Local Action of Sodium Alendronate in Bone Repair of Spontaneously Hypertensive Rat (SHR)

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Summary

Background: The arterial hypertension is a disorder characterized by relevant alterations in the bone tissue. The sodium alendronate is indicated in the treatment of bone diseases, because of its affinity with the hydroxyapatite, inhibiting the bone reabsorptions.

Objective: To analyze local action of the sodium alendronate in the bone repair of spontaneously hypertensive rat (SHR).

Methods: A bone defect was created in the left femur of 80 rat. In agreement with the material used at the place, four groups were created: control (C), starch (St), alendronate 1 mol (A1) and alendronate 2 mol (A2). After 7 and 21 days, the animals were sacrificed. Histomorfometrical and histological analyses were accomplished and the data were submitted the variance analysis (ANOVA) and test of Tukey (5%).

Results: At 7 days, in the area of the defect, conjunctive tissue with hemorrhage and inflammation in all of the groups was observed. Some presented osteoid matrix. The groups A1 and A2 presented, further, a fibrin net. At 21 days, the bone trabeculae practically closed the extension of the defect in the groups C and St. In the group A1 of male animals, trabeculae were observed that irradiated from the medullary canal to the area of the defect. In the groups A1 and A2, only presence of conjunctive fabric with low osteoid deposit was observed. An outstanding histological discovery was the formation of extracortical subperiosteal bone tissue in animals of the groups A1 and A2.

Conclusion: The administration of sodium alendronate did not contribute to bone repair in SHR rat, but possibly has been responsible for the extracortical bone formation observed. (Arq Bras Cardiol 2008; 90(4): 239-246)

Key words: Alendronate; rats, inbred SHR; hypertension; bone regeneration.

Introduction

The systemic arterial hypertension consists of a disorder of great prevalence in the world population, that can cause metabolic alterations in the bone tissue of the indivíduals[1-4].

The existent relationship between the alterations of the calcium metabolism and the tendency to bone loss and decrease of bone mineral density in hypertensive individuals is proven. The main alterations of calcium metabolism evidenced in the systemic arterial hypertension are represented by elevation of parathormone serum and ionized calcium levels, by the decrease of bone calcium content and by hypercalciuria[1-4]. Spontaneously hypertensive rat (SHR) have been used as study model of the arterial hypertension, as these animals reproduce the disease with a lot of similarity to manifested in humans, as they present the same abnormalities in the calcium metabolism[2,5,6]. Besides, the SHR rat are considered bone defficient animals, as they present high rate of skeleton remodeling, with prevalence of the bone reabsorption over the process of new bone formation[7].

In spite of studies demonstrating that the systemic arterial hypertension affects the metabolism of the bone tissue, little is known regarding the influence of this disease specifically in the bone repair. Pereira at al[8] verified that the bone formation in hypertensive animals was larger than observed in normotensive animals.

As bone alterations are recognized in individuals with arterial hypertension, in this study the sodium alendronate was used as medication in the place of the bone defect, in order to observe their effects in the bone repair in SHR rat.

The sodium alendronate is a medication of the bisphosphonates group, largely used in the treatment of diseases characterized by bone reabsorption[9,10]. However, little are the researches regarding its use, local or systemic, stimulating the bone formation[11-14]. The form of administration and the dosage are important factors to be elucidated, since controversies exist regarding the real mechanism of action of this medication and of its action on the bone tissue of hypertensive individuals.

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Methods

This study was approved by the Institutional Review Board of *Universidade Estadual Paulista Júlio de Mesquita Filho - São José dos Campos* (protocol nº 013/2006-PA/CEP).

A monocortical bone defect of 2,5 diameter mm was created in the area proximal of the tibial shaft of the left femur of 80 SHR rat (40 males and 40 females). The animals were six months of age and they weighed 300 g approximately.

