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Study of the Osteoconductive Capacity of Hydroxyapatite Implanted into the Femur of Ovariectomized Rats

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ABSTRACT Osteoporosis is a global public health that affects postmenopausal women due to the deficiency of estrogen, a hormone that plays an important role in the microarchitecture of bone tissue. Osteoporosis predisposes to pathological bone fracture that can be repaired by conventional methods. However, depending on the severity and quantity of bone loss, the use of autogenous grafts or biomaterials such as hydroxyapatite might be necessary. The latter has received increasing attention in the medical field because of its good biological properties such as osteoconductivity and biocompatibility with bone tissue. The objective of this study was to evaluate using histologic and radiographic analyses, the osteogenic capacity of hydroxyapatite implanted into the femur of rats with ovariectomy-induced osteoporosis. Eighteen rats were divided into three groups with six animals in each: group nonovariectomized, bilaterally ovariectomized not receiving estrogen replacement therapy, and bilaterally ovariectomized submitted to estrogen replacement therapy. Defects were created experimentally in the distal epiphysis of the femur with a surgical drill and filled with porous hydroxyapatite granules. The animals were sacrificed 8 weeks after surgery. The volume of newly formed bone in the implant area was quantified by morphometrical methods. The results were analyzed by ANOVA followed by the Tukey test ($P < 0.05$). The hydroxyapatite granules showed good radiopacity. Histological analysis revealed less quantity of newly formed bone in the ovariectomized group not submitted to hormone replacement therapy. In conclusion, bone neoformation can be expected even in bones compromised by estrogen deficiency, but the quantity and velocity of bone formation are lower. *Microsc. Res. Tech. 75:133–137, 2012.*

INTRODUCTION

The World Health Organization defines osteoporosis as a systemic skeletal disease that is characterized by a reduction of bone mass and deterioration of bone microarchitecture, with a consequent increased of bone fragility and susceptibility to fracture (1993). The frequency of osteoporosis and its associated fractures vary according to gender and race/ethnic background. White women with a low weight and body mass index, associated with estrogen deficiency especially during the postmenopausal period, are more predisposed to the loss of bone mass and, consequently, to the development of osteoporosis (NIH Consensus Development Panel on Osteoporosis Prevention, 2001).

Female gonadal hormones such as estrogen stimulate osteogenic activity and bone mineralization (Lincoln and Tyler, 1999; Riggs et al., 2003), with the observation of a direct association between bone mass loss and estrogen deficiency. The extensive information about this relationship emphasizes the importance of estrogen in bone tissue homeostasis in both laboratory animals and humans (Lormeau et al., 2004). The most important effect of estrogen on bone remodeling is a reduction of bone resorption. The drop in hormone levels seen during the postmenopausal period, leads to a predominance of cell resorption and consequent osteoporosis, predisposing the patient to pathological bone fractures (Hawker, 1996; Ito et al., 2002). As a treatment alternative, depending on the volume of bone loss, implants of natural or synthetic biomaterials can be used that act as a support for bone regeneration (Hench, 1998).

The biomaterial implanted should meet some requisites, such as a stable structure, being able to promote osteoconduction, rapid incorporation into bone tissue, and causing few or no complications (Rosen and McFarland, 1990). One of the materials that fulfills these requirements is calcium phosphate, a ceramic biomaterial that has been commercialized in the form of hydroxyapatite and calcium triphosphate since 1970 (Costantino et al., 1992). Therefore, since hydroxyapatite resembles the mineral apatite present in human bone, this biomaterial has been widely used in dental and orthopedic surgeries (LeGeros, 2002; Schmitz et al., 1999). The advantages of this biomaterial

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include its biocompatibility, controllable porous microstructure, and its ability to facilitate the infiltration of newly formed bone, characterizing its osteoconductivity (Ono et al., 2000). However, a good health status of the recipient bone is necessary for a good interaction between bone and biomaterials; otherwise, the results are unsatisfactory (Cunha et al., 2008).

The objective of this study was to evaluate by histological and radiographic methods, the osteogenic capacity of hydroxyapatite Ca10(PO4)6(OH)2 (GenPhos HA TCP Genius-Baumer S.A.) implanted into the femur of rats with osteoporosis due to estrogen deficiency induced by bilateral ovariectomy.

