Bone healing of mandibular critical-size defects in spontaneously hypertensive rats

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Abstract: Few articles have shown changes in bone metabolism caused by hypertension. The objective of this study was to investigate the relationship between hypertension and bone healing. Circular critical-size defects 5 mm and 2 mm in diameter were created, respectively, on the left and right side of the mandible in 40 spontaneously hypertensive and 40 control Wistar-Kyoto rats. Five animals from each strain were killed 2, 3, 5, 10, 15, 30, 60 and 90 days after surgery. The macroscopic evaluation showed great mandibular angle deformation on the left side and non-healed defects on both sides and groups. Histological evaluation revealed similar bone healing on both sides, with initial necrosis in the central area, and fibrosis and angiogenesis within the first 5 days. From the 10th postoperative day on, the newly formed bone displayed progressive thickening until the 90th postoperative day, when the defect margins presented a compact bone structure. Furthermore, the statistical analysis of the histometric data did not reveal any significant hypertension effect on bone healing in the defect area. These results suggest that bone healing was not different between spontaneously hypertensive rats and control rats.

Descriptors: Bone Regeneration; Hypertension; Rats, Inbred SHR; Rats, Inbred WKY.

Introduction

According to a World Health Organization (WHO) report, one in three adults worldwide has raised blood pressure, and the projections for 2025 are that one third of all adults will have this condition.[1,2] Hypertension is important not only because of its high prevalence, but also because it is an asymptomatic disease whose diagnosis and treatment are frequently delayed. As pointed out by the WHO,[2] several diseases—especially obesity, hyperglycaemia and hyperlipidemia—are also related to or have their risk modified by the presence of high blood pressure.

Abnormalities in calcium metabolism have been also associated with essential hypertension, but the results are controversial. Hypercalciuria and a higher calcium/creatinine ratio are found mainly in osteoporotic groups, but bone mineral density (BMD) and osteoporosis percentages in the postmenopausal period are similar between hypertensive and normotensive women.[3] Urinary calcium excretion is inversely correlated to BMD, whereas serum total and ionized calcium levels in hypertensive

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women are not different from those observed in normotensive women.[4,5] Diastolic hypertensive men have reduced bone mineral content when compared with normotensive subjects.[6,7] On the other hand, some reports have failed to reveal a consistent relation between urinary calcium excretion and hypertension in men as well.[8] Recent data show reduced BMD in hypertensive subjects with arterial stiffness.[9]

Investigations with spontaneously hypertensive rats (SHR) have added to the knowledge of calcium metabolism and its relation to bone status. During growth and maturity, SHRs have a smaller body and skeletal mass, as well as lower calcium content, irrespective of age or gender; however, when skeletal mass is calculated as a percentage of body mass, these values prove greater among normotensive rats.[10,11] Resorptive activity seems to be reduced in old animals and shows different rates between genders. Between the ages of 8 and 24 weeks, SHR males have shown a 60% to 70% reduction in the resorption rate, whereas the percentages for females have ranged from 15% to 35%. However, 24-weeks-old, female SHR have shown higher bone turnover rates than male rats.[12,13] This resorptive rate is still high when compared to that of normotensive animals.[11]

Old SHR have hypercalciuria and low serum ionized calcium, but normal serum total calcium.[14] Bone density and bone calcium content are significantly reduced in male SHR compared with Wistar-Kyoto rats (WKY).[11,15] Old female SHR possess a higher percentage of cancellous bone, less net cortical bone, smaller tissue area, and thinner cortex than those of the WKY strain; they have also presented age-related cancellous and cortical bone loss.[16]

Since several changes have been described in the bone metabolism of hypertensive subjects, the purpose of this study was to evaluate bone healing in defects created in the mandibles of SHR and control WKY rats.

Methodology

This study was approved by the Research Ethics Committee (Subcommittee for Animal Bioethics) of our institution, under protocol number 05/05. All procedures were carried out according to the Ethical Principles of our National Board of Animal Experimentation.