Four experimental groups of 20 animals were created: C (control), St (starch), A1 (alendronate 1 mol) and A2 (alendronate 2 mol). In the control group (C), the rat were submitted to surgery only, not receiving filling of the defect. The animals of the starch (St group), after surgery, only received the starch, excipient used in the experiment. The animals of group A1 received 1 mol of sodium alendronate, associated to the starch, for the complete filling of the bone defect. Finally, the group A2 had the surgical defect totally filled out by 2 mol of sodium alendronate, also associated to the starch.

Following the protocol developed by Fernandes[12], in 2005, the local administration of sodium alendronate was accomplished in the amount of 1 mol (A1) and 2 mol (A2) for the filling of the bone defects. The proportions of the materials were calculated in agreement with the molecular weight of the sodium alendronate, that is equal to 325 g/mol. The calculation of the amounts in weight was accomplished using 0,01 mol to measure the appropriate volumetric amounts to the size of the bone defect, that is, 3,25 g were used in the experimental group A1 and 6,5 g in the experimental group A2. With this amount of material, 26% of the defect were filled out with sodium alendronate in the group A1 and 52% in the group A2. The remaining of the bone defects were filled out with 74% and 48% of starch, respectively.

The animals were sacrificed through anesthesia and decapitation by guillotine, 7 and 21 days after surgery. The femurs were removed, preserved in formaldehyde solution to 10% and decalcified in solution of formic acid to 20%, during approximately 10 days. After the histological processing, blades stained with hematoxilin and eosin (HE) were examined under light microscopy and the histological aspects of the newly formed bone were appraised in the whole extension of the defect. The characteristics of the bone trabeculae, medullary spaces and remodelation of the tissue were observed. The morphology of the bone cells and periosteo was also analyzed and described.

For the histomorfometrical analysis, six cuts of each specimen were selected and the blades were photographed in the center of the defect, with original increase of 100 times. The digitalized images were transferred to a microcomputer and submitted the histometrical analysis of the newly formed bone, through the program of image nalysis Image-J (version 1.32 for Windows; National Institutes of Health, Bethesda, United States). A retícule (small net) with 100 points of equidistant intersection (total area of 3.000 μm^2) was positioned over the image and the points that were placed upon the newly formed bone tissue were counted. The results were transformed in percentual averages of bone new formation and submitted to descriptive statistical analysis and the variance analysis to three factors (ANOVA), using as significance level the margin of error of 5%. The three analyzed factors were: the treatment accomplished in each experimental group (C, St, A1 and A2), the gender of the animals (male and female) and the observation period (7 and 21 days). When statistically significant differences were found, the test of multiple comparison of Tukey was applied (5%). For the accomplishment of statistical analysis and making of the graph, the softwares Statistica 6.0 and Excel 2003 were used.

Results

Histological analysis

At 7 days, in all experimental groups a slack conjunctive tissue, with hemorrhage focuses and infiltrated inflammatory occupying the area of the bone defect were found. In this same period, some specimens of the groups C and St presented focuses of osteoid tissue amid the conjunctive tissue (fig. 1a). All of the specimens regarding the groups A1 and A2 presented a fibrin net in the conjunctive tissue of the bone defect (fig. 1b). Some specimens of the groups treated with sodium alendronate also presented hydropic degeneration in the conjunctive tissue (fig. 1b). In this phase, the organized periosteo covering again the area of the bone defect was not observed.