MATERIALS AND METHODS

Animals

Eighteen female rats (*Rattus norvegicus*) maintained under standard conditions of housing and feeding at the animal house of the Faculty of Medicine of Jundiaí (FMJ), Jundiaí, São Paulo, Brazil, were used. The animals were divided into three groups: not ovariectomized (NO), bilaterally ovariectomized not receiving hormone replacement therapy (BOWHR), and bilaterally ovariectomized and submitted to hormone replacement therapy (BOHR).

The study was conducted in accordance with institutional ethical guidelines and was approved by the Ethics Committee of FMJ, Jundiaí, São Paulo, Brazil (protocol 17/2009–2010).

Ovariectomy

All rats, except for NO animals, were weighed and anesthetized by intramuscular injection of ketamine (Francotar, Sespo Ind. e Com., Jacareí, São Paulo, Brazil) and xylazine hydrochloride (2% Virbaxyl, Virbac Brasil Ind. e Com., São Paulo, Brazil) at a proportion of 1:1 and a dose of 0.10 mL/100 g body weight. A 2-cm incision was made in the skin with a scalpel lateral to the spine to completely remove the ovary from the pelvic cavity. The musculature and skin were repositioned and closed with No. 4.0 cotton suture sterilized (Nylon, Technofio).

Surgical Procedure for the Implantation of Hydroxyapatite

Particles of 0.5–0.75 mm of hydroxyapatite Ca10(PO4)6(OH)2 (GenPhos HA TCP Genius-Baumer S.A.) were implanted 4 months after surgery, according to (Wronski et al., 1989). After anesthesia as described above, an incision was made in the skin on the medial side of the thigh, exposing the femoral quadriceps muscle. The muscle was sectioned longitudinally in its distal third and separated anterolaterally. With the distal end of the diaphysis of the left femur exposed and the periosteum separated, a bone defect was created with a 3-mm hand-held surgical drill. Next, the defect was filled with hydroxyapatite. The periosteum, musculature and skin were repositioned and closed with No. 4.0 cotton suture. The animals were sacrificed 8 weeks after implantation and the femurs containing the recipient area were removed and submitted to radiological, histological, and morphometric analysis.

Hormone Replacement Therapy

Estradiol (β-estradiol, Sigma, St. Louis, MO) was used for hormone replacement therapy. The drug was diluted in peanut oil (All Chemistry, Sao Paulo, Brazil) and the animals were injected s.c. with 20 mg at mean intervals of 48 h from the time of hydroxyapatite implantation to the day of sacrifice after 8 weeks.

Radiography and Histological Analysis

Radiographs of the left femurs were obtained with a Rigaku RU-200 apparatus at a focal point of 0.8 × 0.8 mm. Kodak radiographic films measuring 7.6 × 5.7 mm were used. After radiological analysis, the samples were submitted to routine histological processing and semiserial 5-μm cross-sections were stained with hematoxylin and eosin.

Morphometric and Statistical Analysis

The quantity of newly formed bone at the site of the hydroxyapatite implant was calculated for each recipient area using a 100-point square grid coupled to the eyepiece of a light microscope, following histomorphometrical methods (Mandarim de Lacerda, 1999). The results were analyzed by ANOVA ($P < 0.05$) followed by the Tukey test, using the software BIOESTAT 5.0.

RESULTS

Macroscopic Analysis

In the studied groups, it was not observed in the grafted area, pathological alterations such as inflammatory processes, pseudoarthrosis, bone erosions, ulcerative lesions, cysts, or other rejection signs like as infection, suggesting a biocompatibility of the hydroxyapatite used (Fig. 1).

Radiological Analysis

Good radiopacity of the implant recipient area and hydroxyapatite granules was observed in the studied groups (NO, BOWHR, and BOHR). In addition, the presence of a cluster of granules indicated the absence of their migration to distant sites. Discrete points of radiolucency were noted between granules, indicating connective tissue infiltration (Fig. 2).

Histological Analysis

Formation of new bone around the hydroxyapatite granules was observed in the studied groups, in addition to the proliferation of connective tissue. However, projections of more voluminous newly formed bone were more evident in the NO and BOHR groups and containing cavities filled with bone marrow (Figs. 3A and 3C). In the BOWHR group, the quantity of newly formed bone was lower (Fig. 3B).

