BONE REPAIR PROCESS IN NORMAL AND OSTEOPENIC FEMALE RATS' TIBIAE: A COMPARATIVE STUDY

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SUMMARY

The purpose was to compare tibial bone union in normal and osteopenic female rats. Forty-nine Wistar albino female rats weighing 160 g (±20g) and 100 days were distributed into 2 groups: Oophorectomized (OOF) and Pseudo-oophorectomized (SHAM). Thirty days later, a cortical injury was produced in all the animals. They were sacrificed in the 2nd, 4th, 6th and 8th weeks. Osteoblasts count was performed. Progressive weight increase was observed, but the OOF group was shown to have gained more weight (p£0.05) than the SHAM group, at the time of the second surgery. After 15 days post-injury, the animals in the OOF group presented a higher number of osteoblasts (p£0.05) compared to the SHAM group. Thirty days after injury, the number of osteoblasts was reduced, but both groups showed similar amounts. Forty-five days after injury, despite a constant reduction, the number of osteoblasts in the OOF group remained high when compared to SHAM (p£0.05) group. After 60 days, we found less osteoblasts in the SHAM group, suggesting an advanced bone repair process. The osteopenic animals showed an early accelerated response, which became equivalent between both groups 30 days after injury. However, after that period, they showed a delayed osteoid mineralization, suggesting delayed late bone repair process.

Keywords: *Osteoporosis, Osteopenia, Ovariectomy.*

Citation: *Sartori AR, Moreira J, Santos AMM, Cintra DEC, Sartori LR, Baraúna MA, Canto RST. Bone repair process in normal and osteopenic female rats. tibiae: a comparative study. Acta Ortop Bras. [serial on the Internet]. 2008; 16(1):37-40. Available from URL: http://www.scielo.br/aob.*

INTRODUCTION

Osteoporosis is defined as an osteometabolic disorder, of multifactorial etiology, characterized by reduced bone mineral density and degradation of its microarchitecture, with a resultant increased weakness and a higher susceptibility to fractures. In Brazil, about 10 million people are affected by the disease, where fractures are frequent[1,2]. It is classified as primary postmenopausal osteoporosis (Type I), Senile (Type II) and secondary[3]. Bone represents a rigid form of connective tissue subjected to continuous remodeling process, thus being a highly plastic tissue. The shape and density of a bone tissue are maintained throughout life by a balance of mechanical and physiological aspects[4]. On a normal adult skeleton, the new bone deposited by osteoblasts corresponds exactly to the osteoclastic bone resorption[5]. As a result of the hypoestrogenemia, which is characteristic of the postmenopausal period, osteoblasts as well as osteoid deposit speed are reduced. Consequently, bone resorption increases comparing to the deposit, thus reducing bone mass, especially on trabecular bones[4,6-9]. Fractures resulting from osteoporosis are regarded as a major socio-economic issue due to the high incidence of mortality and morbidity, reduced life expectation and high healthcare costs[10-12]. Some authors agree with the fact that fracture union process in the elderly population is slower when compared to young adults[13]. However, recent studies have demonstrated that bone repair in the elderly is no different than it is in young adults in terms of union speed, unless regarding the quality of the newly formed bone[1,2]. The objective of this study was to compare bone repair after tibial injuries on osteopenic and normal female rats by osteoblasts counting and by the assessment of histological aspects involving bone unions in both situations.

MATERIALS AND METHODS

Animals: 49 female albino Wistar rats, weighting 160 ± 20g in average and 100-day old sourced by the University of Alfenas – Unifenas Central Animal Lab. The animals were kept in a scrub environment receiving water and a commercially avail-

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Received in: 08/04/06; approved in: 10/03/06

able ration *ad libitum* over the experiment period. The animals were randomly assigned to two groups: Ooforectomized (O) and Pseudo-ooforectomized (Control group) - SHAM (S). This protocol was approved by the Committee of Ethics in Research for Animal Experiments at UNITRI – Triangle University Center.

