Calcium Phosphate Cement Plus 10% Wollastonite Whiskers: An In Vivo Study

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Abstract. Biomaterials can be used in several areas of regenerative bioengineering and is a viable option in the repair of bone injuries. A number of different types of biomaterials have been studied in relation to bone repair. Ceramics such as α-TCP have low fracture toughness compared to natural bone, so reinforcements such as wollastonite whiskers are developed so that they can be used in places with greater overload. This study aimed to evaluate the biocompatibility and bone neoformation of α-TCP plus 10% wollastonite whiskers, in vivo.

To obtain the cement, α-TCP powders with or without 10% wollastonite whiskers were added to an aqueous solution containing 2.5% by weight of Na2HPO4 (anhydrous bibasic sodium phosphate). The biomaterial then became a paste, which was molded into the critical 5 mm defect made in the parietal bone of Wistar rats. Ten rats were divided into two groups. The animals from each group were euthanized within 30 days. Calvaries were removed and subjected to histological processing with Eosin and Hematoxylin.

The implementation of the whisker biomaterial revealed the formation of intensely vascularized connective tissue in the implemented region; however, animals with the biomaterial α-TCP showed the formation of this tissue around the implemented region. On the other hand, intense bone resorption was observed only in the animals with Wollastonite Whiskers, but new bone formation in both groups.

The biomaterial evaluated was shown to be non-cytotoxic, resorbable, and capable of inducing bone neoformation; however, more studies should be carried out to assess the application of this biomaterial in bone injuries.

Introduction

Bone is a mineralized connective tissue formed by four types of cells: osteoblasts, bone lining cells, osteocytes, and osteoclasts. Its functions include locomotion, support, and protection of soft tissues, storage of calcium and phosphate, and protection of bone marrow. The bone is continuously reabsorbed by osteoclasts and newly formed by osteoblasts. Also, osteocytes act as mechanosensors of the bone remodeling process. Bone lining cells appear to play an essential role in coupling bone resorption to bone formation. [1]

In the event of an extensive injury with substantial bone loss, it is often necessary to use a bone graft or to implant a biomaterial for the regeneration to occur.[2] One of the most used approaches today is autologous graft, which consists of removing the patient’s bone tissue from healthy bone and grafting it at the site of injury, giving the advantage of a lower risk of tissue rejection and disease transmission. However, this technique can lead to complications such as infections, chronic pain, bruising, besides being limited. Because it is necessary to have an additional surgery to remove bone...
to be grafted, increasing the costs with the procedure, risks of vascular and neurological injuries and postoperative morbidity, in addition to subjecting the individual to additional pain.[3,4]

Due to these complications, another option for treating bone injuries is the implantation of biomaterials that can be composed of metals, polymers, ceramics and compounds. These classes can be used alone, or in combination, to form most implantation devices available today.[5]

For a material to be used as a biomaterial it must have important characteristics such as biocompatibility (not inducing adverse biological responses, such as allergic and inflammatory reactions not tolerable by the body) high osteoconductivity (stimulating the growth of bone cells); bioactivity (which is the material’s ability to join with biological tissue) and biodegradability (phenomenon in which the material is degraded or solubilized in tissue fluids, disappearing from the implantation site). [6,7]

In the group of ceramics is the α Tricalcium Phosphate (α TCP), one of the most widely used biomaterials in clinical practice, for it is bioactive, non-toxic both in vivo and in vitro, osteoconductive and bioreabsorbable [8]

However, it has low mechanical resistance, restricting its use to buccomaxillofacial defects and covering of metallic prostheses. [9]. To suppress these disadvantages and improve the mechanical strength and bioactive properties of ceramics, some materials such as fibers and whiskers (very short fibers with an aspect ratio of 10 to 100 microns) have been developed.[10]

Whiskers are formed by small elongated single crystals with high mechanical resistance. They can be obtained through phosphates (hydroxyapatite whiskers), carbonates (aragonite whiskers), and calcium silicates (wollastonite whiskers).[11]

The insertion of various types of fibers in a matrix, in this case, calcium phosphate cement, increases the ability of the ceramics to withstand mechanical stress, since the mechanical load is transferred from the matrix to the fibers. This occurs as a result of the differences between the physical properties of fiber and the matrix. Therefore, this composite resulting from fibers to cement would have higher fracture resistance. [12,13]

Whiskers have high resistance and high purity, leading to increased resistance to crack propagation and improving the properties of biomaterial. [10] They may be composed of calcium phosphates, carbonates, and silicates. Silicon (Si) has been drawing attention because it is an element that influences the bone formation and calcification, as well as stimulating cellular activities such as osteoblast proliferation and differentiation of mesenchymal cells into osteogenic cells[14]. Also, studies show that Wollastonite (CaSiO3), a mineral belonging to the silicate group, has in vitro bioactivity, and the rate of apatite layer formation on its surface is faster when in a simulated biological fluid [15]. Due to these characteristics, Wollastonite whiskers can be added to ceramics to enhance their physical and biological properties.