Forty SHR and 40 WKY rats were enrolled in this investigation. All animals were males, and about 4 months old, an age when hypertension is effectively established. They were housed in a colony room, 5 animals per cage, at 25°C, under a 12h light/dark cycle, and had free access to rodent chow and tap water. For the surgical procedures, they were anesthetized with 100 mg/kg of ketamine hydrochloride and 10 mg/kg of dihydrothiazine hydrochloride; both drugs were injected by the intraperitoneal route. While they were in an unconscious state, the skin on the ventral region below their mandibles was shaved and washed with alcohol 70%.

A 15 mm linear incision was then made on the skin along the line of the mandibular basal bone; the masseter muscle below was blunt dissected to expose the lateral surface of the angle region. A full-thickness round defect 5 mm in diameter was created on the left side with a rotary drill, and a similar defect 2 mm in diameter was created on the right side. The muscle was then repositioned and only the skin layer was sutured. Benzylpenicillin (16 000 IU) was administered by the intramuscular route, and acetaminophen (1 mg/ml) was given through drinking water in the first 2 days after surgery.

Five animals of each strain were killed on the 2nd, 3rd, 5th, 10th, 15th, 30th, 60th and 90th postoperative day (POD), with a lethal dose of carbon dioxide, delivered at a flow rate of 6 L/minute. The animals were exposed for at least one minute after apparent clinical death.[17] The entire mandible was removed by careful disarticulation, preserving the masseter muscle and the soft tissue around the mandibular angle. The specimens were fixed with 10% buffered formalin for at least 24 hours, and then decalcified in 20% formic acid for 7 days. The samples were embedded in paraffin, and 7 μm thick serial sections were cut parallel to the ramus surface. The sections were then processed for hematoxylin-eosin staining.

The histological analysis was performed with an Eclipse E100 light microscope (Nikon, Sendai, Japan) using 100× magnification, coupled to a