A histological characteristic observed at 7 days in the groups A1 and A2, specially in the latter, was the presence

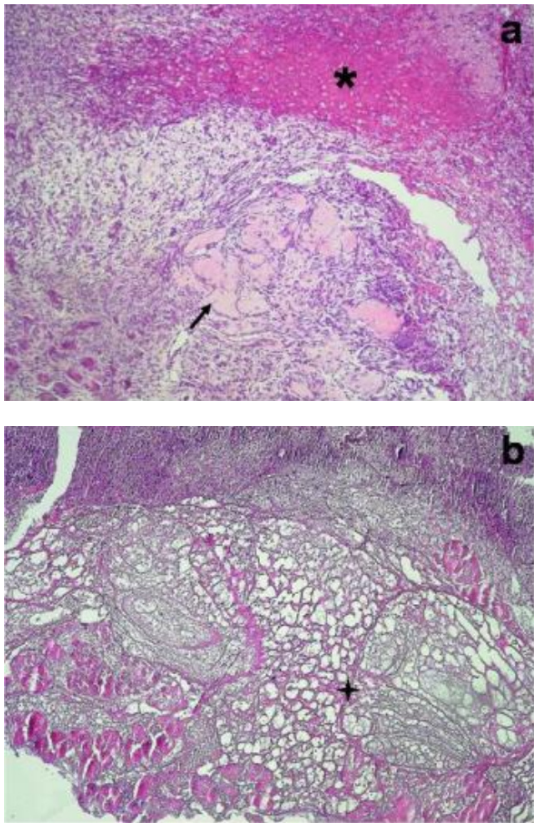

Figure 1 - Defect at 7 days: (a) Group St: hemorrhage (✱) and focuses of osteoid matrix (→). Increase 200x. HE. (b) Group A1: fibrin net (✦) and hydropic degeneration. Increase 200x. HE.

of small extracortical subperiosteal bone formations in the region opposite to the bone defect (figs. 2a-b). The extracortical subperiosteal newly formed bone tissue presented characteristics of immature bone tissue, with thin bone trabeculae, joined by anastomosis and limited wide medullary spaces containing slack conjunctive tissue. This formation was more evident in the females when compared to the male animals. The newly formed tissue contained bulky osteocytes in great quantity, that were sheltered in wide gaps. The periosteo and the muscular tissue covered again the newly formed bone tissue.

At 21 days, the closing of the defect was verified in continuation to the cortical tissue adjacent to the defect in the groups C and St (fig. 3a). The tissue appeared to be formed by lamellar bone with gaps containing less bulky and flat osteocytes (fig. 4a). The osteoblasts of flat format bordered the bone trabeculae. In some cases, already the presence of basophilic reverse lines was evidenced, suggesting the occurrence of bone remodelation.

The periosteo appeared to be very organized in animals of the groups C and St. This was constituted by a superficial layer of fibrous conjunctive tissue and a deep layer formed by flat osteoblasts (fig. 4a).

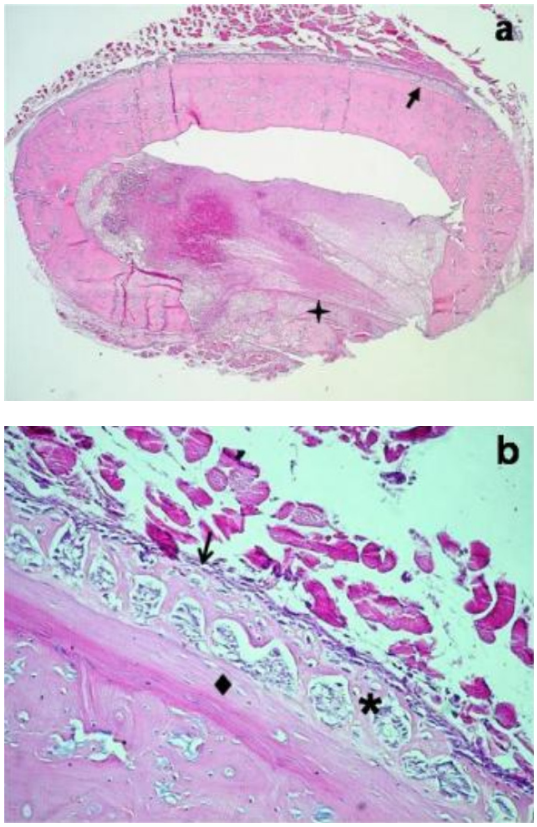

Figure 2 - Group A2 at 7 days: (a) Panoramic view of the defect at 7 days (✦) and of the extracortical subperiosteal bone formation (➙). Increase original 100x. HE. (b) Details of the extracortical subperiosteal bone formation: newly formed bone tissue (✱) covered by the periosteo (➔) and the external cortical bone (♦). Increase original 200x. HE.