For this reason, the present article aimed to evaluate the biocompatibility and the ability to stimulate bone neoformation of a new α TCP-based biomaterial plus wollastonite whiskers in vivo.

**Material and Methods**

This experimental protocol follows the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation (COBEA), having been approved by the Animal Use Ethics Committee - CEUA / Unicamp (number 2810-1).

For the in vivo study, the model chosen was the Wistar strain rat from the Multidisciplinary Center for Biological Research in the area of Laboratory Animal Science (CEMIB) - UNICAMP. Albino rats (Rattus norvegius), eight weeks old Wistar male, were used.

Tab. 1. Population distribution according to the experimental group and the euthanasia period of the animal.

| OBSERVATION PERIOD | GROUP α- TCP | GROUP α- TCPW |
|--------------------|---------------|---------------|
| 30                 | 5 RATS        | 5 RATS        |
Preparation of Biomaterials

The material used in this study consists of a Wollastonite whisker-reinforced \( \alpha \)-TCP-based CFC that was developed by the Institute of Chemistry of UNICAMP.

The \( \alpha \)-TCP was obtained through the solid-state reaction of calcium carbonate (CaCO\(_3\), CC-RC, Synth) and monetite (CaHPO\(_4\), M-RC, Synth) at a temperature of 1300°C for 6 hours. The wollastonite whiskers were synthesized using the reactive salt fusion method, starting with CaCO\(_3\) calcium carbonate obtained in the laboratory and silicon dioxide, SiO\(_2\). The CaCO\(_3\) was transformed into calcium oxide, CaO, by the calcination process at 800 ºC / 12 h. The saline flow was prepared by dry mechanical mixing of NaCl and KCl in a 1:1 mass ratio. Then, CaO and SiO\(_2\) were mixed in an agate mortar in equimolar proportion. Finally, through a dry mechanical mixing, the precursors of wollastonite, CaO, and SiO\(_2\), were dispersed in the saline flow in a mass ratio of saline flow/precursors of 6:1. [16]

After synthesis, samples were washed with deionized water at 90 ºC and magnetic stirring until the salts' total elimination. The crystalline phases formed after synthesis were determined using X-ray diffraction (Shimadzu XRD7000, 10-40º, 30 mA, 40 kV). The whiskers' morphology and aspect ratio were analyzed by scanning electron microscopy (Jeol JSM6360-LV and Jeol 5900LV).[16]

The \( \alpha \)-TCP powders and wollastonite whiskers were mixed in the proportion of 10% by weight of whiskers. Finally, the powders were added to an aqueous solution containing 2.5% by weight of Na\(_2\)HPO\(_4\) (bibasic sodium phosphate anhydrous) to obtain the cement. Figure 1. [17]

Fig 1. (A) Biomaterial powder. (B) Aqueous solution containing 2.5% by weight of anhydrous bibasic sodium phosphate. (C) Paste resulting from mixing the powder with the aqueous solution.

Intraosseous Implant

For the placement of the intraosseous implant, the animals were anesthetized with Ketamine Hydrochloride and Xylazine at a ratio of 1:1 with a dose of 0.15 ml / 100g of weight; skull trichotomy and assepsis with 70% alcohol were performed. Then, a median sagittal incision of the skull was made, bending the periosteum and performing a parietal bone defect with a 5 mm critical diameter surgical punch (Fig. 2A); The paste-shaped biomaterial was then molded into the defect shortly after the lesion (Fig. 2B), after that the periosteum and skin were sutured (Fig. 2C, Fig. 2D).
Once sutured, the antibiotic Rifamycin was administered to prevent infections. The analgesic Dipyrone Sodium at a dose of 875mg / kg was administered orally in the five days following the surgery. The animals were divided into 2 groups of 5 animals each. In one of the groups (G1), the α TCP was implanted without any reinforcement (group α-TCP); in a second group (G2) the α TCP reinforced with 10% wollastonite whiskers (α-TCPW group) was implanted.

