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Estudante
Mariana do Souto Fontes Antunes Lopes

Orientador
Professor Doutor Mário Ramalho de Vasconcelos

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Preliminary Study In Vivo Of The Bone Regenerative Capacity Of Hydroxyapatite When Associated With Omeprazole In Critical Size Defects

Dissertação apresentada à Faculdade de Medicina Dentária da Universidade do Porto para obtenção do Grau de Mestre em Medicina Dentária

Mariana do Souto Fontes Antunes Lopes *

* Estudante da Faculdade de Medicina Dentária da Universidade do Porto

Correio Electrónico:
marianasoutolopes@gmail.com
miana.lopes@iol.pt
cmd07002@fmd.up.pt

Endereço:
Rua Eng.º Adelino Amaro da Costa
114, R/C Dt.º
4400-134 Vila nova de Gaia

Orientador
Professor Doutor Mário Ramalho de Vasconcelos

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(Regras de submissão de manuscrito em Anexo)

Mariana do Souto Lopes[1], Mário Ramalho Vasconcelos[2], Ana Portela[2]

Preliminary study *in vivo* of the bone regenerative capacity of hydroxyapatite when associated with omeprazole in critical size defects

1 Dental Medicine Integrated Master Degree Student, Faculty of Dental Medicine, University of Oporto, Oporto – Portugal

2 Department of Conservative Dental Medicine, Faculty of Dental Medicine, University of Oporto, Oporto – Portugal

Abstract

The vacuolar-type H^+-ATPases (V-ATPases) are abundant enzymes in the ruffled border of the osteoclast that have a fundamental role in the bone resorption process. Consequently are considered crucial therapeutic targets in the treatment of lytic bone diseases.

In the present study, 80 mg of omeprazole, a gastric proton pump inhibitor (PPI), was evaluated *in vivo*, in calvarial critical size defects in Wistar rats, whether it increases bone regeneration when associated with the hydroxyapatite granules, Osteopatite®, and modified exoskeleton of shrimp (MES) membrane. In the same animal there were both test and control defects (paired sample design). Although the results show slightly more bone regeneration and more Osteopatite® granules osseointegration in the test defects than in the controls, the results must be considered inconclusive. This issue will need further studies and to adequate the methodologies, in order to verify the omeprazole therapeutic effects as a potential local osteoclast function inhibitor.

Corresponding Author: Prof. Dr. Mário Ramalho Vasconcelos, Faculdade de Medicina Dentária da Universidade do Porto, Rua Dr. Manuel Pereira da Silva, 4200-393 Porto PORTUGAL, Phone: +351 220 901 100, Fax: +351 220 901 101, E-mail Address: mvasconcelos@fmd.up.pt

Introduction

Bone is a dynamic tissue that is continuously remodeling itself by a process called coupling, in which the oldest tissue is reabsorbed by the osteoclasts and new bone is formed by the osteoblasts in a coordinated and proportioned way. This process enables the repair of microdamage, maintenance of mature bone mass and quality, adaptation to the load placed upon it and calcium homeostasis [1-3].

The osteoclasts are large, highly movable, multinucleated cells derived from the fusion of hematopoietic mononuclear cells [4-6]. According to Xu *et al*. (2007) [4] resorption includes 4 stages and begins with the osteoclast attachment to the calcified bone surface via actin microfilaments and αv_3 integrins. Afterwards, it undergoes a cytoskeletal and membrane polarization dividing it into three parts: the basolateral membrane facing the marrow space and surrounding the basal side of the cell where are most of the organelles and nuclei, the tight sealing zone which is closely opposed to the bone matrix, the ruffled border with its cytoplasm protrusions and encircled by the clear zone that has no organelles [4,7,8]. Consequently, an isolated microcompartment forms (the Howship or resorption lacuna) between the bone and the ruffled border, where the resorption will take place [4,9].

Since bone consists of inorganic (hydroxyapatite) and organic (mostly collagen type I) components, the next stage includes firstly an acidification phase that is a prerequisite for the proteolytic phase [3,9,10]. The osteoclast secretes hydrochloric acid thanks to numerous plasma membrane V-ATPases present on the ruffled border that actively extrude protons to the Howship lacuna, as well as chloride channels ClC-7 that diffuse passively Cl^- ions, maintaining the electrochemical gradients [1,4,3,7,9-11]. The proteolytic enzymes such as cathepsin K and matrix metalloproteinases require an acidic environment to degrade the exposed proteinaceous bone matrix [2-5,9-11]. The resultant products are transported via transcytotic carrier vesicles to the basal zone and released to the extracellular environment [4].

Intracellular V-ATPases are a class of ATP-driven multisubunit proton pumps found in all eukaryotic cells [1,3,6], which are primarily responsible for the acidification of intracellular compartments of both constitutive and specialized secretory pathways [3-6,11], including cell differentiation, endocytosis, intracellular membrane traffic, macromolecular processing, neuronal transmission, degradation and ligand-coupled transport, cell invasion by viruses and tumor metastasis [4,8,11]. The V-ATPases in plasma membranes that face the extracellular environment specifically have been identified only in a few cell types like the osteoclast, the kidney's intercalated cells, the epididymus cells, neutrofils and macrophages [3,5,6,11].

Generally, V-ATPases are composed of at least 14 subunits and several accessory proteins. While the V1 domain (A to H subunits) carries out ATP hydrolysis, the V0 domain (a1 to e2 subunits) anchors the complex to the membrane (especially through the *a* subunit) and translocates the protons [4-6,8]. In the osteoclast, the structures of the subunits have been shown to differ from those in other cells [12], and it contains a specific a3 subunit, responsible for severe osteopetrosis when there is loss of function [1,4,6,8].