In all groups, a bone layer around the implant showing characteristics of trabecular and cortical bone were observed. A tendency of new bone tissue migration partially covering the bone defect was observed in NO and BOHR (Figs. 3A–3C). The newly formed bone presented characteristics of immaturity; lacunae harboring osteocytes arranged in various directions as well as mature (dense texture) bone (Figs. 3D–3F). No fibrous tissue capsule or inflammatory process in the implant recipient area was observed in any of the groups.

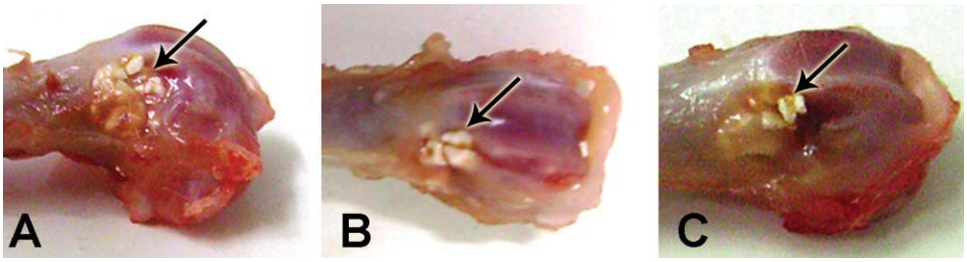

Fig. 1. Anterior view of the implant recipient area in the left femur of nonovariectomized rats (**A**: NO), animals submitted to bilateral ovariectomy without hormone replacement therapy (**B**: BOWHR), and bilaterally ovariectomized animals submitted to estrogen replacement therapy (**C**: BOHR). Note the recipient area (arrow) grafted with hydroxyapatite granules without evidence of infectious foci.

Hydroxyapatite presence presented similar aspects of the amorphous materials, acellular, well defined shape and with higher affinity to hematoxylin (Fig. 3).

Morphometric Analysis

Statistical analysis showed a similar quantity of newly formed bone in the recipient area of NO (36.6 ± 1.1402) and BOHR (32.4 ± 0.8944) animals, which was significantly higher than that observed in the BOWHR group (12.6 ± 1.1402).

DISCUSSION

Despite the applicability of hydroxyapatite as a bone implant, some factors, including diseases associated with bone mineral deficiency such as osteoporosis, may compromise the promising results reported in the literature. According to Frayssinet et al. (1992) the health conditions of bone are fundamental for its interaction with the implant biomaterial. Factors that increase the risk for osteoporosis development include a low calcium intake, smoking, alcoholism, lack of physical activity, and hormonal disorders resulting from early menopause or late menarche (Pinheiro et al., 2008; Shen et al., 2000). The risk of osteoporosis is also increased with ovariectomization (Lincoln and Tyler, 1999), considering that female gonadal hormones such as estrogen stimulate bone growth and mineralization (Wronski et al., 1989). Therefore, this research was able to evaluate the osteoconductive capacity of hydroxyapatite in bone tissue affected by the hormone deficiency effects through ovariectomy procedures.

Ovariectomized animals as it was used in this research are frequently employed for the study of postmenopausal osteoporosis. However, some discrepancies are reported in the literature regarding the interaction between bone and implants of biomaterials such as titanium and ceramics. Some studies have shown significant bone loss around these biomaterials, whereas others reported no difference in bone growth in the recipient areas of ovariectomized animals (Fini et al., 1997; Hayashi et al., 1994; Pan et al., 2000). This divergence might be due to the difficulty in standardizing methods for the correct quantification of bone tissue formed on the implant (Ozawa et al., 2002). In addition, few studies have characterized hydroxyapatite biomaterials introduced into bone defects created in ovariectomized rats in terms of the quantity and mechanical strength of the bone formed in the recipient area.

Several studies have reported the deleterious effects of ovariectomy on the characteristics and properties of bone tissue, such as a reduced strength to withstand external mechanical loads (Giavaresi et al., 2004). According to Mizutani et al. (2000) hypoestrogenism increases bone resorption and reduces trabecular bone mass, thus affecting the biomechanical strength of bone. However, the authors cited above did not study any type of implant. However, Ozawa et al. (2002) implanted titanium into bone defects created in the femur of ovariectomized rats. Biomechanical tests performed after 2 weeks showed that the mechanical strength in the recipient area of ovariectomized rats was half that found in nonovariectomized animals.