In the male animals of the group A1, at 21 days, presence of immature bone trabeculae was observed, interposed by slack conjunctive tissue, that irradiated from the interior of the medullary canal to the area of the defect, however without closing it entirely (figs. 3b and 4b). This finding was not observed in the females, that presented, in the area of the defect, only dense conjunctive tissue, with deposit of collagen fiber bunches and, in some cases, deposit of osteoid matrix. In the animals of the group A2, no new bone formation with the closing of the bone defect in the period of 21 days was observed.

The extracortical subperiosteal bone formation observed in the area opposite to the bone defect were present in the groups A1 and A2 at 21 days and they were more expressive in the females comparad to the males. The tissue, covered by periosteo and muscular tissue, contained osteocytes in great quantity, that were sheltered in gaps of smaller proportions, compared to the groups of 7 days. The medullary spaces containing slack conjunctive tissue and blood vessels were also reduced. The bone presented greater maturation (figs. 5a-b), with evident characteristics of bone remodelation, by the presence of basophilic reverse lines.

Histomorfometrical and statistical analysis

The result of the descriptive statistical analysis is represented

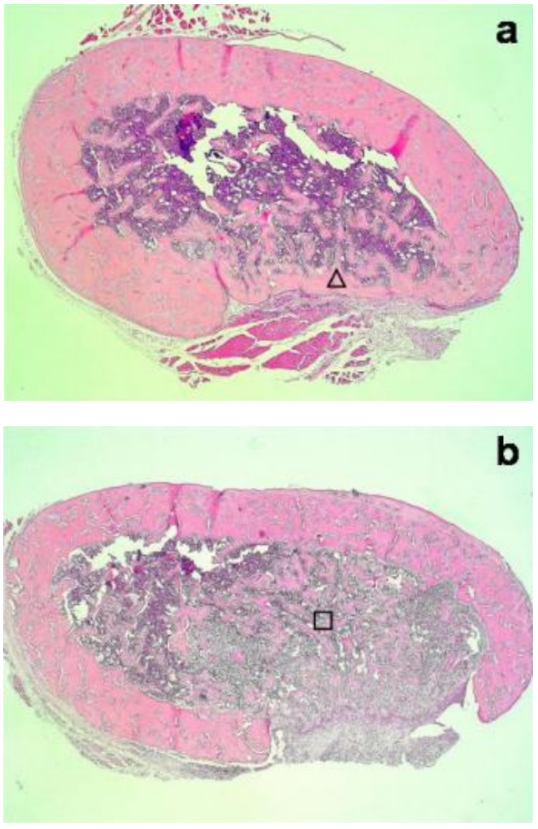

Figure 3 - Defect at 21 days: (a) Group C: total closing of the defect with newly formed bone tissue (△). Increase original 100x. HE. (b) Group A1 of male animals: bone trabeculae (□) starting from the bone marrow to the defective area.