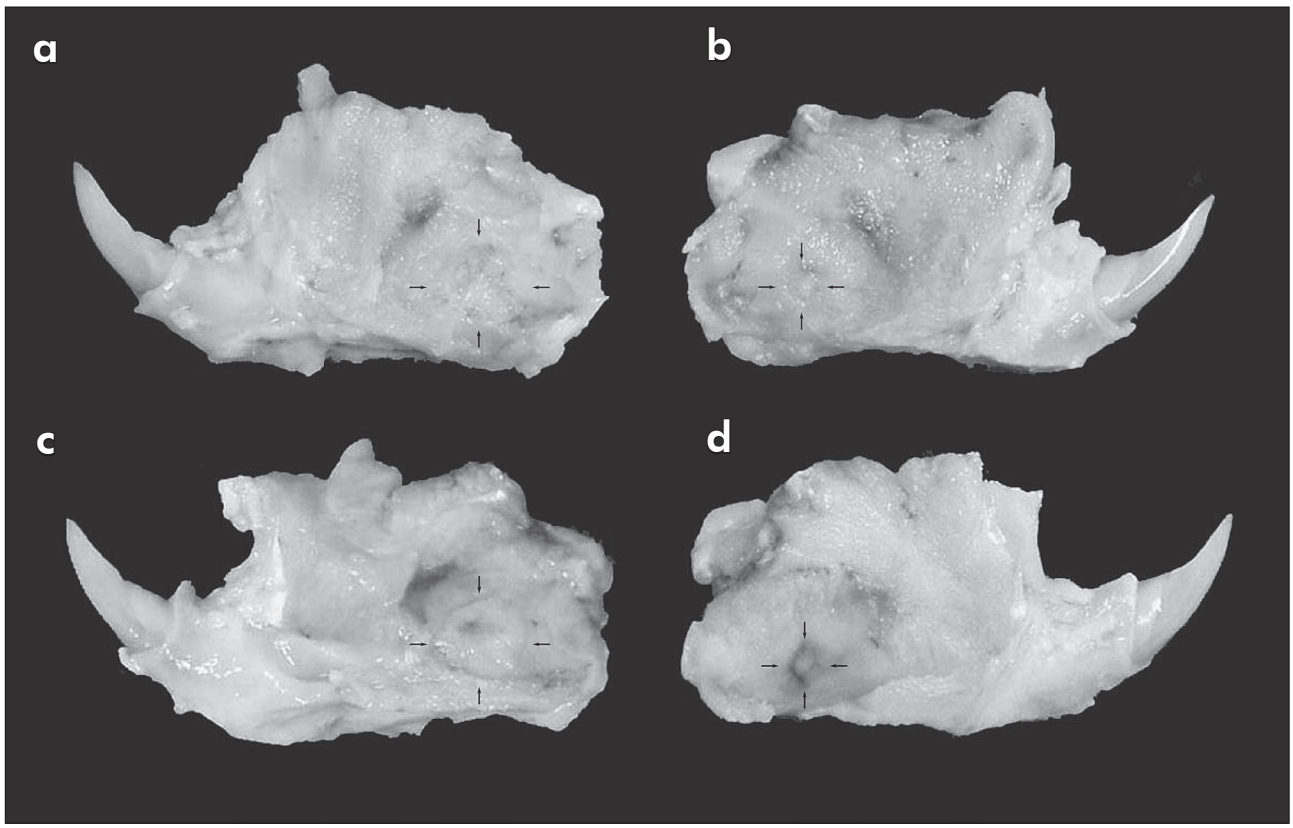

Figure 1 - Specimens on the 90th postoperative day. (**a**) left side SHR. (**b**) right side SHR. (**c**) left side WKY. (**d**) right side WKY. The defect contours are still visible with few signs of bone closure.

C720UZ charge-coupled device digital camera (Olympus, Tokyo, Japan), used to capture section images containing all margins of the round defect in JPEG file form for histometric evaluation. The remaining defect area was delimited and measured with Image J 1.4 software (National Institutes of Health, Bethesda, USA). The resulting values were expressed in square pixels.

The defect area values within the same side were compared between SHR and WKY animals after each postoperative period (POP) of time, and also according to each side among the POP for each strain. Data analysis was performed with Minitab 15® Statistical Software (Minitab Inc., State College, USA) and, whenever the *p* value was below 0.05, statistical differences were considered significant.

Results

During anaesthesia induction, 5 animals died in the WKY group and 4 animals died in the SHR group. Recovery from anaesthesia occurred after 4–6 hours and full motion was recovered within the first 24 hours. Rats showed some facial swelling, that lasted no more than 4 days, and no signs of infection or postoperative sequelae.

Gross macroscopy

In both groups, WKY and SHR, a great bone deformation was observed on the left side, which became particularly evident as of the 30th POD. On the 90th POD, neither the 5 mm defect on the left side nor the 2 mm defect on the right side had shown complete bone closure in both groups (Figure 1).

Histological findings

A similar bone healing process occurred on both sides in both groups, control WKY and SHR. On the 2nd and 3rd POD, there was cellular debris, fibrin and necrosis in the central area, and reactive oste-

