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ORIGINAL ARTICLE

Evaluation of strontium-containing hydroxyapatite as bone substitute in sheep tibiae.

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KEY WORDS

Strontium, Durapatite, Sheep.

With the advancement of research in biomaterials, it has been suggested that the best osteoconductivity of hydroxyapatite would be achieved if its crystal were closer to the structure, size and morphology of biological apatite, that is why nano-hydroxyapatite (nano-HA) is of great importance. current interest. Strontium ions are known to reduce bone resorption, induce osteoblastic activity and stimulate bone formation. The aim of this study was to evaluate biocompatibility and osteoconduction in surgical defects filled with nano-hydroxyapatite microspheres containing 1% strontium (nano-SrHA), stoichiometric nano-HA microspheres (nano-HA) compared to the clot (control) . Four Santa Inês sheep, weighing an average of 32 kg, were anesthetized and submitted to three 2 mm diameter perforations in the medial face of the tibia. The surgical defects were filled with blood clot, microspheres of Sr-HA 1% and microspheres of HA. After 30 days the samples were drawn (6 mm), decalcified, processed for inclusion in paraffin and stained with hematoxylin and eosin (HE) for histological evaluation with light microscopy. All groups revealed bone neoformation from the periphery to the center of the defect, with the nano-SrHA group being less intense among those studied. Presence of a discrete mononuclear inflammatory infiltrate in all experimental groups. Giant foreign body cells were only observed in the HA group. Areas of bone neoformation were observed in close contact with both biomaterials. According to the results obtained, microspheres of HA and SrHA 1% are biocompatible and have osteoconductive properties.

Avaliação da hidroxiapatita contendo estrôncio como substituto ósseo em tíbias de ovelhas.

Com o avanço das pesquisas em biomateriais, tem sido sugerido que a melhor osteocondutividade da hidroxiapatita seria alcançada se o seu cristal estivesse mais próximo da estrutura, tamanho e morfologia da apatita biológica, por isso a nano-hidroxiapatita (nano-HA) é de grande interesse atual. Os íons estrôncio são conhecidos por reduzir a reabsorção óssea, induzir a atividade osteoblástica e estimular a formação óssea. O objetivo deste estudo foi avaliar a biocompatibilidade e a osteocondução em defeitos cirúrgicos preenchidos com microesferas de nano-hidroxiapatita contendo estrôncio a 1% (nano-SrHA), microesferas de nano-HA estequiométrica (nano-HA) em comparação ao coágulo (controle). Quatro ovelhas Santa Inês, pesando em média 32 kg, foram anestesiadas e submetidas a três perfurações de 2 mm de diâmetro na face medial da tíbia. Os defeitos cirúrgicos foram preenchidos com coágulo sanguíneo, microesferas de Sr-HA 1% e microesferas de HA. Após 30 dias as amostras foram trefinadas (6 mm), descalcificadas, processadas para inclusão em parafina e coradas com hematoxilina e eosina (HE) para avaliação histológica com microscopia de luz. Todos os grupos revelaram neoformação óssea da periferia para o centro do defeito, sendo o grupo nano-SrHA com menor intensidade dentre os estudados. Presença de discreto infiltrado inflamatório mononuclear em todos os grupos experimentais. Células gigantes do tipo corpo estranho só foram observadas no grupo da HA. Áreas de neoformação óssea foram observadas em íntimo contato com ambos os biomateriais. De acordo com os resultados obtidos, microesferas de HA e SrHA 1% são biocompatíveis e apresentam propriedade de osteocondução.

Palavras-chave: Estrôncio. Durapatita. Ovinos.

INTRODUCTION

Localized bone defects can occur as a result of infections, pathological processes, congenital injuries, traumatic injuries or even due to tooth extractions and generally interfere with the installation of implants and prosthetic rehabilitation. In recent years, with the objective of restoring and preserving the alveolar bone morphology, new materials and techniques have been the target of research aimed at providing the development of a set of procedures and biomaterials that optimize the performance of this rehabilitation [10,12] .

