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Biological Evaluation *In Vivo* of Synthesis of Silicated Apatites

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ABSTRACT

The incorporation of silicon into the apathetic structure gives significant results in terms of mechanical properties and the bioactive behavior. The Bioactivity promotes the reaction between the bone and the implant, leading to excellent osteointegration and assures then the success of the implant.

The biological study was performed on adult dogs. The choice of the experiment focuses on the tibias because these latter represent interesting models for evaluating substances stimulating bone formation. The surgically created defects have been filled by our biomaterials and followed by clinical, radiological and histological studies. The follow-up period was determinated so as to be close to the bone healing time. Therefore, the duration of the experiment was 12 weeks.

Different biological studies reveal the biocompatibility, bioactivity and osteoconduction phosphocalcic silicate apatites which are gradually resorbed and replaced by immature bone after three months post filling.

Keywords: Apatite phosphate silicate, Bioactivity, Biocompatibility, Biological evaluation, Bone filling.

INTRODUCTION

The calcium phosphate apatite are a family of ionic compounds described by the chemical formula (Me)x (XO_4)y(Y)z wherein Me is a divalent metal (Ca^{2+}, Sr^{2+}, Ba^{2+}, Pb^{2+} ...), XO_4 is a trivalent anion (PO_4^{3-}, AsO_4^{3-}, SiO_4^{3-},..) and Y is a monovalent anion (F^-, Cl^-, Br^-, I^-, OH^-..) [1]. The bioactive behavior of the apatite may be improved by approaching the general formula of the bone that is: $Ca_{8.3}(PO_4)_{4.3}(CO_3)_{1.0}(HPO_4)_{0.7}(CO_3, OH)_{0.3}$ as it was declared in Britel et al. [1], the non-stoichiometry, illustrated by the presence of gaps on Me and Y sites, is generated by the poly substitution of XO^{2-}_4 sites by species of different vacancies [2]. This approach can be achieved by incorporating the components (Si, Mg, V, As ...) [3]. To this end, we are interested in the insertion of Si on phosphate apatite. The model chosen for this substitution is:

$$Ca_{10}(PO_4)_{6-x}(SiO_4)_x(OH)_{2-x} \text{ with } 0 \leq x \leq 2$$

The septic complications and the unavailability of bone grafts justify the development of synthetic bone substitutes [4-6]. Many synthetic biomaterials have been available to surgeons for many years [7,8]. According to the physicochemical properties of these synthetic biomaterials, we distinguish those used for filling bone defects and those intended to replace a skeletal piece called prosthetic. The biomaterials that we have developed, as part of this work, are the silicate biomaterials intended for bone filling, that are synthesized by wet process [9-11].

The objective of this work is the evaluation of the biocompatibility, bioactivity and osteoconduction to the silicated apatites made from calcium carbonate $CaCO_3$, phosphoric acid H_3PO_3, and a silicate precursor.

MATERIALS AND METHODS

Sample preparation

The powders were prepared by an aqueous precipitation method using $CaCO_3$ (SOLVACHIM), H_3PO_4 (SOLVACHIM) and $Si(CH_3CH_2O)_4$ TEOS as reagents. The quantities of reagent were calculated assuming that one mole of phosphate was substituted by one mole of silicate, while maintaining our stoichiometric apatite [12-14], molar ratio Ca/(P+Si)=1.67 and the substitution mechanism proposed for this synthesis rests on this equation:

$$10Ca^{2+}+(6-x)PO_4^{3-} + x(SiO_4^{4-})+(2-x)OH^- \longrightarrow Ca_{10}(PO_4)_{6-x}(SiO_4)_x(OH)_{2-x} \quad (1)$$

with $0 \leq x \leq 2$

The reaction was maintained in a 50% ethanol water medium, the ethanolic aqueous solution at 50% and H_3PO_4 and TEOS was added to $CaCO_3$ solution and the number of moles of $CaCO_3$was kept constant(equation1) [15-17]. The precipitation reactions were carried at 80°C and the pH was maintained at 10 by the addition of ammonia solution. After a complete mixing of the reactants, the suspensions were agitated for 4 h. The resulting precipitates were filtered, washed and dried at 900°C for 4 h.

