data in normotensive males (NM) and hypertensive males (HM), normotensive females (NF) and hypertensive females (HF), at days 7 and 21.*

Twenty-one days after the bone defect was created, male and female SHR presented a higher percentage of neoformed bone than the normotensive animals of both sexes, and histologically, the first presented flattened osteoblasts in the periphery of the bone trabeculae, characterizing their quiescent aspect[14-15]. This fact led us to suppose that in SHR, the osteoblasts reduced or even ceased the production of bone matrix during this period, whereas in the normotensive animals, the large osteoblasts were still active and could reproduce the matrix for a longer period. Consequently, we can suppose that, over a longer experimental period, bone healing in SHR might be inferior to the one observed in normotensive animals.

At the end of the 7-day period, no difference was observed regarding the amount of neoformed bone when the normotensive animals were compared to the SHR. However, some distinct histological characteristics were observed between the groups. Most of the SHR did not show hemorrhagic sources in the superficial area of the defect, suggesting an accelerated substitution of blood clots by granulation tissue. In addition, the presence of flattened osteoblasts in the periosteum of hypertensive animals also suggests a more advanced healing process in these animals, when compared to the normotensive ones.

Factors related to tissue neoformation, degradation or inflammation can exert a major role during tissue repair.

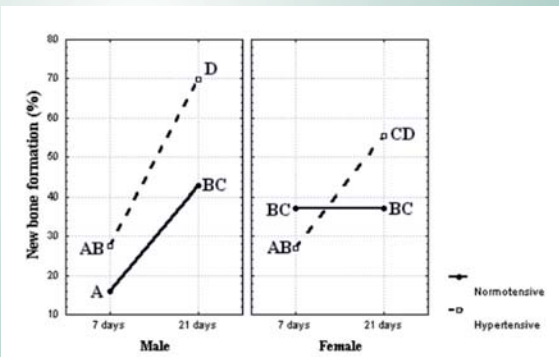

Fig. 6 - *Means of data of the experimental conditions (neoformed bone expressed in %) in male and female, normotensive and hypertensive rats, on day 7 and 21. Values with similar letters are not significantly different (Tukey with p=0.05).*

Recent studies have investigated the role of angiotensin II (Ang II) in the inflammation and tissue repair processes[16-18]. Thus, the higher amount of bone formation in hypertensive animals on day 21 after the creation of the defect might have occurred due to other metabolic factors, apart from the bone-related ones.

Hypertensive humans present increased levels of Ang II, which is related with the initial phases of the inflammation process through the recruiting of inflammatory cells[19,20], and also with tissue repair and remodeling through cell growth and matrix synthesi[21]. Recent *in vitro* and *in vivo* studies have shown that Ang II presents a pro-inflammatory action[16] and fibrinogenic effect on the kidney[22].

In cultures of cells that are similar to osteoblasts, Ang II stimulates cell proliferation by DNA synthesis[23,24] and increases the formation of collagen by the osteogenic cells[24].

Therefore, one can hypothesize that the increased levels of Ang II (present in hypertension) could stimulate the formation of bone matrix in SHR. However, the degree of mineralization of the neoformed tissue is questionable, as Hagiwara et al[25] demonstrated that Ang II reduces the process of mineralization of the matrix formed by osteoblasts *in vitro*. In the present study, as the material was demineralized, it was possible to evaluate the degree of mineralization in the neoformed bone tissue, but it can be suggested that although the hypertensive animals presented a larger amount of bone tissue, the quality of this tissue is uncertain, due to the possibility of its presenting less mineral content.

The differences between the bone characteristics of male and female SHR are scarcely reported in literature. DeMoss et al[26] evaluated the dry weight and calcium content in the skeleton of male and female normotensive and SHR rats, and observed that the dry skeletal mass presented higher values in females when compared to males; however, the calcium content per skeletal unit was similar among the different groups. On the other hand, Wright et al[9] observed an increase in bone resorption in female SHR, while our findings showed similar bone neoformation means between the genders, as well as similar histological characteristics.

Therefore, we conclude that the presence of hypertension interferes in the process of bone healing in rats, considering that 21 days after the surgical procedure, the percentage of bone formation in hypertensive rats was higher than that observed in normotensive animals, although there was no difference between the healing process of bone defects between males and females from the same lineage.

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