Bone grafts are classified according to their origin, as autogenous, allogeneic, xenogenous and synthetic or alloplastic. The limitations and difficulties existing to obtain autogenous bone grafts, such as post-operative patient discomfort, morbidity of the donor site, limitation of the amount of graft, ethical and religious issues, and the possibility of transmitting diseases from allogeneic and xenogenous grafts keeps them stimulated researchers to develop synthetic biomaterials, to assist in the regeneration of lost bone tissue [24] .

Calcium phosphates have been studied as materials used in bone repair for the past 80 years. Of the compounds based on calcium phosphate, the most extensively investigated are hydroxyapatite (HA) and tricalcium phosphate. HA has been widely used as an important bone substitute and is distinguished from other calcium phosphate-based ceramics because it is similar to the inorganic portion of bone tissue, biocompatible, mechanically resistant, bioactive, non-toxic, radiopaque, allowing periodic monitoring through imaging tests, cause little tissue reaction and not be antigenic or carcinogenic, in addition to having a great capacity for protein adsorption on its surface [15]. The synthetic HA generally used is in the form of coarse particles, which have a size and shape of the crystal quite different from the morphology of the biological bone apatites [18] . It has been suggested that the best osteoconductivity of AH would be achieved if its crystal were closer to the structure, size and morphology of biological apatite [8,16,19] . The high stability and flexibility of this apatite structure, allows the wide variety of possible cationic and anionic substitutions, thus having the presence of a number of foreign ions associated with biological apatites [18] . Strontium (Sr) is present in the mineral phase of bones, especially in regions with greater metabolic function [2]; its content in the new compact bone is three to four times higher than that of an old compact bone, and approximately 2.5 times higher in the new cancellous bone than in the oldest [11] . As it increases the activity of osteoblasts and decreases the activity of osteoclasts, Sr has an anti-resorptive and bone-forming effect *in vitro* [3] . Calcium phosphate ceramics containing Sr have been shown to increase the proliferation and differentiation of osteoblasts *in vitro* [23] . A previous study revealed *in vivo* an increase in the thickness of the bone layer formed at the bone-

cement interface and a better osseointegration of SrHA cement, compared to pure HA cement [20]. The aim of this study was to perform a subjective histological evaluation of bone repair in sheep tibias 30 days after implantation of microspheres of nano-hydroxyapatite (nano-HA) and nano-HA containing 1% strontium (nano-SrHA) in comparison clot (control).

METHODOLOGY

Four Santa Inês ewes of both genders weighing between 30 and 55 kg and deprived of food 24 hours before the surgical procedure were used in this research. The animals were previously weighed and operated under general anesthesia, and received as preanesthetic medication acepromazine (0.1 mg.kg-1) IV, diazepam (0.2 mg.kg-1) IV and morphine (0.4 mg.kg-1) IM. Induction was performed with propofol (4 mg.kg-1) IV and diazepam (0.1 mg.kg-1) IV (dose effect), and anesthetic maintenance with isoflurane in the appropriate concentration so that autonomic responses did not occur.

After performing the trichotomy and degerming on the medial face of the tibia, the animals were installed on the operating table in lateral decubitus. Then, an incision of approximately 6 cm was made to allow the detachment until exposure of the skeletal plane to make three perforations distant 6-8 cm from the 2 mm diameter tibial crest (2 mm / spear and spherical cutters) SIN - Implant System, São Paulo, SP, Brazil) (Figure 1A). The bone defects were filled with nano-HA microspheres, clot and nano-SrHA microspheres (Figure 1B). The internal and external planes were sutured with Vycril 3.0 and Nylon 5.0, respectively. The surgical wound was left uncovered and all animals received as a postoperative protocol to prevent infections and meloxicam pain control at a dose of 0.5 mg.kg-1 for five days and antibiotic enrofloxacin 5 mg.kg-1, before surgery and for five days after all surgical procedures.