1st Surgery – Surgical ooforectomy procedure and sham ooforectomy surgery: Twenty-five animals were submitted to ooforectomy procedures. The rats were weighted and anesthetized with intraperitoneal ketamine hydrochloride and thiasine hydrochloride injection at a ratio of 3:1, respectively at a dosage of 0.002mL/g of body weight. Then, the low belly region was trichotomized with razor and antisepsis was provided with iodine alcohol. Surgical incision was made with a knife blade and a small suture with reabsorbable Catgut-Chromed wire (3-0) was provided on the uterine base followed by bilateral ooforectomy and hysterectomy. Finally, internal sutures were made with reabsorbable wire and the external ones with non-absorbable nylon wire. The animals on control group (pseudo-ooforectomized – SHAM – S) were submitted to the same surgical steps, except for uterus and ovaries removal[14,15].

2nd Surgery – Surgical procedure for producing a cortical bone defect: Thirty days after ooforectomy (1st surgery), the animals were submitted to a new surgery for producing a cortical bone defect. The animals were weighted and anesthetized as described above. During the procedure, the animals were kept at supine position with left limb in external hip rotation and triple flexion (hip/ knee/ ankle). With a pachymeter positioned from the knee joint interline to medial malleolum, the proximal tibial third was measured in order to produce the injury. A corresponding skin incision was provided and the myotendinous fascia of the region was averted and the bone injury was produced by scarification, using a Carbide dental drill attached to a low-rotation engine. In a single movement, this was introduced at 90º from longitudinal axis, at cortical bone core on tibial medial surface (diaphyseal region) in order to penetrate cortical bone and injury the trabecular mesh of the medullary channel, promoting a 1.5 mm-wide scarification. During this procedure, continuous irrigation with saline solution was maintained. Then, skin suture with non-absorbable wire and local asepsis with PVPI (Polyvinylpyrolidone Iodine) were provided, with no kind of subsequent immobilization of the segment[16,17].

Sacrifice: The animals on subgroups I, II, III, and IV were sacrificed on the 2nd, 4th, 6th and 8th week, respectively, after bone injury[18]. The animals were initially weighted and anesthetized with high doses of the same anesthetic agents as described previously. – ketamine hydrochloride and thiasine hydrochloride – at a ratio of 3:1 and a dosage of 0.005mL/g of body weight.

X-ray analysis:

After sacrifice, animals' right paws were disconnected at hip level, and X-ray images were taken with the paws positioned at triple flexion. Kodak® 3 x 4 films previously identified were used. The X-ray images were used for excluding samples with total bone injuries.

Histological analysis:

Right tibiae were dissected and fixed into 10% formalin solution for five days and subsequently rinsed on tap water for 24 hours. The pieces were then included into MORSE decalcification solution (20% sodium citrate and 50% formic acid) for 3 days and again washed on tap water for 24 hours. The, the pieces were dehydrated with increasing 70% alcohol solutions, diafinized in xylol alcohol, then in xylol and immersed into paraffin. Longitudinal serial 5µm-thick sections were made, 5-7 were discarded on each section until the next one was obtained, totaling 3 sections/ piece. The slides were stained with Hematoxylin/ Eosin (HE)[19] and assessed by light microscopy. Photographs were taken of the right and left ends of each section, totaling 6 photos for each sample, using 40x magnification. The morphometric analysis based on the osteoblasts count[20] was made by using a graded screen of the *Adobe Photo-Paint* software – release 6.0. The histological analysis was made by the same examiner in a random and blinded fashion.

Statistical analysis: The Wilcoxon's test was used for the body weight variable, the Mann-Whitney and Kruskal-Wallis tests were used for the osteoblasts count variable. In significant cases, the Duncan test was used for discriminating differences. For all analyses, a significance level of 5% was adopted.

RESULTS

Animals' weight

No significant differences were seen for animals' body weight ($p>0.05$) when submitted to the first surgery (OOF and SHAM) – Weight 1. In both groups, a progressive weight gain was noticed between the 1st and the 2nd surgery ($p> 0.05$), but the OOF animals gained additional weight ($p\leq 0.05$), when compared to those on control group SHAM at the time of the 2nd surgery – Weight 2 (Figure 1).

Osteoblasts count and Histological aspects

OOF-I and OOF-III subgroups showed a significantly higher amount ($p \leq 0.05$) when compared to SHAM subgroups (Figure 2). Osteoblasts count can only be made on groups I, II and III. This was not possible on group IV, since bone injury ends were no longer outlined, suggesting the existence of total bone union (Figures 3 and 4).