Given the essential role of the V-ATPase in the osteoclast function, it becomes a fundamental therapeutic target [4,8,11,12] in the treatment of lytic bone diseases like

osteoporosis, bone aseptic loosening, tumor-induced bone destruction, Paget's disease or periodontitis [2,4,8]. Many drugs have already been studied as anti-resorptive agents (Table 1).

Table 1. V-ATPases inhibitors

V-ATPase Inhibitors	Acting targets	Sources
Bafilomycin A1	V0 a subunit	Streptomices species
Concanamycin A	V0 c subunit	Streptomices species
SB 242784	Osteoclast V-ATPase	Bafilomycin derivatives
FR167356	Osteoclast V-ATPase	Novel chemical structure
Salicylihalamide A	V-ATPase	Benzolactone enamide core structure
Lobatamides	V-ATPase	Benzolactone enamide core structure
Oximidines	V-ATPase	Benzolactone enamide core structure
Destruxin B	V-ATPase	Fungus Metarhisium anisopliae
Omeprazole	Gastric H^+/K^+ATPase	Thiol reagents

Adapted from Xu *et al.* (2007) [4]

The first specific V-ATPase inhibitor to be studied was bafilomycin A1, a macrolide antibiotic with a specific and potent inhibitory effect on all V-ATPases *in vitro* and *in vivo* [4,12]. It interferes with the proton transport by binding to subunit C of the V-ATPase [12]. However, being a nonspecific inhibitor of all V-ATPases, bafilomycin A1 could exhibit unacceptable levels of toxicity of the cellular physiology, thus making its use as a systemic anti-resorptive agent unfeasible [2,4,11]. Therefore, for the treatment of excess bone resorption, it is necessary to modify the structure of bafilomycin A1 to confer higher selectivity for the osteoclast V-ATPase enzyme [4]. Since 1998 that Gagliardi and his investigation group [11] have been trying to synthesize a drug as potent as bafilomycin A1, but selective to the osteoclasts' V-ATPases and, consequently, not as toxic. The same investigators came up in 2000 [13] with a derivative of bafilomycin A1, SB 242784, which was subsequently investigated in 2001 [14] and 2002 [15], reporting that it prevented bone loss in ovariectomized rats [13] and it showed osteoclast V-ATPase selective inhibition [14]. In 2005 Niikura *et al*. [2] compared a novel osteoclast V-ATPase inhibitor, FR167356, with bafilomycin A1 and SB 242784 *in vitro* and *in vivo* and concluded that it might be more appropriate for clinical application. Moreover, in another 2005 study, Niikura *et al*. [16] prevented alveolar bone destruction in experimental periodontitis in rats with oral administration of FR167356. In addition, in 2007, Sørensen *et al.* [1] studied a natural compound, diphyllin, as a novel inhibitor of the human osteoclast V-ATPase, concluding that it strongly inhibited acidification of the lysosomes and the resorption lacunae. However, in all of these experiments the drugs were studied having in mind a systemic approach of their potential applications, especially for the treatment of osteoporosis. The only exception goes to an experiment by Rzeszutek *et al.* (2003) [12], where bafilomycin A1 was incorporated in the chemical composition of calcium phosphate rods, which were implanted *in vivo* in osseous defects, to observe the local effect of the PPI on the rods resorption by the osteoclasts. They ended not only with a slower resorption of the graft material but also of the newly formed bone.

Omeprazole is a selective and irreversible inhibitor of the gastric parietal cells' H^+/K^+ATPases and is currently used to treat peptic ulcer, *Helicobacter pylori* infection, gastroesophageal reflux disease, non-steroidal anti-inflammatory drug-induced gastrointestinal

lesions and Zollinger-Ellison syndrome [17]. It was the first gastric PPI to be introduced in the pharmaceutical market in 1989 and since then it has demonstrated an excellent safety profile for long-term use [17]. It is generally well tolerated, even by pregnant women and children, and the incidence of adverse effects is relatively low, approximately 1-3% [17,18]. In the active parietal cells' acidic environment it converts to the active cationic sulfonamide that binds to the H^+/K^+ATPase's α subunit, thus inhibiting acid secretion up to 36 hours [17,19].

Although the gastric proton pump is different from the osteoclast's V-ATPase, *in vitro* and *in vivo* studies have demonstrated that omeprazole inhibits systemic bone resorption [19]. Back in 1986, Tuukkanen and Väänänen [20] cited a demonstration of immunological cross-reactivity between the gastric H^+/K^+ATPase and "proteins" from the osteoclast ruffled border (later on named V-ATPase) in rats. As a consequence, they performed an experiment *in vitro*, where they demonstrated that both the basal resorption of mouse calvarial bones and their prostaglandin or parathyroid (PTH) stimulated resorption could be inhibited by doses of omeprazole sufficient to block the gastric acid secretion *in vitro*. However, in 1990 Väänänen *et al.* [19] did not find any immunoreactions in osteoclasts when antibodies against gastric H^+/K^+ATPase were used.

The aim of this preliminary study is to evaluate if the hydroxyapatite granules, Osteopatite®, and MES membrane's bone regeneration capacity increases when associated with a PPI like omeprazole, in rat calvarial critical size defects.