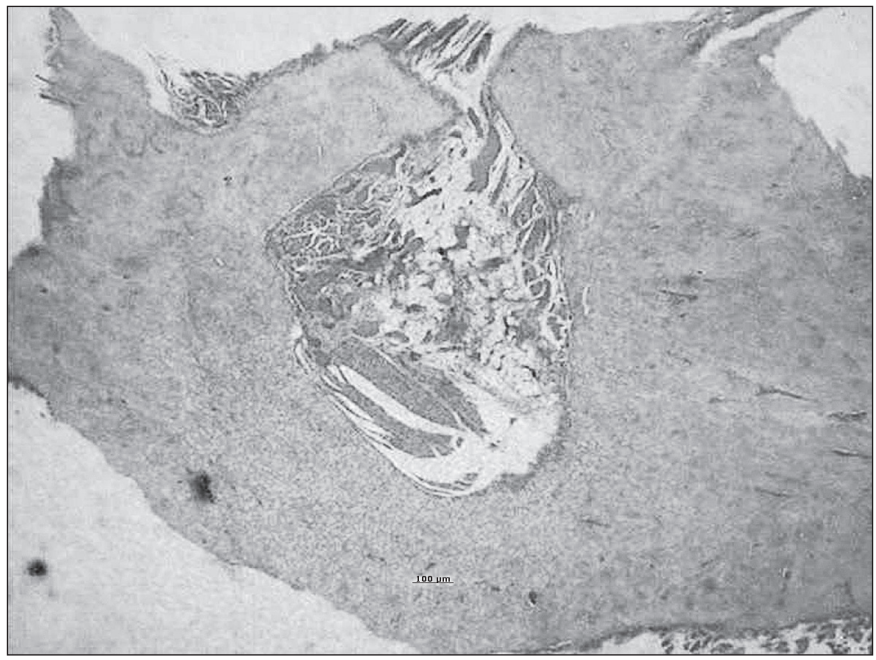

Figure 2 - Histological image of the entire 5 mm defect at the 90th postoperative day, showing evident deformation around the defect area. In this SHR specimen, evident deformation can be seen around the defect area without complete closure. There are no signs of bone neoformation, suggesting a permanent defect.

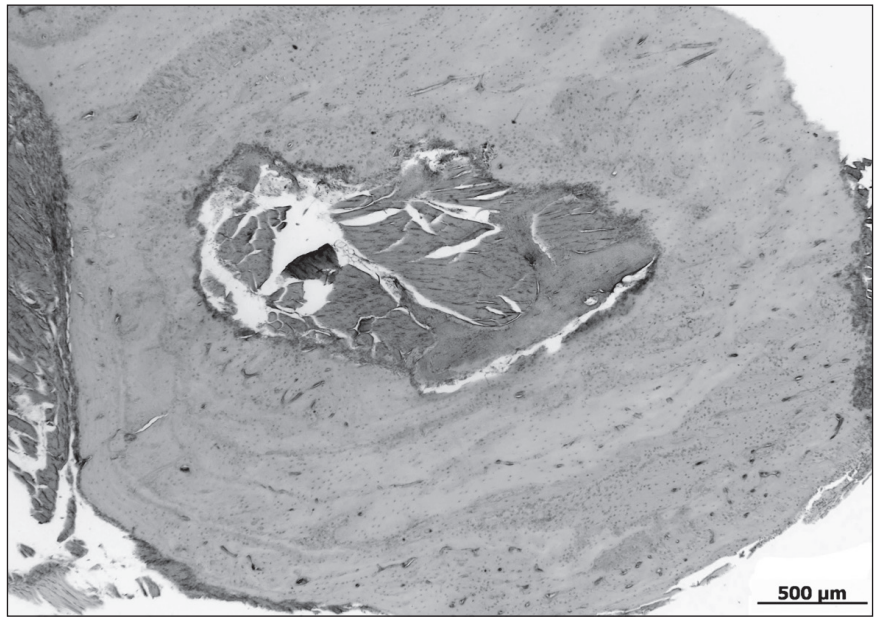

Figure 3 - Histological image of the entire 2 mm defect at the 90th postoperative day. In this WKY specimen, some osteoblast activity can still be seen in proliferative regions around the defect, although insufficient to achieve closure of the defect.

oblasts on the defect margins with disorganized collagen deposition. On the 5th POD, there were giant cells, fibrosis, angiogenesis, and the first osteocytes in the central area, the latter surrounded by an extracellular matrix resembling immature bone; some tissue sections also displayed immature cartilage, most of which were on the right side. On the 10th and 15th POD, a newly formed trabecular bone layer displayed progressive thickening, reduction of active osteoblasts, and lamellar deposition of collagen. From the 30th to the 90th POD, the defect margins presented a compact bone structure with haversian systems and osteocytes (Figures 2 and 3).

Histometric and statistical analysis

The defect area mean values are presented in Ta-

Table 1 - Defect area ($\times 10^3$ pixels^2) and percentage (%) in relation to the initial value.