To study the bone-implant interaction in cases of osteoporosis induced by hormone alterations, some investigators performed ovariectomy in rats and then implanted titanium into the bone cavities produced in these animals. The authors noted poor interaction between the material implanted and bone tissue, as well as a low amount of newly formed bone, and attributed the findings to the osteoporosis-like conditions as a result of gonadal deficiency induced by ovariectomy (Hayashi et al., 1994; Lugero et al., 2000). Similar results have been reported by De Benedittis et al., (1999); Fini et al., (1997); Hayashi et al., (1994); Jung et al., (2001); and Pan et al., (2000), who used hydroxyapatite as implant material. Cunha et al., (2008) confirmed the effects of estrogen deficiency on the delay of bone regeneration in defects created experimentally in the femur of ovariectomized rats and filled with collagen membranes. According to Albrektsson et al. (1981), bone health status is essential for the interaction of bone with biomaterial implants.

In this study, a lower quantity of newly formed bone was observed in ovariectomized animals not submitted to hormone replacement therapy (BOWHR) and similar quantity of newly formed bone was observed in nonovariectomized (NO) animals and ovariectomized animals submitted to hormone replacement therapy (BOHR), with the quantity of newly formed bone being higher than that observed in the BOWHR group. These findings suggest the influence of estrogen on both the process of osteogenesis and the type of new bone. Similar results have been reported by (Cho et al., 2004), who studied the bone-dental implant interface in ovariectomized rats and observed a reduction of the cortical layer and an increase of trabecular bone in the recipient area. The authors concluded that osteoporosis

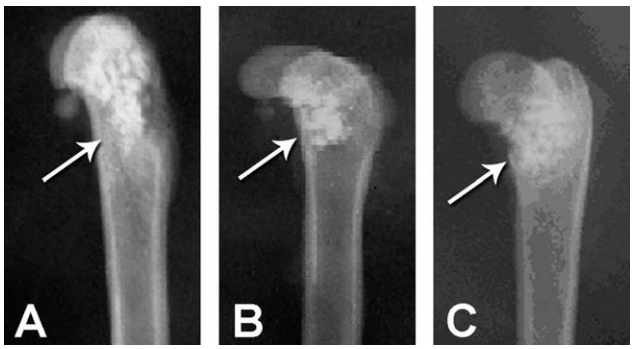

Fig. 2. Radiographs of the implant recipient area in the left femur of nonovariectomized rats (**A**: NO), animals submitted to bilateral ovariectomy without hormone replacement therapy (**B**: BOWHR), and bilaterally ovariectomized animals submitted to estrogen replacement therapy (**C**: BOHR). Note that the arrow represents the radiopacity of hydroxyapatite granules implanted into the distal epiphysis of the femur.

induced by estrogen deficiency might compromise the mechanical stability of the implant area.

Aiming to evaluate the bone-implant interaction considering the osteoporosis condition induced by hormonal alterations, some researches performed ovariectomy in rats and after this surgical procedure, implanted titanium into the bone gaps created in these animals. The histological findings presented a poor interaction between the inserted material and bone tissue, and a low amount of newly formed bone, and were attributed to the osteoporosis-like conditions as a result of gonadal deficiency induced by ovariectomization procedure (Li et al., 2004; Lugero et al., 2000; Pan et al., 2000; Wang et al., 2005). Similar findings have also been reported by De Benedittis et al. (1999), Fini et al. (1997), Hayashi et al. (1994); Jung et al. (2001), who, however, used hydroxyapatite as implant material. Statistical analysis did not revealed statistical difference between the BOHR and NO, but these two groups were different in relation to the BOWHR group. This analysis confirms the effects of estrogen deficiency on bone metabolism in response to the implant used. Thus, bone neoformation can be expected even in ovariectomized animals, but it is slower and the bone volume is smaller. In addition, we noted the presence of trabecular bone in the implanted areas of group BOHR. Similar results have been reported by (Cho et al., 2004; Cunha et al., 2010), who studied the bone-implant interface in ovariectomized rats. The authors concluded that osteoporosis-like conditions induced by hormone deficiency might compromise the mechanical stability of the implant in view of the results demonstrating a reduction of the cortical layer and an increase of trabecular bone in the recipient area.