The biological study of this apatite has developed a clinical, radiographic and histological study for the filling of bone defects surgically created. The choice of the animal and the procedure to be followed in this study depends on the nature of the used biomaterial.

The choice of the animal, the site and the operating channel

The dogs and cats are interesting models. The bones of these carnivore animals are osteon and haversian; they have a bone structure similar to that of human. The modalities of healing and bone remodeling are also very close [18,19]. Furthermore, the highly treatable nature of dogs may be beneficial during the postoperative healing phase, where they can be trained to take an active part in recovery [20,21].

The choice of the test site focuses on the radius and the tibia [22,23] which represents interesting models for assessing substances stimulating osteogenesis. The main animals used in test studies of bone defects are rats, rabbits, dogs and sheep. The model is selected according to the biomaterial and the evaluation criteria (Figure 1).

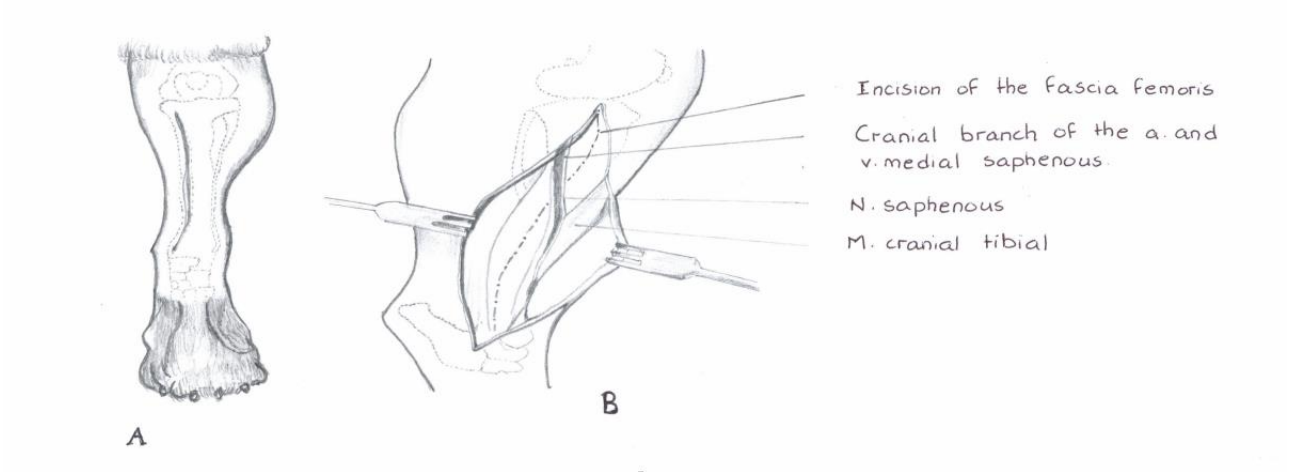

Figure 1: The medical approach of the tibia shaft. A: Place the incision; B: Anatomical structures of the medial tibia

The operating procedure

To evaluate the behavior of the apatite on the bone tissue, a surgical protocol has been defined; it includes the preparation of the animals for surgery, by creating bone defects, the preparation of materials and finally the monitoring after surgery [24-26]. The adopted protocol is described as follows:

Preparing animals for surgery

The study was performed on male common race dogs, aged from 1 year and a half to 3 years and weighing between 15.60 and 18.60 kg. The dogs are dewormed and vaccinated against rabies and they are free of the heart worms of rickettsial and borreliosis. They all have a good condition and show no sign of musculoskeletal disorder.

Anesthesia

The animals were premedicated with an intramuscular injection, 15-20 min before the induction of acepromazine at a dose of 0.2 mg/kg. An intravenous administration of thiopental sodium at a dose of 15 mg/kg was then performed. The maintaining of anesthesia is provided by adding the same anesthetic doses as needed.

Drip

Animals were perfused during these procedures with saline (0.9% NaCl) in order to compensate the blood loss related to the surgery, and to ensure the hydric maintenance requirements. The affected members of dogs (radius, tibia), were degreased with alcohol and then disinfected with Betadine, to ensure strict asepsis.