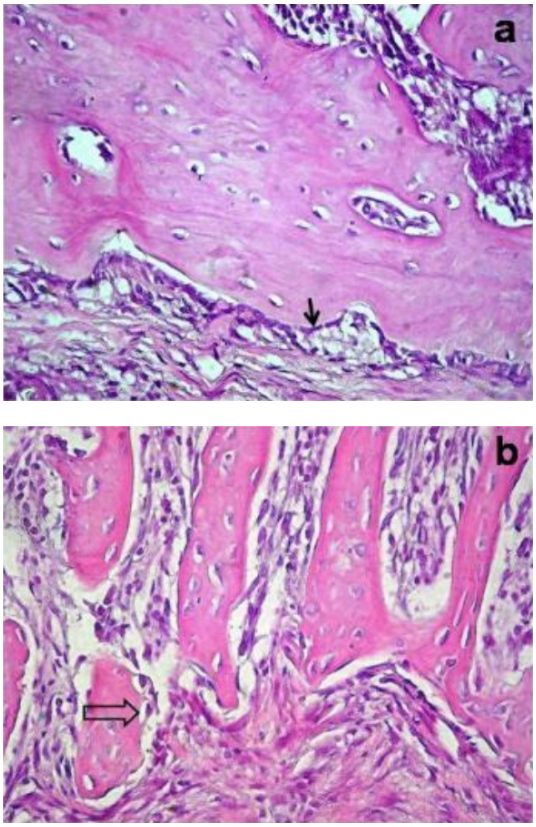

Figure 4 - D defect at 21 days: (a) Group C: mature bone with lamellar organization, flat osteoblasts forming the periosteo (→). Increase original 400x. HE. (b) Group A1 male: thin trabeculae delimiting spaces with conjunctive tissue, bulky osteocytes in wide gaps (⇨). Increase original 400 x. HE.

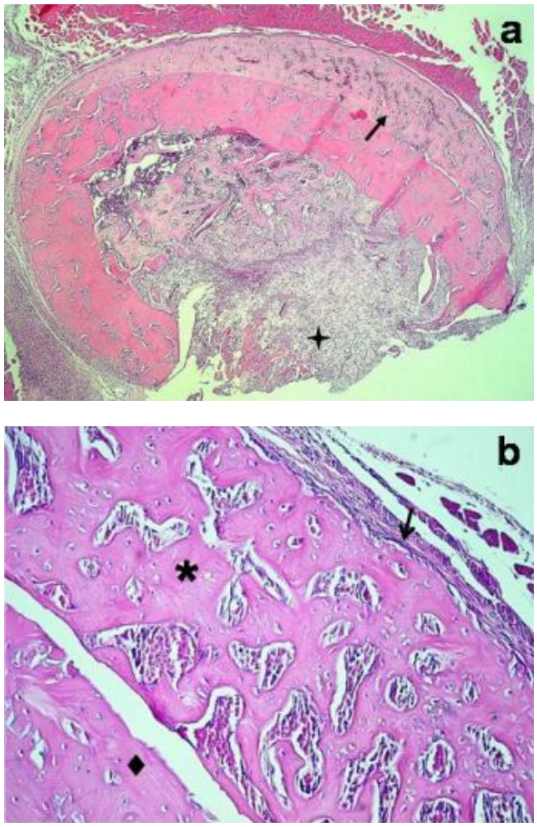

Figure 5 - Group A2 at 21 days: (a) A panoramic view of the defect at 21 days (✦) and of the extracortical subperiosteal bone formation (→). Increase original 100x. HE. (b) Details of the extracortical subperiosteal bone formation: newly formed bone tissue (✱) covered by the periosteo (➔) and the external cortical bone (♦). Increase original 200x. HE.

in illustration 6, through graph of columns. The graph refers to the percentual averages and the standard deviation-pattern of the newly formed bone data in the different experimental groups (C, St, A1 and A2), at 7 and 21 days, in male and female SHR rat.

The ANOVA variance analysis demonstrated that the effect interaction among the gender of the animals ($p = 0,34$) and between gender and observation period ($p = 0,06$) was not significant. The test of Tukey (5%) was accomplished and the formation of the homogeneous groups is represented in table 1.

The results demonstrated that, at 7 days, the group C of male animals presented larger bone formation. At 21 days, the animals of the groups C and St exhibited larger bone formation. The group A1 of male animals was the only that demonstrated bone formation in the area of the defect at 21 days, with result closer to the groups C and St. The remaining of the animals treated with alendronate at 21 days demonstrated smaller new bone formation in the area of the defect.