Materials and methods

Hydroxyapatite granules

In this study it was used Osteopatite® granules, which can be considered a modified hydroxyapatite, developed at the Institute of Biomedical Engineering (INEB) at the University of Oporto. The basic apatite formula $Ca_{10}(PO_4)_6(OH)_2$ is chemically altered by the addition of usual bone cations such as Fe^{3+}, Mg^{2+}, Na^{2+} and K^{+}, which partially substitute the cation Ca^{2+}.The chemical composition of Osteopatite® by weight is summarized at Table 2 and it matches that of bone natural hydroxyapatite [21]. The granules of Osteopatite® are microporous, presenting a granule size of -20/+ 100 mesh (ASTM) (0.15-0.85 mm) [21] (Figure 1).

Table 2. Chemical composition of Osteopatite® by weight

Chemical compound	% w/w
P_2O_5	43,1
K_2O	1,0
Al_2O_3	0,21
CaO	Residual
MgO	0,7
Na^2O	3,1
Fe_2O_3	0,34
Adapted from Vasconcelos *et al*. (1997) [21]	

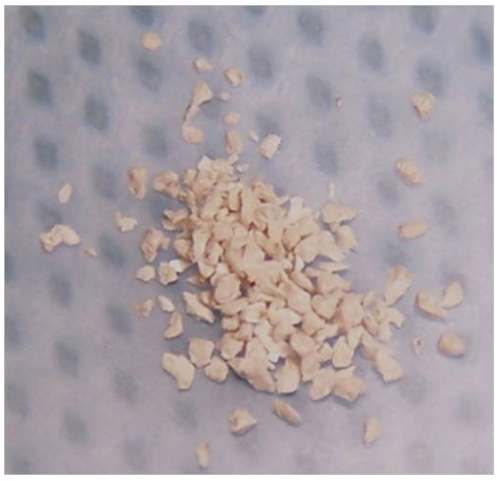


Fig. 1. Osteopatite® granules

MES Membrane

Chitin is a linear beta 1,4-linked polymer of N-acetyl-D-glucosamine (GlcNAc) and is the second most abundant biopolymer in nature, which is extracted primarily from the

exoskeleton of crustaceans, but also from insects and fungi [22]. In the last 40 years it has aroused the interest of the scientific community for its potential biomedical applications as a dressing material, drug delivery vehicle and, increasingly, a candidate for tissue engineering [23]. The reasons are its versatile biological activity, excellent biocompatibility and complete biodegradability in combination with low toxicity [22].

In order to fix the Osteopatite® granules and to increase the osseous regeneration it was used MES membranes from the shrimp´s chitin exoskeleton (*Palaemonetes spp.*), which were also developed at INEB (Figure 2). The crustaceans are from the Atlantic ocean weighted between 12,5 and 17,0 g. The exoskeleton was detached from the abdomen and washed in a sonicated NaOH 3% (w/w) solution at 70º C in order to remove its proteins. This solution was substituted 10 times, until the exoskeletons looked white and then were washed with distilled water to remove the NaOH residues. Afterwards, the samples were superficially modified by immersing in a sodium silicate 25% (w/w) solution for 1 hour and drying, so as to add silicon to its composition. This superficial treatment allows the formation of an apatite layer on the membranes surface after immersion in a simulated body fluid at 37º C, where Ca was substituted for Sr (SBF-Sr) (composition on Table 3) for one week [24]. Just before its surgical application, the MES membranes were boiled until sterilization and were stored in a saline solution.

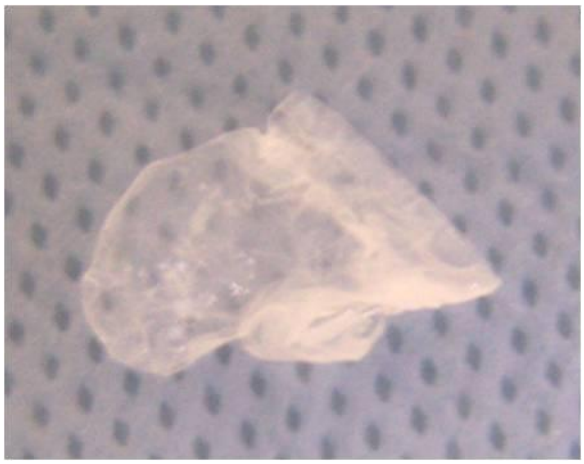


Fig. 2. Boiled MES membrane

Table 3. Chemical composition of SBF-Sr (pH 7)

Chemical compound	mmol/L
NaCl	136,8
$NaHCO_3$	4,2
KCl	3
$KHPO_4$	3
H_2O	1
$MgCl_2$	6
Na_2SO_4	0,5
$SrCl_2$	2,5
HCl	1 M
Adapted from Mesquita *et al*. (2004) [24]	

Omeprazole

The pharmaceutical form of the omeprazole (Laboratórios BIAL - Portela & Cª SA, S. Mamede do Coronado, Portugal) used was powder (Figure 3). Omeprazol was stored in a plastic bottle at 4º C. 80 mg ± 0,1 mg of omeprazol for each test defect were weighted using an analytical balance (Kern® 770-13, Kern & Sohn GmbH, Balingen, Germany).

Fig. 3. Omeprazole powder

Experimental procedures

The experimental models were six specimens of 5 months old female Wistar rats (*Rattus norvegicus*) weighting 209 to 266 g.