Creation of bone defects

Bone defects were created at tibias and radius of dogs using an orthopedic drill, five holes with 4 mm of diameter and spaced by about 1 cm were made at the dorsal side of the shaft radial and the medial side of the tibial diaphysis [27]. Witness holes were also made for each animal (the animal under control) (Figure 2) [28]. Hemostasis was carried out by applying sterile gauze and injecting a few drops of adrenaline *in situ*. Different created holes were filled by apatite silicate (SiHa) developed as blocks, while others have not been filled, they served us as witnesses.

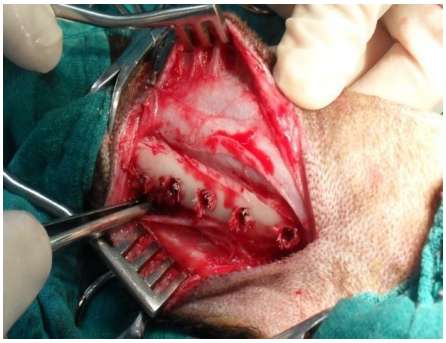

Figure 2: Photograph taken during surgery, showing bone defects creation protocol

Preparation of the material for filling created bone defects

The phosphocalcic apatite silicated has been developed in blocks and it is sintered at 900°C. While filling bone defects, we noticed that the phosphocalcic apatite silicated takes perfectly the form of surgically created hole.

RESULTS

Chemical analysis

The Ca/P ratio of apatite silicated was found to be equivalent to the theoretical value of the pure phase of apatite (1.67). The Ca/P molar ratio of apatite silicate synthesis (1.81) (x=0.5) (Table 1) was much higher than the value of stoichiometric apatite, this increase in Ca/P ratio for SiHa is due to the substitution of silicate ions for the phosphate site in apatite, in order to accommodate silicate ions, the phosphorus content was decreased [16]. The X-ray Diffraction (XRD) patterns of sintered Ha and SiHa using a D8 advance Eco de Brucker diffractometer. Data were collected over the 2θ range 5°C-70°C with a step size of 0.06°and a count time of 1s, as shown in (Figure 3). The XRD for the two materials appeared to be similar; the incorporation of silicon into the Ha did not have a direct effect on the phase composition, with no secondary phase (Figure 4). The Fourier Transform Infrared (FTIR) spectra using the iraffinity-1s spectrometer, at a resolution of 4cm^{-1} averaging 100 scans, of sintered Ha and Si Ha are similar to those observed by Gibson et al and Palard et al. [12,16,29] (Figure 3). The most notable effect of silicate substitution on the FTIR spectra of apatite being the changes in PO_4^- bands between 500 and 700 cm^{-1}, 800 cm^{-1} and 1100 cm^{-1}.

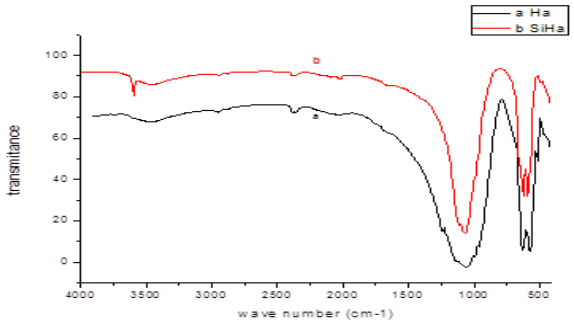

Figure 3: FTIR spectra of sintered (900°C; 4 h) hydroxyapatite (Ha) and silicate substituted apatite (SiHa)

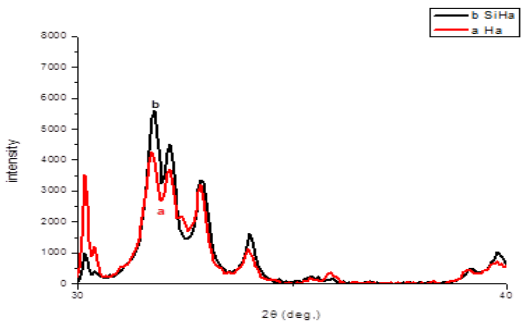

Figure 4: XRD patterns of sintered (900°C; 4h) hydroxyapatite (Ha) and silicate substituted apatite (SiHa). No secondary phases were detected

Table 1: chemical analysis of sintered Ha and SiHa, showing the measured silicon content compared to the calculated value and the Ca/(P+Si) molar ratio

Sample	X	Ca/P molar ratio	Ca/(P+Si)
Ha	0	1.67	1.67
SiHa	0.5	1.81	1.67

Tracking protocol for biological evaluation

The time of experimentation may be varied according to the species and to the age of used animals. The follow-up period was determined so as to be close to the duration of the bone healing. Therefore, the duration of the experiment is 12 weeks.