Biomaterials as hydroxyapatite may be performed for clinical application aiming to improve the bone healing process. The porous of these substances are well tolerated by bone tissue in addition to facilitate osteoblasts infiltration and proliferation of blood vessels, essential phenomena of regenerative process (Shirane et al.; Thomson et al., 1998). Hydroxyapatite granules become possible the migration and union of the osteoblastic cells, resulting in new bone formation (LeGeros, 2002).

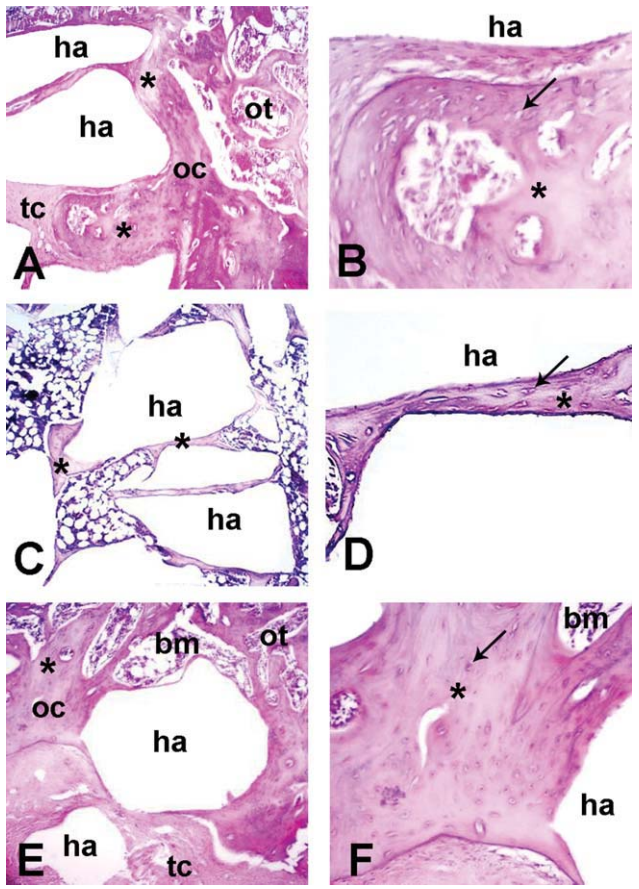

Fig. 3. Histological images of the implant recipient area in the left femur of nonovariectomized rats (**A** and **D**: NO), animals submitted to bilateral ovariectomy without hormone replacement therapy (**B** and **E**: BOWHR) and bilaterally ovariectomized animals submitted to estrogen replacement therapy (**C** and **F**: BOHR). Note the formation of new bone (*) around the hydroxyapatite granules (ha), especially in the NO and BOHR groups. Note thin layer the newly formed bone ingroup BOWHR. OC, cortical bone; OT, trabecular bone; TC, connective tissue; BM, bone marrow. Arrow shows osteocyte in lacunae. Figures 3A–3C (×4) and Figures 3D and 3E (×10). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

In this research, hydroxyapatite granules with particles of 0.5–0.75 mm of diameter were used.

According to the macroscopic results of this research, it was not found signs of rejection of hydroxyapatite, confirming the biocompatibility of this substance (Oonishi et al., 1997; Schmitz et al., 1999; Shirane et al.; Yamamoto et al., 2000).

Radiological observations showed that the radiopacity of hydroxyapatite was more intense than that the normal bone, which was also described by Rezende et al. (1998) and Vital et al. (2006). This can be explained by chemical formula of the hydroxyapatite, which depends on its chemical constituents (Ono et al., 2000). Between the hydroxyapatite granules, it was observed radiolucency due to the presence of the connective tissue; however, did not differ visually between groups.

It was concluded that the hydroxyapatite used in this study showed to be biocompatible, because there any clue for rejection of the implant. Considering the

animals of the NO and BOHR groups, the bone healing process occurred faster confirming the osteoconductive properties of the hydroxyapatite in health bone tissue. In addition, this study showed that bone neoformation even occurs in bone compromised by osteoporosis due to gonadal deficiency, especially when the defect is filled with hydroxyapatite like those used in this study but the quantity and velocity of bone formation are lower.

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