Before starting the surgical procedure, the anesthesia was induced with isoflurane 100% (IsoFlo®, veterinaria Esteve) by inhalation during approximately 5 minutes (Figure 4) and then they were anesthetized with an intraperitoneal injection of ketamine (Imalgene®, Merial) (0,2 mg/100 g of body weight) plus xylasine 2% (Rompum®, Bayer) (0,065 mg/100 g) in a 1:1 ratio. The animals took approximately 10 minutes to reach the anesthetic level, which could be recognized and monitored by their slower and deeper breathing pattern. Whenever necessary the anesthesia was reinforced with a ¼ of the volume of the initial anesthetic dose.

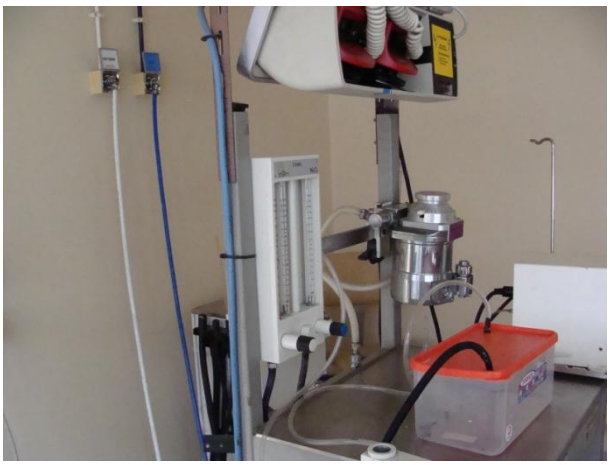


Fig. 4. Equipment for the induction of anesthesia by inhalation with isoflurane 100%

The animals were submitted to a surgery at the experimental operation room at the Faculty of Dental Medicine, University of Porto, under sterile conditions, in which the surgical field was disinfected with a topical antiseptic povidone-iodine 10% (Betadine®, MEDA Pharma). A sagittal incision from the coronal suture until the lambdoidal suture was performed with a surgical scalpel blade #15 on a handle #3on the cranium skin and periosteum (Figure 5) and these were retracted until the osseous surface of both parietals was completely uncovered (Figures 6 and 7). 2 circular osseous defects with 3 mm of diameter (critical size defects) were made, one in each parietal bone, with a 4 mm bone carbide tapered bur on a low speed handpiece under constant sterile saline solution irrigation (NaCl 0,9%®, Braun), without injuring the dura mater or the sagittal sinus. Avoiding unnecessary trauma was a main concern during the whole surgery.

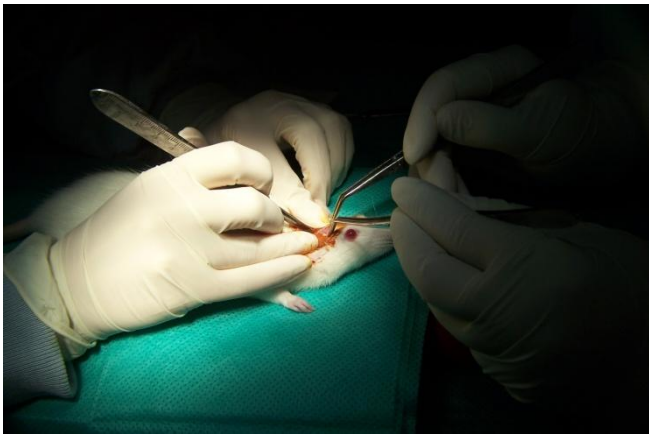
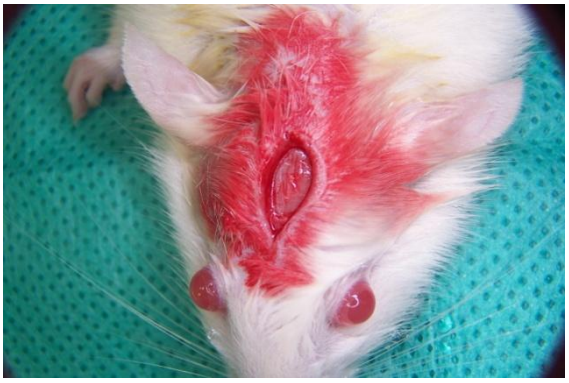


Fig. 5. Operators performing a sagittal incision on the rat‘s cranium skin under sterile conditions



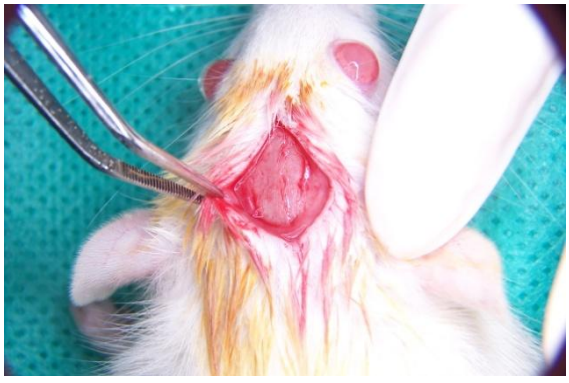


Fig. 6, 7. Appearance of the cranium osseous surface after skin and periosteum retraction

The control group defects were made at the most posterior area of the left parietal bone (Figure 8), whereas the test defects were made at the most anterior zone of the right parietal (Figure 9), so that the defects would be as far as possible and their contents would not mix. The control defects (n = 6) were filled with Osteopatite® granules and covered by a MES membrane previously wet and shaped with a sterile scissors, in order to make it more manageable and easier to adapt to the wound (Figure 10). The test defects (n = 6) were also filled with Osteopatite® granules, as well as with 80 mg of omeprazole and were covered with the wet

MES membranes. The surgical wound was closed with interrupted silk 5-0 stitches (Surgisilk®, Sutures Ltd, U.K.) and was abundantly cleaned and disinfected with povidone-iodine 10% (Figure 11). The animals were housed in individual cages, so that injuries would not occur, until they woke up by themselves (Figure 12).