Post-operative followed protocol

After waking, the animal is placed in an individual cage for the rest of the observation period. The surgical wound is disinfected using Betadine and covered by a healing cream. The animal receives a daily antibiotic therapy with penicillin for 6 days and undergoes a comprehensive review including the evaluation of rectal temperature, heart and respiratory rate, the state of the glands, the mucous membranes and the capillary refill time. Clinical examination of the animal postoperatively doesn't show any obvious signs of pain or locomotive difficulties following the introduction of the biomaterials. The dog was able to walk with moderate limping in the hours following the intervention. Surgical wounds don't show any healing problem or complications of sutures or secondary infections. These results show a good tolerance of the considered biomaterials.

Radiographic control

The follow-up radiographic (the type of radiation: 50 kV, 50 mA, 1.2 ms) is made in the following postoperative periods:

- Immediately after surgery.
- 4 weeks after surgery.
- 8 weeks postoperatively.
- 12 weeks after surgery.

This control allows us to determine the radiographic density of the biomaterial used and to observe the responsiveness of bone tissue with biomaterial, including: the radiopacity of the biomaterial and the appearance of its edges, the site radiographic appearance receiver, the radiographic appearance of bone interface/substitute, and the resorption of the biomaterial used and the formation of the new bone.

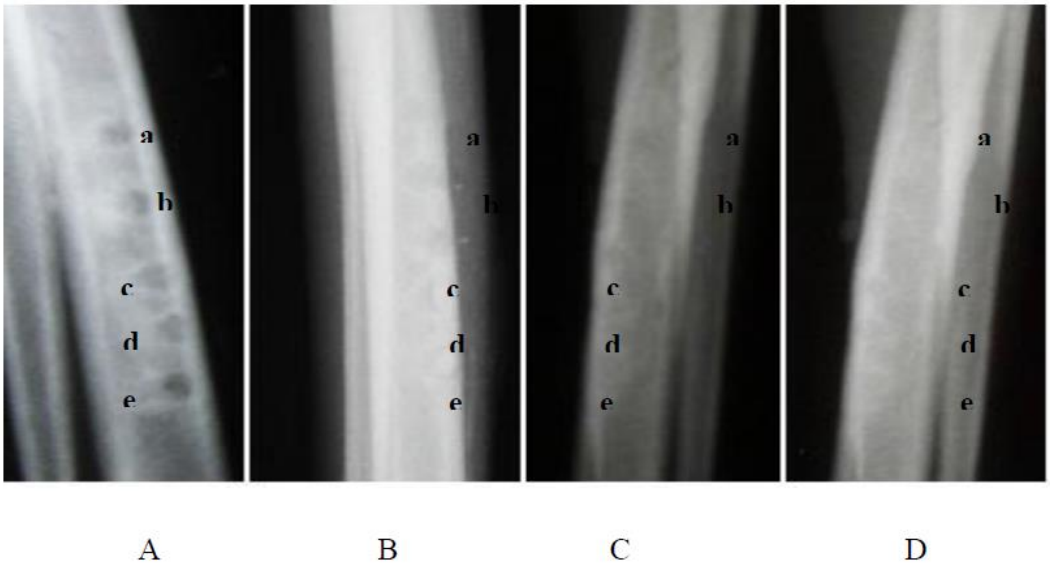

Figure 5: Oblique radiographs. a and b: witness holes and c-e: holes filled by SiHa; A: Immediately after surgery; B: 4 weeks after surgery; C: 8 weeks postoperatively; D:12 weeks after surgery

A radiograph of the tibia profile was performed immediately after the operation. It shows the presence of SiHa at the holes c, d and e, the holes a and b are witness holes. The radiograph shows that the apatite phosphacalcic silicate is radiopaque (Figure 5A).