![Fig. 2 (A) Defect made in the skullcap. (B) Biomaterial inserted into the experimental cavity. (C) Periosteum suture. D) Skin suture.](image)

**Euthanasia and Specimen Collection**

The rats were euthanized after 30 days after the surgical procedure through deepening anesthesia. Inspection showed that there was no sign of inflammation or infection in the operated region.

The right parietal was then removed. After 30 days, it was still possible to observe macroscopically, the remaining biomaterial.

Histopathological analysis of bone tissue or calcified requires a step of descaling after fixation, which allows removal of calcium salts, necessary for obtaining cuts in this type of sample.\[18\] Therefore after removal, the samples were placed in 10% 0.1 M formalin solution pH 7.3 for 48 hours at room temperature. After that, they were washed with running water overnight and placed in 70% alcohol and then placed in 5% EDTA® solution. The decalcifier was changed every two days for 8 weeks.

**Light Microscopy**

Most tissues are made of water, so increasing concentrations of alcohol baths are necessary to achieve dehydration. The fragments were dehydrated in batteries of alcoholic solutions at gradual and increasing levels totaling 6 hours. Diaphanized in xylol (2 hours) bleaching to replace the ethanol present in the sample and soaked in liquid paraffin to make the microtomy blocks.

For the preparation of the histological slides, the blocks were cut to 6 µm thickness in a Leica RM2125RT manual rotary microtome and then stained with Hematoxylin and Eosin H&E.
The histological sections were examined with the aid of a Nikon model 80i photomicroscope with 4x, and 10x objectives and their images were captured by a Nikon DS-Ril camera. (Fig. 3).

Fig. 3 Assembly of the different pictures of the same blade obtained by the Adobe Photoshop CS program.

Results

The animals were submitted to a surgical procedure to implement the biomaterial Wollastonite Whiskers or α-TCP in the skullcap. After about 30 days, they were euthanized, and the tissue containing the biomaterial was removed for analysis with HE. The implementation of the Whisker biomaterial revealed the formation of intensely vascularized connective tissue in the implemented region; however, animals with the biomaterial α-TCP showed the formation of this tissue around the implemented region. On the other hand, intense bone resorption was observed only in the animals with Wollastonite Whiskers, but new bone formation in both groups (Figure 4A and 4B).

Due to the expected time of 30 days for the collection of samples for analysis, no points of tissue inflammation were observed, since the inflammatory process occurs in the first days after the implementation of the biomaterial, indicating resolution of the inflammation.
A significant increase was observed both in the number of blood vessels and in the number of osteoblasts in the animals that had the α-TCP biomaterial implemented (Figure 5A). There were no significant changes in the number of osteoclasts among the groups that had the implementation of different biomaterials. However, it seems that the group with the biomaterial α-TCP has a propensity to decrease the observed osteoclasts (Figure 5B).

![Graphs demonstrating vascular formation, osteoblastic, and osteoclastic activity.](image-url)

**Fig. 5A-5B-5C.** Graphs demonstrate vascular formation, osteoblastic, and osteoclastic activity.

### Discussion

In the fields of medicine and dentistry, success rates with the use of biomaterials are around 95%, which encourages both surgeons and patients to seek this technology for the treatment of specific conditions. [19] Biomaterials, such as ceramics, are viable options in the choice of treatment, as they are the best alternatives when compared to autogenous bone graft because there are no limitations on the amount available and do not require an additional surgical procedure. [20]

Calcium phosphates, belonging to the ceramics group, are used in applications involving the entire skeletal system, such as craniomaxillofacial reconstruction and treatment of bone defects; however, the main limitations of the use of these biomaterials are the fact that they are very brittle and have a low resistance to fatigue. [21]

However, the development of techniques such as the insertion of ceramic, polymeric, or whisker fibers to increase their resistance to fatigue may increase their applicability. [22]

Recent studies reveal that the addition of various amounts of wollastonite can improve bioactivity, mechanical properties, and biodegradation. [23]

In this study, Wistar rats were used as an experimental model, in which a critical parietal defect was performed to evaluate the implantation of a new biomaterial. Critical defect refers to an injury that does not regenerate spontaneously unless a graft is used. Animal studies show that a defect of 5 mm in diameter can no longer be regenerated. The concept of critical defect allows the evaluation of the osteogenic potential of the material tested. When there is a critical defect in which there is no implantation of bone grafts or implants, tissue repair occurs through fibrous connective tissue instead of bone tissue. [24,25]