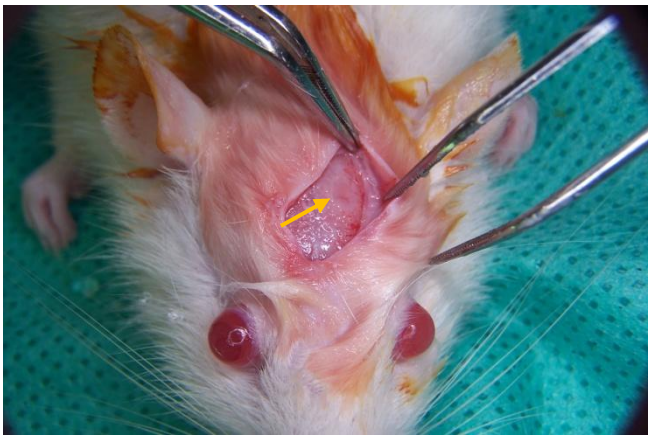


Fig. 8. The control defect (arrow) was positioned at the most posterior area of the left parietal bone

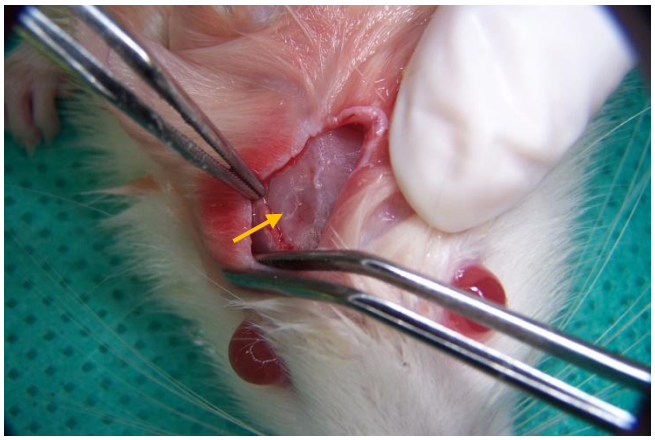


Fig. 9. The test defect (arrow) was located at the most anterior zone of the right parietal

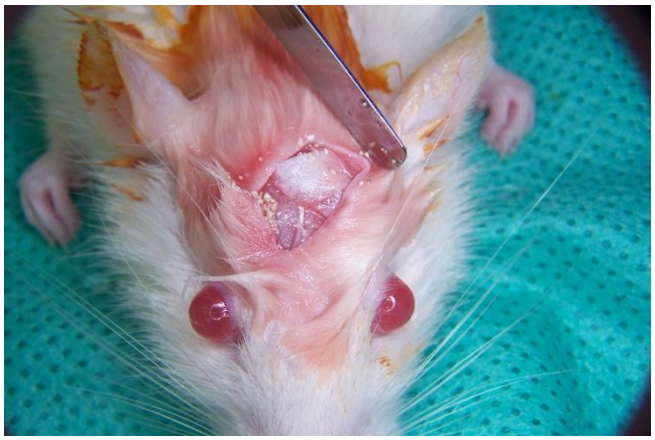


Fig. 10. Adaptation of the Osteopatite® granules and the wet MES membrane to the defect

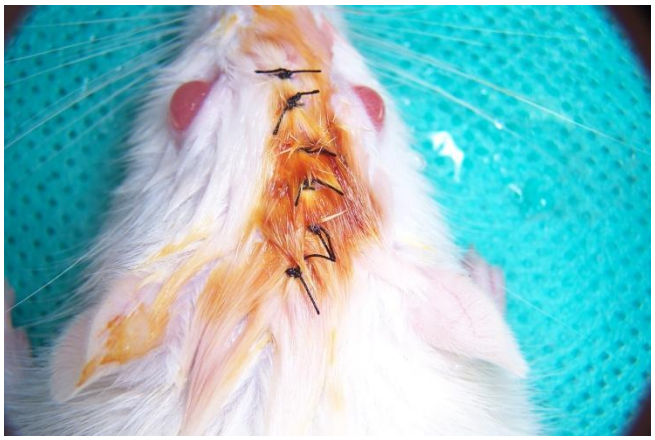


Fig. 11. Suture of the incision with interrupted silk 5-0 stitches and disinfection of the wound with povidone-iodine 10% solution

Fig. 12. The animals rested in individual cages during the anesthesia recovery period

The animals were housed in a standard light–dark schedule, standard relative humidity, air pressure, air ventilation and temperature. Stock diet and tap water were available *ad libitum* (Figures 13 and 14). 8 weeks afterwards, the specimens were euthanized with an overdose of anesthetic (ketamine and xylzine), preceded by a sedation with isoflurane. A new sagittal incision was made, the skin and periosteum were retracted (Figure 15) and the parietal bones were removed from the cranium using a saw on a low speed handpiece under constant sterile saline solution irrigation (Figures 16 to 18). The samples were washed with saline solution, cataloged and stored in sterile plastic bottles for the fixation process in a formaldehyde solution 4% in a phosphate buffer solution (pH 7,4) for 48 hours (Figures 21 to 35).