Postoperative day	Side			
	Left		Right	
	WKY	SHR	WKY	SHR
2	204.43 (100.0)	243.06 (100.0)	120.02 (100.0)	103.16 (100.0)
3	151.79 (74.2)	193.90 (79.8)	94.04 (78.4)	98.72 (95.7)
5	257.07 (125.7)	231.00 (95.0)	60.34 (50.3)	92.82 (90.0)
10	201.82 (98.7)	268.95 (110.7)	89.92 (74.9)	85.39 (82.8)
15	138.79[a] (67.9)	220.87 (90.9)	57.65 (48)	127.79 (12.9)
30	199.30 (97.5)	176.82 (72.7)	52.25 (43.5)	49.29 (47.8)
60	286.47[a,b] (140.1)	191.52[b] (78.8)	87.20 (72.7)	38.86 (37.7)
90	218.36 (106.8)	214.71 (88.3)	66.42 (55.3)	63.91 (62.0)

* Same letters indicate significant differences ($p < 0.05$, ANOVA) between values.

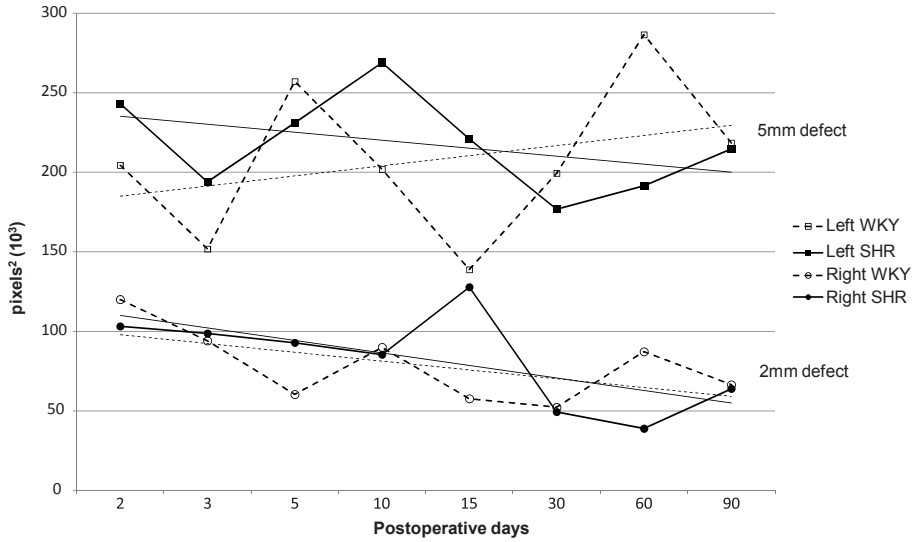

Figure 4 - Progression of the defect area across the postoperative days. The tendency lines of the 2 mm defect show similar bone closure behavior between the two strains. On the other hand, the 5 mm defect of the WKY strain seems to increase, but its final value is similar to that of the SHR strain.

ble 1 and postoperative progression of the defect can be seen in Figure 4. Two-way analysis of variance and Tukey multiple comparison tests revealed significant differences only on the left side:

- in the WKY group, the defect area was smaller on the 15th POD than on the 60th POD ($p = 0.0042$);
- between strains, the defect area on the 60th POD was larger in the WKY group than in the SHR group ($p = 0.0491$).

Discussion

In similar conditions, dogs and rats with induced renal hypertension display an immature trabecular pattern during alveolar bone repair after tooth extraction, suggesting some interference in the mineralization phase.[18,19] In monocortical defects on rat femur, SHR presented faster granulation tissue growth and bone healing than did normotensive rats, although bone matrix production was reduced at later periods because osteoblasts showed low cellular activity.[20] Recently, it has been found that untreated male SHR show alveolar bone with a high expression of RANKL (receptor activator of nuclear factor kappa-B ligand, a protein involved in osteoclast activation), a high ratio of RANKL/OPG (osteoprotegerin, a decoy receptor for RANKL), a high number of positive tartrate resistant acid phosphatase cells (an enzyme highly expressed by osteoclasts), greater bone loss, and reduced bone density.[21]