Although some studies use critical defects of 8 mm in diameter in rats, others also use defects with diameters of 6 and 5 mm to evaluate different grafts and biomaterials with or without the addition of growth factors.[26] In (2004 Aalami and collaborators) [27] evaluated critical defects of 3, 4 and 5 millimeters in diameter in adult rat calvaria and found by radiographic analysis that after eight weeks, there was a bone repair of minus five percent. (Mulliken; Glowacki, 1980) [28] performed 4 mm defects in the parietal of rats and found that even after six months, there was no complete repair of the defect. However, (in 2004, Porto and collaborators) [24] found that there was a full closure of the defect in 2 of the 5 rats with a critical defect of 5 mm after 60 days of the surgical procedure.

The main characteristic for the biomaterial to be implanted is biocompatibility; that is, the material must not contain elements that cause tissue damage[29]. The success of the implant is subject to tissue response of the receptor site and the type of interface and adhesion that occurs between the implant and the living tissue of the host. Any material implanted in a living organism stimulates some
reaction; if the biomaterial is non-toxic and biologically active, the formation of a continuous bonding interface between tissue and implant occurs, as with glass-ceramics. [30]

The biomaterial plus 5% wollastonite whiskers was analyzed \textit{in vitro} by (Domingues, 2017) [31] Who found the increase alkaline phosphatase activity is known to be an early marker of osteoblast differentiation and enable cell adhesion, spreading, and increased viability; that is an essential process for the formation, growth, and maintenance of a tissue-biomaterial interface, as well as for proliferation and bone matrix synthesis.[32]

The characteristics of biocompatibility, osteoconduction, and bioactivity are essential for choosing a biomaterial in the biomedical field.

In relation to biodegradation, one of the major goals of bioengineers and scientists is the production of biomaterials capable of replacing injured tissues for a certain period during which the natural healing process of the affected area is being promoted. In addition to the biodegradation capacity, the ideal material should stimulate the regeneration of the matrix tissue and present degradation kinetics of the mechanical properties compatible with the tissue repair kinetics. In this way, the new tissue would progressively replace the implant in the required functions. [33]

For several years, hydroxyapatite was the only ceramic in the calcium phosphate system used to replace bone tissue. However, due to its slow biodegradation, other phosphates such as TCP have begun to draw attention due to their better solubility, which leads them to being degraded more rapidly in the biological environment.[34] Among the calcium phosphates used as biomaterials, $\alpha$ TCP is more resorbable than $\beta$ TCP, making it an excellent material for implantation, being able to be replaced with new bone faster than other materials available on the market today. [8]

In this study, the materials provided greater stimulation of osteoblasts and osteoclasts in 30 days, corroborating with the data of (Azevedo et al. 2012) [35], who implanted TCP / HA in rabbits. These results occur due to osteoconductive action and osteotransductivity of these biomaterials, which implies simultaneous absorption and replacement by new bone. Its speed depends on the implantation site, the age of the recipient, and the type of calcium phosphate cement used.[36]

For repair to occur, bone formation depends on an adequate vascular supply with osteoblasts performing their functions in the regions adjacent to blood vessels, where the elaboration of highly organized bone tissue needs a mechanically stable surface, so that the newly formed bone is deposited in a solid foundation. [37]

Corroborating with the research data of (Carlo et al 2009) [37] that implanted a hydroxyapatite-based biomaterial in rabbits recovered the normal bone repair process with bone neoformation starting from the medial region to the pattern in the group with wollastonite whiskers, with a large number of active osteoblasts and osteoclasts.

In addition to the presence of dense vascularized connective tissue, according to (Vital et al.2006) [38], the connective tissue is replaced by bone tissue in the healing process, considered a typical event in bone surgery.

It is important the detailed knowledge about the interactions between material and living cells for creating and developing new materials that can carry living cells or growth factors that allow regeneration and not just the repair of damaged or worn structures and tissues[39]

As they present characteristics of biocompatibility and bioactivity, whiskers can be a viable option to reinforce calcium phosphate cement, improving their mechanical properties without harming patients' health. [16]

### Conclusion

An addition of wollastonite whisker in other biomaterials becomes a viable option because it is not cytotoxic; it is resorbable and stimulates bone neoformation, as found in this study. However, further studies are needed for these biomaterials to be improved and available for the treatment of bone injuries.
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