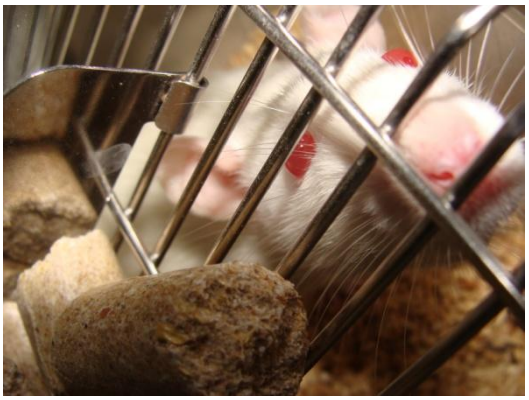
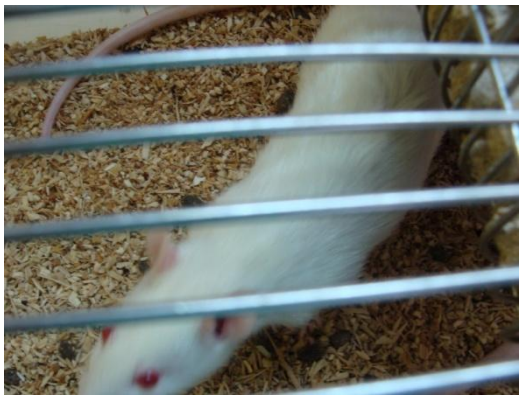



Fig. 13,14. For 8 weeks the animals were housed in proper conditions of space, light, temperature, humidity, food and water

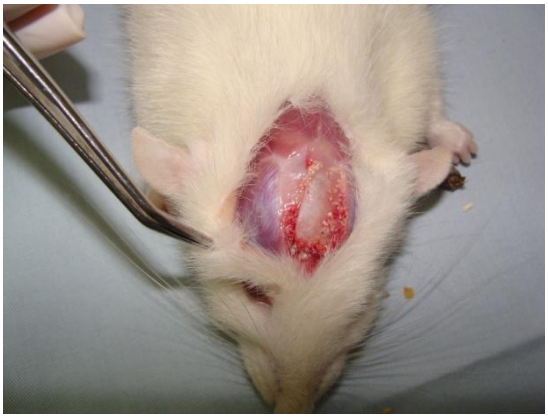


Fig. 15. Upon the rats sacrifice, a new sagittal incision was performed to show the cranium surface

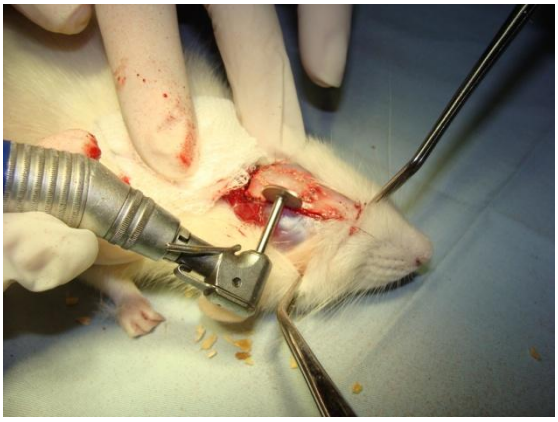
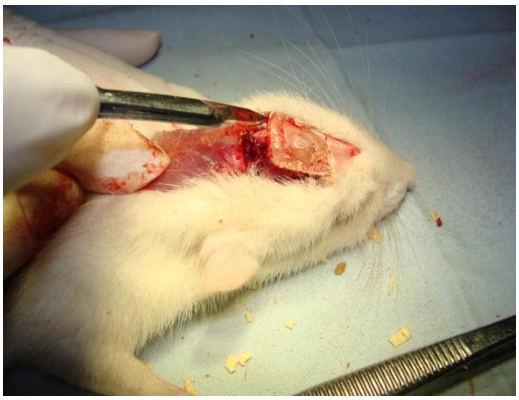


Fig. 16. The bone was cut using a stainless steel saw on a low speed handpiece under saline solution refrigeration with a security distance in order to avoid the destruction of the study areas



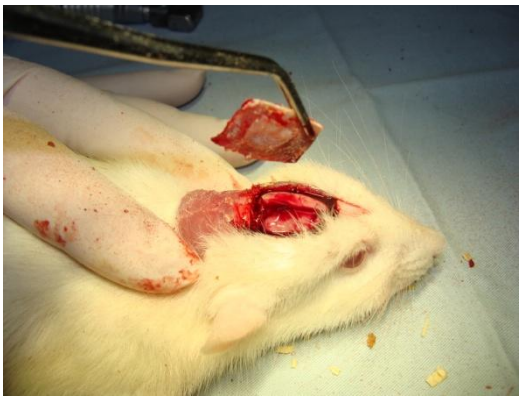
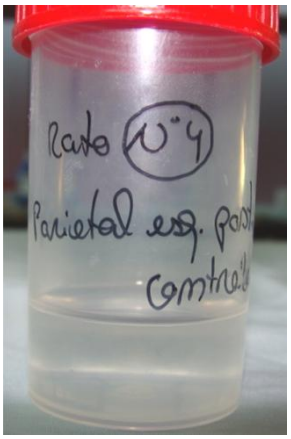
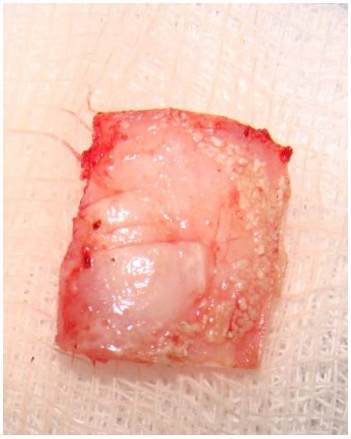