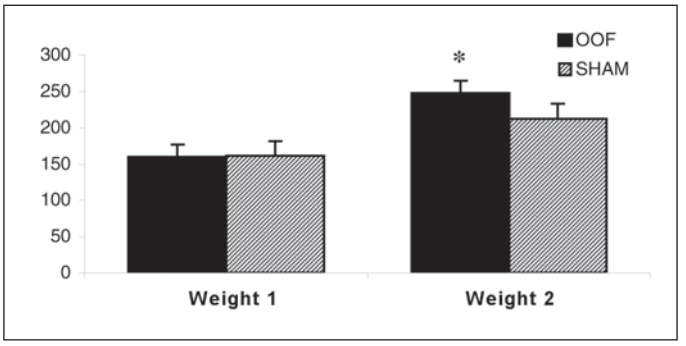


***Figure 1** - The bars represent mean and standard deviation values for animals' body weight. * Different from SHAM Group, $p \leq 0.05$.*

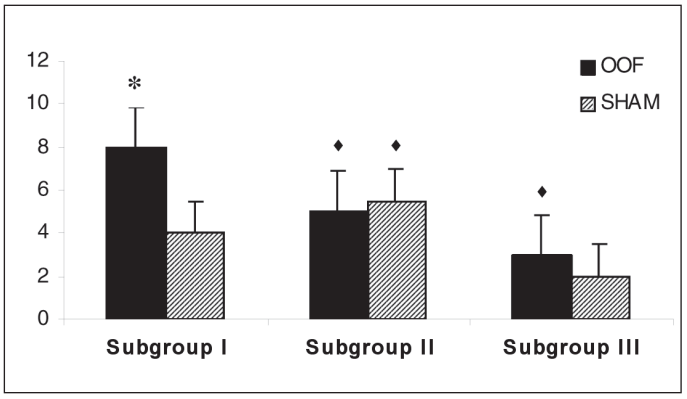


Figure 2 *- The bars represent median and standard deviation values for the number of osteoclasts in the experimental subgroups.*
** p ≤ 0.05 different from all SHAM subgroups.*
♦ p ≤ 0.05 different from SHAM subgroup III.

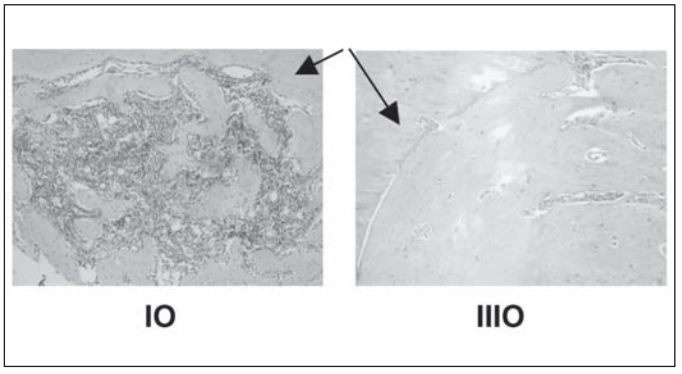
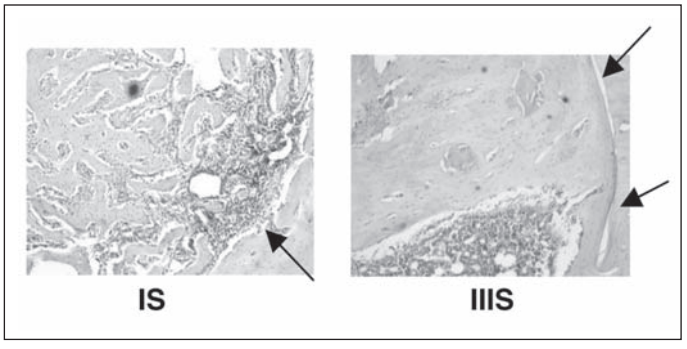


Figure 3 *- Histological photographs of ooforectomized subgroups (OOF) I (left) and III (right). Arrows point to bone lesions ends.*



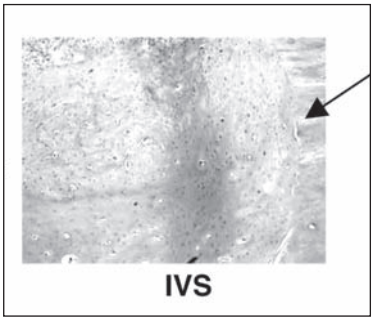


Figure 4 *- Histological photographs of the non-ooforectomized subgroups (SHAM) I (left), III (center) and IVS (right). Arrows point to bone lesions ends. Note that in the IVS group, this end is almost undistinguishable.*