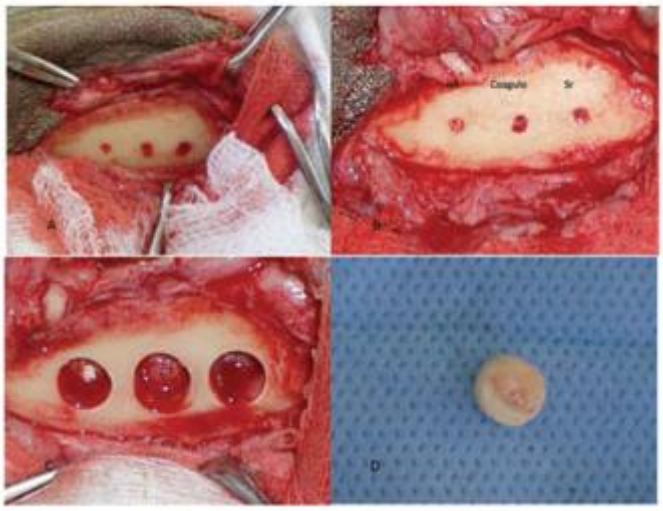


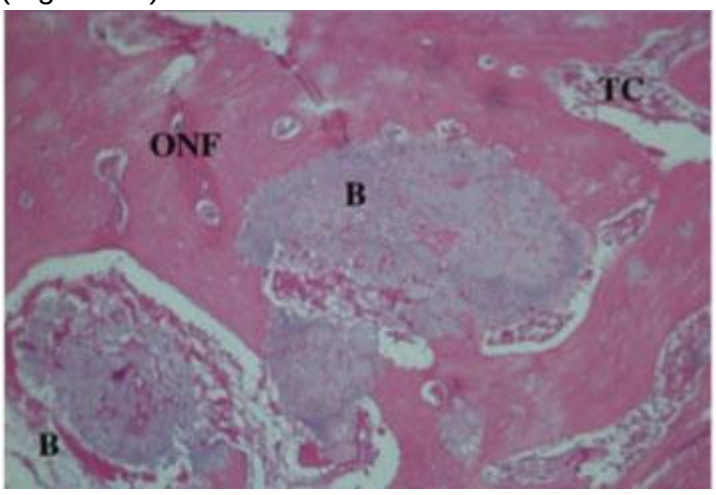
Figure 1- Surgical procedures for implantation and removal of samples. A. Three 2 mm diameter perforations; B. Perforations filled with nano-HA, clot and nano SrHA 1%; C. Bone samples removed through local drawing; D. Bone block with a perforation filled with biomaterial.

After the experimental period of 30 days, the animals were anesthetized, as previously described, and performed: incision, detachment, removal of the samples with a 6 mm internal diameter milling cutter (Figure 1C and 1D) followed by planar suture. The animals were kept alive and the suture and postoperative protocol adopted was the same as the previous surgical time.

The samples obtained from the 4 animals were fixed for 48 hours in 10% formaldehyde buffered with pH 7.4, washed in running water for 6 hours, demineralized with the Allkimia® bone softener at room temperature, for 48 hours. After the demineralization of the blocks, they were dehydrated, cleared and embedded in paraffin. Transverse sections with a thickness of 5 µm and stained with hematoxylin and eosin (HE) were obtained and analyzed under light microscopy. In the microscopic analysis, the type and intensity of the inflammatory process in response to the procedure, the presence of connective tissue and newly formed bone in the surgical defect were described through subjective analysis.

RESULTS

Histological analysis revealed that in the group containing a clot (control) bone neoformation was observed from the periphery to the center of the bone defect, consisting of large anastomosed bone trabeculae, however, in the central portion of the defect, areas of loose connective tissue and rare inflammatory cells were observed. (Figure 2A).