Fig. 17, 18. The surgical piece was carefully detached from the rest of the body, preventing its fracture





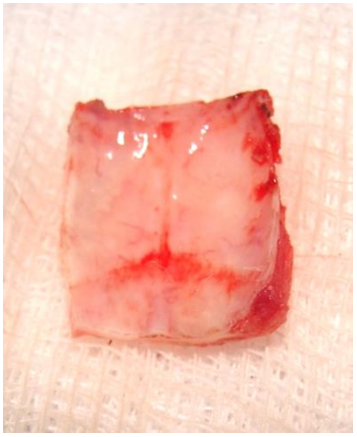


Fig. 19, 20, 21. Plastic identified bottle with formaldehyde solution 4% in a phosphate buffer solution and the surgical pieces seen from the external and internal surfaces

Histological preparation

The histological procedures were performed at the Dental Anatomy Laboratory of the Faculty of Dental Medicine, University of Porto.

After the 48 hours fixation, the samples were dehydrated in a sequence of increasing concentrations of ethanol solutions, beginning with ethanol 70% for 48 hours at room temperature (20-25º C). Afterwards, the samples were placed successively in 70, 80, 90 and 100% ethanol solutions for 48 hours each. The 100% ethanol solution was substituted 3 times for 48 to 48 hours.

During the inclusion procedure, each sample was placed in an individual sterile glass bottle containing 800 mL/L of methyl methacrylate (Methyl Methacrylate BDH®), 200 mL/L of Plastoid (Plastoid N®, Rohm Pharma) and 1 g/L of Perkadox (Akzo® Chemicals Bv, Netherlands). The bottles were left unclosed, put in a vacuum equipment for 15 minutes, in order to remove every air bubbles, and then were hermetically closed and placed in a 37º C bath apparatus GFL (Gesellschaft fur Labortechnik GmbH, Germany) for 48 hours for the complete polymerization of the inclusion material.

The blocks where the samples were included were cut with a microtome (Accutom, Struers, Denmark) for non-decalcified tissues at 2500 rpm and low pressure, along with copious

lubrication and refrigeration to avoid mechanical or thermal alterations, in order to obtain sections with an average thickness of 150-200 μm. In order to reduce that width to 40 μm, the samples were polished (DAPS®, Struers, Denmark) with a series of discs of decreasing granulometry, starting with #1000 (P1000, 3M 734®, UK) and #1200 (P1200, 3M 714®, UK) and finishing with an ultra-thin grain (SF737, 3M®, UK). The final thickness was corroborated with a digital microtome (Digimatic Micrometer®, Mytotuyo, Japan). The samples were put in an equipment of ultrasound bath ULABO USRI (Julabo Labortechnick, Germany) for 10 minutes, in order to remove the residues and impurities due to the cutting procedures. In some samples the cuts were longitudinal and in others were made transverse cuts.

The staining chosen was the Solochrome Cyanine R method, which differentiates osteoid from newly laid-down bone and older bone. The mineralized bone stains light blue, whereas the calcification front becomes dark blue. The osteoid bone is light red-orange, as well as the wide osteoid, which also has pale blue and orange bands. The nuclei become blue [25].

The specimens were mounted on glass slides bonded with Permacol (Ind Permacol UV®, Adhesive 327/3, Permacol Ind. Netherlands), which is polymerized with ultraviolet light. In the same way, the glass coverslip was placed upon the specimen, avoiding the enclosure of air bubbles. The glass slides were appropriately identified and observed at the laboratory's optical microscope (Leica DMLB®, Wetzlar, Germany). The images were captured by a video camera coupled to the microscope and were visualized in a computer screen (LG W1943SB, LG Electronics®, Germany), through a specific software of image acquisition and analysis (Leica Lida, Leica Imaging Systems®, Cambridge, UK, 1996). The area of osseous defect which was filled with new bone was observed in each sample obtained.

Results

Just before the experimental surgeries, each specimen's weight was determined, as it is stated at Table 4.

Table 4. The specimen's weight

Specimen #	Weight (g ± 1g)
1	226
2	213
3	209
4	231
5	226
6	266

The animals recovered from the surgeries and healed uneventfully until the end of the experiment, with exception to the animals # 4 and 6, which wound healing took a little longer to close. However they did not show any signs of infection.

Although the MES membranes were used upon all the defects to fix the granules as much as possible, in some samples these are spread all over the calvaria surface, especially those granules of bigger size (Figures 22a and b).