A critical bone defect is the smallest defect that cannot heal spontaneously during the whole life of an animal; in rats, it was determined that the minimum diameter of such a defect on the mandibular angle is 4 mm. Thus produced, the defect is well-delimited by the ramus and lower border of the mandible, with 1 mm of safety margin.[22-23] Although SHR are small animals, we chose to perform defects with 5 mm, not only because most investigations with Wistar rats perform a 5 mm defect, but also because SHR show fast angiogenic proliferation, thus posing a greater risk of early closure of the defect. Indeed, these defects did show incomplete closure, as shown by the similar defect area values observed along the length of the study, and by the severe mandibular angle deformation, especially from the 30th POD on, which could be explained by bone remodelling owing to functional adaptation. In spite of the statistical differences observed, the variation in defect area across the POP and between strains was unpredictable. The remaining defect area on the 90th POD was practically the same original size, suggesting a similar inability of both animal groups to appose and sustain newly formed bone on the defect margins.

Thus, if any diameter smaller than 4 mm has the potential to close spontaneously, healing of the 2 mm defect should have been observed, but this did not occur. The unexpected result was that none of them had closed completely on the 90th POD, suggesting impairment of the bone healing. Several hypotheses were raised to explain this finding:

- simultaneous defects on either side of the mandible could influence the repair process on the opposite side;
- mandibular angle and ramus have scarce cancellous bone, therefore the number of osteogenic cells available is small and self-limited, and defects of any diameter would not close in this region;
- although the WKY strain is often used as a control, because the SHR strain descended from it and any genetic differences would be limited to mechanisms of blood pressure elevation, the former would not be an appropriate control for the latter, owing to the higher incidence of spontaneous hypertension in the WKY strain than that observed in Wistar rats; and, finally,
- a period of time longer than 90 days would be necessary to complete bone closure, because in both strains there was a tendency towards bone closure as indicated by the 40%–45% closure rate. Future investigations are needed to shed light on the factors involved in this process.

The results of the histological analysis are quite similar to the macroscopical findings, because no significant differences were found in bone repair between the study groups. The amount of angiogenesis was similar at the early sacrifice time points, although some studies report that microvascular rarefaction in SHR could delay the formation of granulation tissue.[24] At the later time points, the rate of bone remodelling was also similar between strains, irrespective of the size of the defect. The progression of bone healing was more coordinated on the right side for both strains, maybe because the reduced size of the defect on that side (2 mm) allowed an enhanced response to the same functional pressure exerted on both sides.

Comparing our results to those of other authors is difficult because there are few studies available on bone repair in hypertensive animals, and they have been conducted either with monocortical defects in SHR rat femur,[20] or with tooth extraction in dogs and rats with induced renal hypertension.[18,25] Although changes in cellular activity have been described in the bone metabolism of SHRs, these changes did not seem to adversely affect bone repair when the hypertensive strain was compared to the WKY strain.

Some aspects of bone healing and hypertension should be addressed in future investigations, namely:

- the use of another strain as a control instead of the Wistar or WKY strains;[26]
- the fact that smoking, hypertensive women and male rats exposed to passive smoking have shown a significant reduction in BMD;[16,27]
- the observation that ovariectomy has induced earlier and greater cancellous bone loss in SHR than in WKY rats, with greater increase in bone turnover rate and eroded surface;[25]

- the observation that the addition of inhibitors of angiotensin-converting enzyme in cocultures of bone cells inhibits the resorptive activity,[28] even though there are no reports of an anti-hypertensive drug protocol for SHR and the use of any medication must be confirmed by investigations on blood pressure control; and, finally,
- the activity of OPG and osteopontin, both bone metabolism biomarkers, because there is evidence that their elevated levels are associated to inflammation and arterial hypertension.[29]

Conclusions

Within the limits of this investigation that compared SHR with control WKY rats, after a critical-size defect 5 mm in diameter was created on the left side of the mandible, and another defect 2 mm in diameter was created on its right side, it could be concluded that there was no statistically significant difference in terms of bone healing between strains or among the evaluated postoperative periods.

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