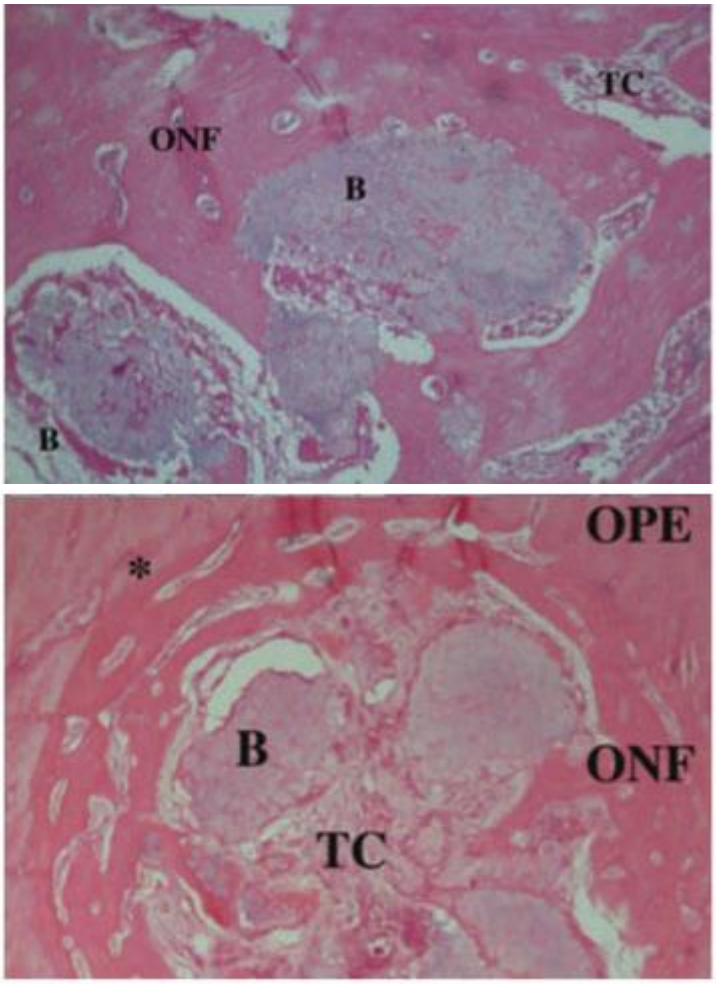


Figure 2- Photomicrographs of the experimental groups:
A- Coagulum
B- Nano-HA
C- Nano-Sr-HÁ

DISCUSSION

The most important aspect during the development of new biomaterials is the experimental and clinical trial to assess their biocompatibility [17] . The use of sheep for research has increased over time due to similarities with humans in weight, bone and joint structure, and bone regeneration [1,13] . It was observed in this study that the sheep drilling model proves to be an excellent animal model to assess the biocompatibility of bone substitute biomaterials, where in the implantation model (femur), it revealed the possibility of implanting up to 8 biomaterials per animal [14] and also by possibility of keeping the animals alive after the end of the experiment.

Nano-HA is of current interest due to its biological properties compared to HA, in addition to its greater similarity to physiological apatite [8] . Previous studies have shown an increase in the motility of osteoblasts exposed to spherical nano-HA particles [25] and

an increase in the migration of these cells when exposed to spherical nano-HA particles [9] . Nano-HA exhibits excellent adhesion, not only for mineralized and non-mineralized tissues [22] . Based on these previous studies, the present study adopted spheres as the material format.