Discussion

The systemic arterial hypertension consists of a disease related to important bone alterations, that can cause mainly the decrease of the bone mineral density[1,4,15-17]. Some authors suggest the existence of positive relationship between the alterations of the factors of regulation of the calcium and the tendency to the bone loss in hypertensive individuals[18,19].

In the present study, the local action of sodium alendronate was evaluated in the repair of bone defects in the femur of SHR rat of both gender, at 7 and 21 days. It was observed that the SHR rat demonstrated more retarded bone repair when compared to study by Pereira et al[8], accomplished in SHR rat, and to the study of Fernandes[12], accomplished in normotensive rat of the Wistar-Kyoto (WKY) lineage. The results of this study confirm data of the literature regarding presence of bone alterations in hypertensive animals.

Studies accomplished in SHR rat demonstrate that, in the course of time, the hypercalciuria and the secondary activation of the parathyroid glands lead to smaller growth of the bone tissue and the decrease of total bone mineral mass[1,5], culminating in bone disorders. In the systemic arterial hypertension, the low levels of calcium ions in the serum, mainly due to the hypercalciuria, can stimulate the compensatory increase in the production of PTH, the main

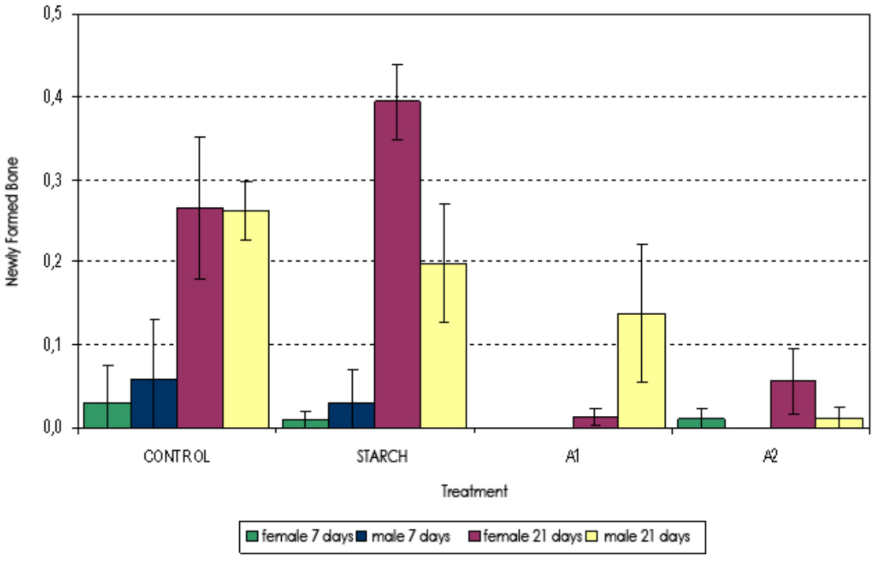

Figure 6 - Illustrative graph of the averages and standard deviation-pattern of the data of the newly formed bone in the different experimental groups, at the 7 and 21 days, in the spontaneously hypertensive male and female rat.

Table 1 - **Formation of homogeneous groups after the test of Tukey (5%)**

Experimental group	Period of observation	Gender	Average	Homogeneous groups				
A1	7 days	Male	0,00	a				
A2	7 days	Male	0,00	a				
A1	7 days	Female	0,00	a				
St	7 days	Female	0,0086	a				
A2	7 days	Female	0,0108	a				
A2	21 days	Male	0,011	a				
A1	21 days	Female	0,0129	a				
C	7 days	Female	0,03	a				
St	7 days	Male	0,0303	a				
A2	21 days	Female	0,056	a	b			
C	7 days	Female	0,0582	a	b			
A1	21 days	Male	0,138		b	c		
St	21days	Male	0,1992			c	d	
C	21days	Male	0,262				d	
C	21days	Female	0,2654				d	
St	21days	Female	0,3936					e

Groups with same letters do not differ statisticallly at 5%.

hormone related to the control of the plasmatic level of calcium in the organism. In this way, high levels of PTH tend to contribute to the process of bone remodeling, as they stimulate the osteoclastic activity and mobilize the calcium in the skeleton, and could lead to the decrease of the bone mineral density[20].