DISCUSSION

In the present study, all animals were of same age (100 days) and no significant difference (P>0.05) was seen between baseline weights, thus evidencing a homogeneous sample. All animals gained weight 30 days after ooforectomy (OOF) and sham surgery (SHAM), with animals on OFF group gaining significantly more weight ($p \leq 0.05$) when compared to SHAM group. Estrogen increases aerobic power while, in hypoestrogenic conditions, aerobic power is lower, resulting in body weight gain[14,21,22]. Ovarian hormone deprivation is associated to weight gain and reduced bone mineral density[2,15,23]. Carvalho and Cliquet[14] reported that, 30 days after surgery, ooforectomized animals showed lower calcium and phosphorus levels compared to non-ooforectomized animals, thus evidencing an effective protocol for inducing osteopenia, therefore selected for this study. The degree of soft tissue damage interferes on the experimental model of bone injury, but rats' tibiae offer an advantage of having only a small portion covered by muscles. The spherical bone injury model produces identical injuries in terms of site and size[24]. In order to assess osteoid formation and mineralization, the osteoblasts were counted. The osteopenic group showed stronger osteoblastic activity in the first 15 days after bone injury ($p \leq 0.05$). These results are similar to those found in literature[14] reporting larger amounts of osteoblasts in ooforectomized animals, perhaps as an attempt to reverse the accelerated increase of bone mass loss after estrogen suppression. There was a reduction of the amount of osteoblasts 30 days after bone injury with comparable values between both groups (OOF-II and SHAM-II), suggesting a balance of the bone repair process in this period, despite of the exaggerated early response of the OOF group. In the subsequent period, 45 days post-injury, despite of the continuous reduction of the number of osteoblasts, the OOF-III group remained slightly high when compared to control SHAM group ($p<0.05$). But, perhaps this fact may be explained by the reduced number of samples collected on group SHAM due to the difficult visualization of injury ends, suggesting that animals on SHAM group were already presenting an advanced stage of bone repair process when compared to animals on OOF group for the same period. In this study, the high number of osteoblasts in the first 15 days post-injury on the OOF group might also be suggestive of initial union delay. Several studies have shown that osteopenic animals present an initial delay in bone injuries repair process[1,2,18]. The comparable osteoblasts number on both groups (OOF and SHAM) 30 days after bone injury suggests that the attempt to revert the bone loss process by adding osteoblasts in early phases may have worked, showing no differences in terms of bone repair between groups during this phase. However, 45 days post-injury – a time that can be regarded as a remodeling phase – persistent high numbers of osteoblasts in the OOF group compared to SHAM group may be suggestive of delayed mineralization of the osteoid matrix and resultant delayed bone repair due to the extensive endochondral calcification phase, thus increasing the time to

remodeling[1,2,15,18]. Bone injuries may show union even under low mineral density and low estrogen levels conditions. However, the deprivation of this hormone delays the process of adding minerals to the osteoid matrix. Thus, the neoformed bone tissue on osteopenic animals after bone injury shows osteoporotic changes, such as, for example, low mechanical properties values, less amount of mineral bone and reduced mineral density, suggesting an inferior bone quality[1,2,15,18,25]. Ooforectomy not only reduces mineral density but also attenuates bone mass gain after fracture[15]. Osteoporosis affects the early repair period and the late callus mineralization. The inferior mechanical properties of the bone callus in osteoporotic animals may reflect a reduced bone quality[26]. The understanding of bone repair in osteoporotic individuals is essential to establish more straightforward therapeutic measures resulting on a reduced treatment time, as well as on better rehabilitation conditions. The results of this study suggest that post-injury repair on osteoporotic bones follows the same sequence of events as normal bones, but presents a slight early acceleration (first 15 days), but showing no difference in the repair of both types of bone within a subsequent period of 30 days according to the variables assessed. However, after that period, osteoporotic bones showed a delayed repair time as these remain longer on the osteoid mineralization phase.

ACKNOWLEDGEMENTS:

The authors acknowledge Professor Helena Chini and Professor Ana Maria Duarte of the University of Alfenas Medical School and Professor Hercílio Martelli Júnior and Professor Luiz Antonio Sartori of the Dental School of Alfenas.

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