The calcium phosphate apatites silicated, due to their chemical composition, are radio opaque facilitating their localization at the radiographic control, and later to track the kinetics of osteointegration [30]. The radiographic brought us a qualitative answer regarding the disappearance of bone defects and the biomaterials used for the filling. This result confirms the biocompatibility of the calcium phosphate apatite silicate [31].

Radiographic control at 30 days (Figure 5B) is based on the results of radiological changes in bone defects filled by our biomaterials. We see that after one month, a part of the holes has shown a lack of endosteal callus, while the majority of the holes showed endosteal callus light and a clear callus.

The radiographic control at 60 days (Figure 5C) showed the disappearance of callus in the majority of holes (at the holes which have submitted a callus 30 days) and the end of the proliferation of callus in a small percentage of holes filled by our biomaterial (the holes that showed a lack of callus (at 30 days).

The radiographic control at 90 days (Figure 5D) showed the holes filled by SiHa: half of them have become invisible, whereas the other half is still visible on the plate after 90 days post operation.

Histological evaluation

The histological studies allow us a quick reading of the morphological evolution of bone defects filled by our biomaterial. This study was conducted, following the last radiographic control, three months after surgery.

The realization of histological sections was made according to conventional techniques by inclusion of paraffin. They were stained with Hematoxylin and Eosin (HE) and also Masson's trichrome method (TM).

As for witnessholes, histology shows a typical trabecular structure of the immature bone during remodelling and the presence of osteoblasts in apposition of newly formed bone tissue. The medullary spaces look normal, containing cellular and vascular elements, without inflammatory infiltrations (Figures 6a and 6b).

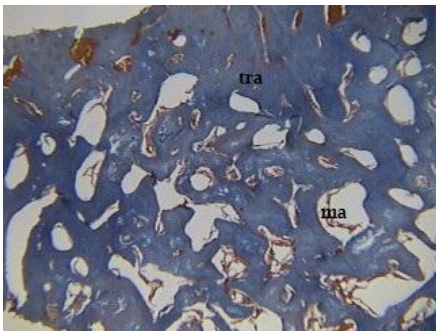

Figure 6a: Histological section of infilled hole showing the presence of a bone newly formed trabecular types (magnification 25 Trichrome Masson). tra: Trabecular, ma: Bone marrow

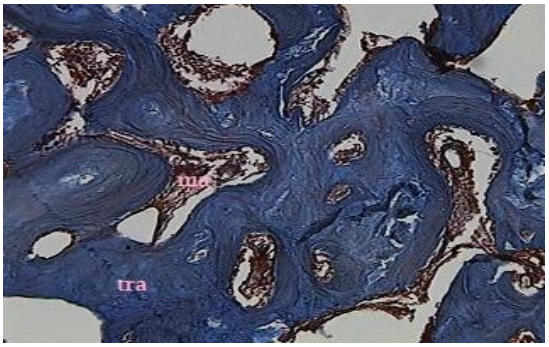

Figure 6b: Histological section showing the new bone trabecular type stage redesign (Magnification 40, Trichrome Masson)

The histological sections of the holes, filled by our product synthesized, demonstrate the new bone formation between the grains of SiHa. The interfaces of SiHa and the bone are characterized by the presence of vascular elements, a new bone trabecular type and the absence of any inflammation or fibrous encapsulation (Figures 7a and 7b). Trabecular density appears to be significant compared to the radiographic control. The trabecularis surrounded by two or three rows of osteoblasts. Marrow spaces additionally contain cellular elements and vascular residues of our biomaterials. We also note the absence of any inflammation or fibrous encapsulation with respect to the product used (Figure 6b).