The histological analysis of this study presented results that differed of those found by Pereira at al[8]. Those authors observed that, at 7 days, the SHR rat presented formation of bone trabeculae in most of the studied specimens. In this study, in the period of 7 days, most of the groups still presented slack conjunctive tissue with hemorrhage focuses in the area of the bone defect, suggesting that the phase of substitution of the clot for granulation tissue was not completed. Some

specimens of the groups C and St presented focuses of osteoid tissue amid the conjunctive tissue, characterizing immature bone formation in the first week of the experiment.

At 21 days, the groups C and St presented closing of practically the whole extension of the bone defect in most of the specimens, with the presence of a newly formed bone tissue in continuation to the adjacent cortical tissue. In these specimens, the presence of mature bone trabeculae was observed disposed amid slack conjunctive tissue. It was also verified that the bone trabeculae, joined by anastomosis, were surrounded by flat osteoblastos, similar to the one was noticed by Pereira at al[8].

Some authors report different bone characteristics between the SHR male and female rat. The studies demonstrate that the female present corporal weight, length and reduced bone volume[15], and higher rate of bone reabsorption[7]. However, Pereira at al[8] observed that the process of bone repair in normotensive and hypertensive rat did not present differences among the gender. In this study, no statistical difference was observed in the bone repair among the gender of the animals, except when the interaction of the gender was evaluated with all the other studied factors.

The sodium alendronate was used as medication in the bone defect, with the objective of making available the molecule in the place and to observe their effects in the bone repair of SHR.

The molecular mechanism by which the biphosphonate inhibits the bone reabsorption is not completely understood. It is known that it is related to the inhibition of the osteoclasts in the reabsorption gaps, affecting the intracellular metabolism and inducing apoptosis of those cells. Through an indirect action, the biphosphonates also act stimulating the osteoblasts to produce chemical mediators that inhibit the recruitment of the osteoclasts. All this contributes to the increase of the bone mineral density[21-22].

In the last ten years, there have been studies regarding the use of sodium alendronate with the objective to inhibit the bone reabsorption due to surgical procedures and to improve tissue repair[23-26], mainly for repair of the bone tissue[11-14]. Within the context of the systemic arterial hypertension, allusive reports do not exist to the effects of sodium alendronate in the bone fabric and few are the studies that use the medication locally to evaluate the processes of bone repair.

Jaime at al[13] demonstrated that the medication did not contribute to the bone repair, when administered locally in the bone defect of the calvaria of female, ovaryectomized rat. Fernandes[12], studying the local action of sodium alendronate, of hydroxyapatite and association of these medication in the bone repair of femurs of rat, also did not observe the positive action of the sodium alendronate.

In this study, it was observed that the group treated with alendronate 2 mol presented lesser bone formation at the end of 21 days, when compared to the group treated with alendronate 1 mol, with significant difference in the SHR male rat. This data can be explained by the fact of sodium alendronate causing cytotoxic effects when administered in higher concentrations[22,23].

Besides, the results of this research demonstrate that the animals of the groups A1 and A2 presented smaller bone new formation when compared to those of the groups C and St, independently of gender or observation period. Similar data were obtained by Fernandes[12], that demonstrated the influence of sodium alendronate delaying the process of bone repair in the area of the bone defect.