The present study demonstrated that HA containing 1% Sr is biocompatible, presenting newly formed bone in close contact with the biomaterial, but revealed less intensity of bone neoformation than nano-HA, results that were not confirmed by a previous study that evaluated a cement of HA containing Sr, which showed better osteoconductivity, biocompatibility and biodegradability than the free HA cement of Sr. In addition, the doses of Sr incorporated in the crystal structure of HA played an active role *in vitro* and *in vivo* . From the data from the biocompatibility tests, they observed that the cement containing 5% Sr was more biocompatible, followed by the HA cement containing 10% Sr and finally the Sr [6] free HA cement.. In another study, by these same authors, it was also found that HA cement containing 5% Sr achieved greater resistance to compression in the analyzed samples (5-10%) [7] . These data indicate that there is an ideal dose of Sr to be incorporated into the HA crystal in order to obtain better physico-chemical and biocompatibility properties [5] . However, the results of the intramuscular implant and the implantation experiments in the rabbit femur show that the average dissolution rate of HA cement containing Sr increases with the increase in the dose of Sr [6]. This can be explained by the fact that an HA containing substitutions is considered a calcium deficient HA and, consequently, more soluble and the greater the incorporation of the metal the more calcium deficient it becomes, therefore the concentration of the incorporated metal is a important control parameter to adjust the properties of HA.

An *in vitro* study evaluated the bioactivity of HA containing Sr in simulated body fluid, and its effect on proliferation, cell morphology, alkaline phosphatase and osteopontin activity in the culture of osteoprecursor cells *in vitro*. The HA ceramics containing Sr exhibited high bioactivity in simulated body liquid, which was clear by the rapid formation of apatite on its surface. The cell culture test indicated that HA containing Sr has good biocompatibility in human osteoblasts. Compared to HA, Sr-HA promoted adhesion of osteoprecursor cells and cell proliferation, and did not present any deleterious effect on the formation of the extracellular matrix and mineralization. It has also been shown that the presence of Sr stimulates the differentiation of osteoprecursor cells, and increases alkaline phosphatase and expression of osteopontin [23]. This study concluded that Mr promotes osteoblastic action and subsequent bone neoformation. These results different from those obtained can be justified by the concentration of the Sr used in this study (1%). However, more research is needed for a detailed understanding of the cellular and molecular mechanisms of the effects of strontium on bone cells.

Another *in vitro* study carried out in order to evaluate the osteoblastic and osteoclastic response of HA containing Sr in different concentrations, showed that osteoblastic cells

cultured in HA, containing Sr grew showing normal morphology, good proliferation and increased values of differentiation parameters, at the same time. At the same time, the number of osteoclasts was negatively influenced by the presence of Mr. The positive effect of the ion on bone cells was particularly evident in the case of HA deposition containing relatively high Sr (3-7%), values that significantly increased the activity of alkaline phosphatase, osteocalcin, type I collagen and osteoprotegerin / TNF related to cytokine receptors, a considerable reduction in osteoclast proliferation has also been observed [4].

An *in vivo* study investigated the bone tissue response of a bone cement of HA containing Sr injected into spongy iliac crest bone of rabbits for 1, 3 and 6 months. Bone affinity to HA cement containing Sr increased from 73.55% ± 3.50% after 3 months to 85.15% ± 2.74% after 6 months ($p = 0.01$) [21] . These results show that the HA cement containing Sr is biocompatible and osteoconductive, confirming the data obtained in this study despite our experimental period having been only 30 days. In another *in vivo* study , osseointegration in cancellous bone was achieved with the use of HA cement containing Sr in rabbits, which stimulated bone formation and union, the fusion of bone with HA cement containing Sr and indicated biocompatibility *in vivo*. Tetracycline staining showed that the mineralization area was in the order: 3 months - 1 month - 6 months. In the 1st month, the increase in mineralization was due to the bone healing process. An additional increase in the mineralization area in 3 months indicated that HA containing Sr has a stimulating effect on bone formation. The mineralization area decreased in 6 months because the healing process was concluded with bone remodeling [20] . Among all the studies analyzed, it was observed that HA containing Sr is biocompatible depending on the concentration of Sr and the period of the experiment.

CONCLUSION

According to the results obtained, the microspheres of HA and SrHA 1% can be considered biocompatible and with osteoconductive potential and can be indicated as bone substitutes.

CONFLICT OF INTERESTS
The authors declare no conflicts of interest.

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