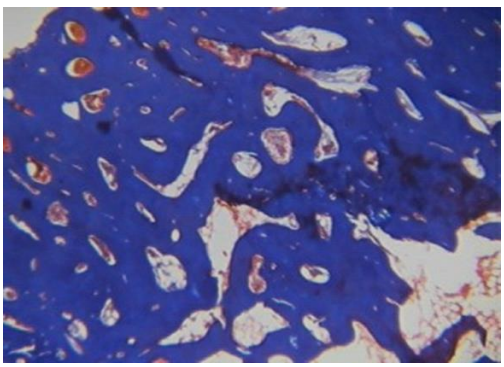

Figure 7a: Histological section at a hole filled by SiHa (Magnification 25 Trichrome Masson). tra: trabecular, ma: bone marrow

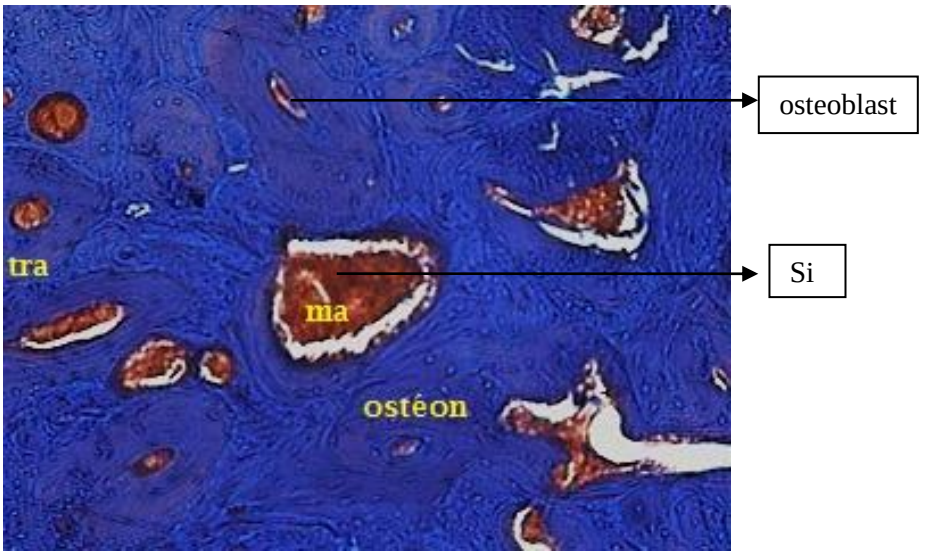

Figure 7b: Histological section at a hole filled by SiHa (Magnification 40) tra: trabecular, ma: bone marrow

DISCUSSION

This study has successfully shown how the *in vivo* bioactivity of phosphocalcic apatite silicated can be enhanced with the incorporation of physiologically relevant ions such as silicate into the apatite lattice. In this *in vivo* study, the pure phase of apatite and of silicate substituted apatite was prepared with similar physical properties. The synthesis route used to prepare the apatite silicated was successfully realized; the chemical analysis and spectroscopy following this synthesis demonstrated positive results. The Ca/(P+Si) molar ratio for SiHa was calculated as 1.67, which is equivalent to that of the phase pure Ha. The chemical analysis confirms that the silicate incorporated into the Ha lattice occupies the phosphorus site according to the reactional mechanism described elsewhere (Equation 1). The incorporation of silicon into the Ha lattice demonstrated by the XRD data (Figure 3) shows no observation of secondary phase for the SiHa. The importance of the granule size, shae, bulk density and packing density of bioceramics when implanted in an osseous environment has been reported by Oonishi et al. and Patel et al. [31,32].

The biological study allows us to evaluate the biological properties of calcium phosphate apatite silicated to assess both the efficiency and the performance of these biomaterials as bone substitutes. The *in vivo* results indicate bone ingrowth into the spaces between the surrounding bone and our product SiHa. We also noted that the SiHa biomaterial is well accepted in the host tissue, with no evidence of inflammatory cells, there is a good fixation of our product. The absolute percentage of bone ingrowth coverage and bone mineral apposition rate for SiHa implant was significantly interesting. These results have established that silicium plays an active role in bone formation and calcification. Finally this study shows clearly, that the biological activity/response of apatite is significantly enhanced by the substitution of low levels of silicate ions into the Ha lattice [33].

CONCLUSION

The *in vivo* study of silicated apatites developed and conducted on dogs for a period of three months allowed us to verify the effectiveness and the simplicity of preparation of the developed materials. Different biological studies reveal the biocompatibility, the bioactivity and the osteoconductive of our biomaterials that are gradually resorbed and replaced by immature bone three months after post filling.

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