Studies accomplished in vitro demonstrate the anabolic action of alendronate, stimulating the bone new formation[27-28]. Giuliani at al[27] observed that the sodium alendronate stimulated the formation of osteoblastic cells in the bone marrow of rat, when administered in low concentrations, and inhibited the process in higher concentrations. Im at al[28] demonstrated that the use of alendronate in the culture of osteoblastic cells stimulated the cellular proliferation in the concentration of 10-8 mol.

The researches have demonstrated that the biphosphonates cause bi-phase action, stimulating the cellular proliferation and the formation of osteoid tissue in the lower concentrations and inhibiting these processes in the higher concentration[27-28]. The higher concentrations of the medication seem to influence the viability of the cells, impairing their metabolic functions[29] and provoking cellular death.

It is possible that the molar concentration used in this study has affected the cellular viability, causing hydropic degeneration of the conjunctive tissue, and, for this, it might have caused cytotoxic tissue action that interfered in the bone repair in the groups A1 and A2. At 7 days the presence of conjunctive tissue with infiltrated inflammatory, frequently associated to a fibrin net in the area of the defect of the groups A1 and A2 (fig. 1b) was evident. However, the histomorfological analysis presented an interesting discovery, which was the formation of extracortical subperiosteal bone tissue, in all of the groups treated with alendronate 1 mol and 2 mol. This fact was more evident in the female SHR of the group A2, at 21 days of the experiment (fig. 5a-b).

The extracortical subperiosteal bone tissue was on the opposite side to the bone defect and it presented characteristics of remodeled mature tissue, with increased medullary spaces in relation to the adjacent cortical bone and with a great number of gaps occupied by bulky osteocytes.

This characteristic was also evidenced by Fernandes[12] in normotensive rat, in all of the groups treated with sodium alendronate. The author suggests that this phenomenon might have been brought on by the penetration of the medication under the periosteo, at the moment of insertion of the material. Starting from this study, it is believed that the sodium alendronate might have been incorporated to the bone fabric, as there was the insertion of the medication inside the medullary canal at time of the surgery. In this way, once deposited in the bone, the alendronate might have been liberated little by little from the bone tissue and stimulated the quiescent osteoblastic cells in the periosteo. This bone formation did not happen in such an evident way in the area of the bone defect, probably due to the disorganization of the periosteo cells at time of the surgical procedure.

The sodium alendronate is a quickly reabsorbed medication when it is not deposited in the bone tissue. However, when incorporated to the hydroxyapatite crystals, it is liberated

slowly over the time[9,10]. This could stimulate the mesenchymal stem cells and the cells of the endosteo and periosteo to produce bone tissue.

Based on this theory, it is also intended to justify the pattern of bone formation found in the group A1, of male rat, at the end of 21 days. In these animals, the formation of the bone trabeculae projected starting from the bone marrow to the area of the defect. Such new formation can be explained by the stimulation of the mesenchymal stem cells and of the osteoblastic cells of the endosteo by the action of sodium alendronate incorporated to the bone fabric. It is believed that alendronate molecules might have been liberated slowly, in viable concentrations, not causing damages to the tissue.

Conclusion

In agreement with the results obtained in this study, it is concluded that the local application of sodium alendronate did not contribute to the bone repair in the SHR; however, the medication possibly contributed to the formation of extracortical subperiosteal bone tissue.

The results demonstrated that the bone repair among the experimental groups was larger in the period of 21 days when compared to the period of 7 days. There was no difference in the process of bone repair among the male and female animals.

In spite of the statistical results having demonstrated that the local application of the sodium alendronate did not contribute to the process of bone repair in SHR rat, it is believed that variations in the methodology, as new forms of administration and different molar concentrations, can generate positive results, mainly due to the histological finding of the extracortical subperiosteal new formation found exclusively in the groups treated with alendronate. It is believed, therefore, that there is need of more profound studies to elucidate the local action of sodium alendronate in the process of bone formation, as well as to evaluate its action on the bone tissue in the presence of arterial hypertension.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

There were no external funding sources for this study.

Study Association

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