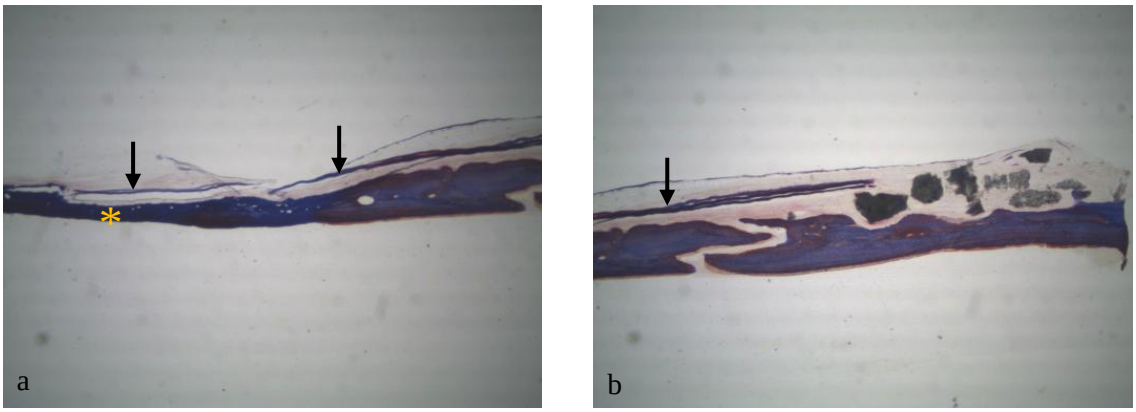


Fig. 22. Test critical size defect in which the membrane (arrows) is placed upon the defect area (asterisk) (a), but the granules, particularly those of wider diameter, are spread in a different place (b) (Solochrome Cyanine R stain; a and b: 2,5x)

Generally in this study, the use of Osteopatite® microporous granules was benefic for bone regeneration, as it was noticed in all specimens and defects (tests or controls) that bone started developing inside or upon the microporosities (Figures 23a and b). Interestingly, the only granules that became completely osseointegrated were those of smaller size (Figures 24a and b), whereas the larger ones were only attached to the calcification front by one side. Some granules distant from the bone surface appeared surrounded by a calcification front of newly formed bone (Figures 25a and b). Frequently, adjacent granules became attached to each other through

thin pink-orange bands of osteoid (Figure 26). The larger granules were usually surrounded by collagen fibers from the conjunctive tissue, which is precursor of the osteogenesis (Figure 27).

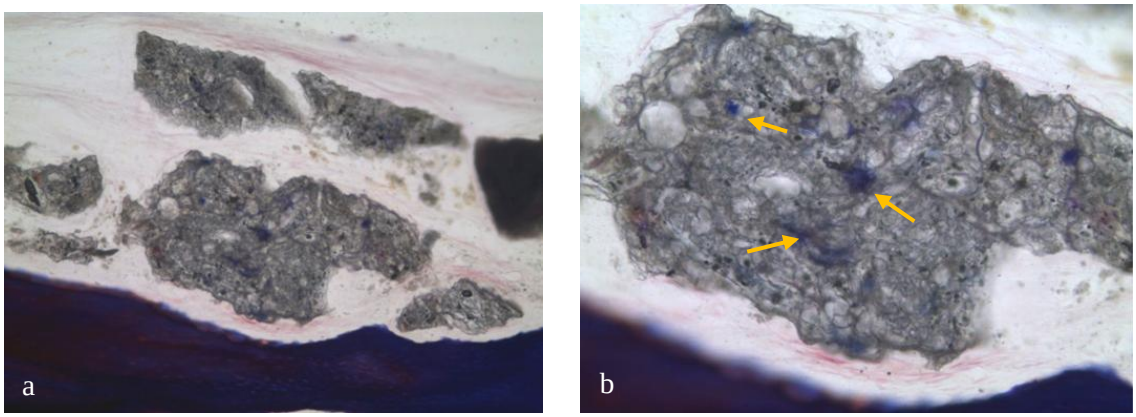


Fig. 23. Bone formation cores (stained blue – arrows) inside the microporosities of the Osteopatite® granules, from the specimen # 2 test defect. (Solochrome Cyanine R stain; a: 10x; b: 20x)

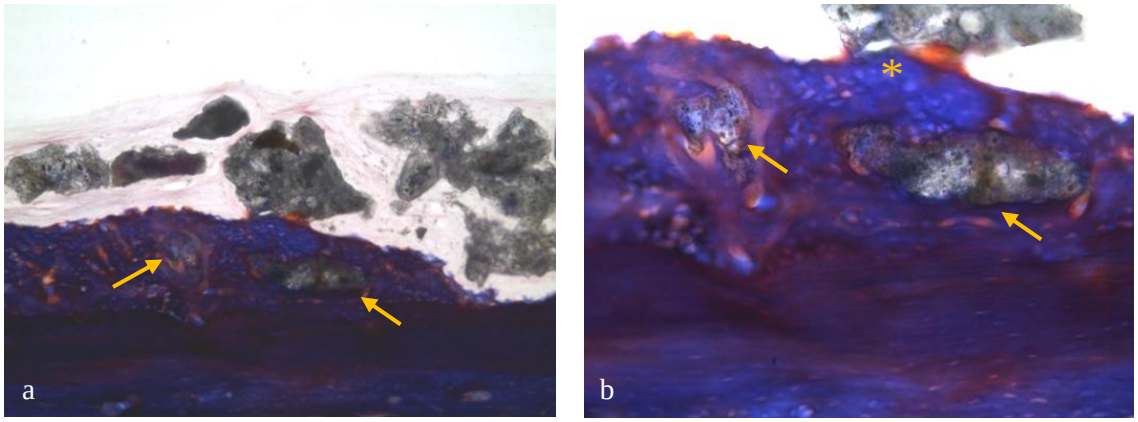


Fig. 24. Complete osseointegration of the smaller granules (arrows), whereas the larger ones only have one side contact with the calcification front (asterisk), from the specimen # 2 test defect. (Solochrome Cyanine R stain; a: 10x; b: 20x)

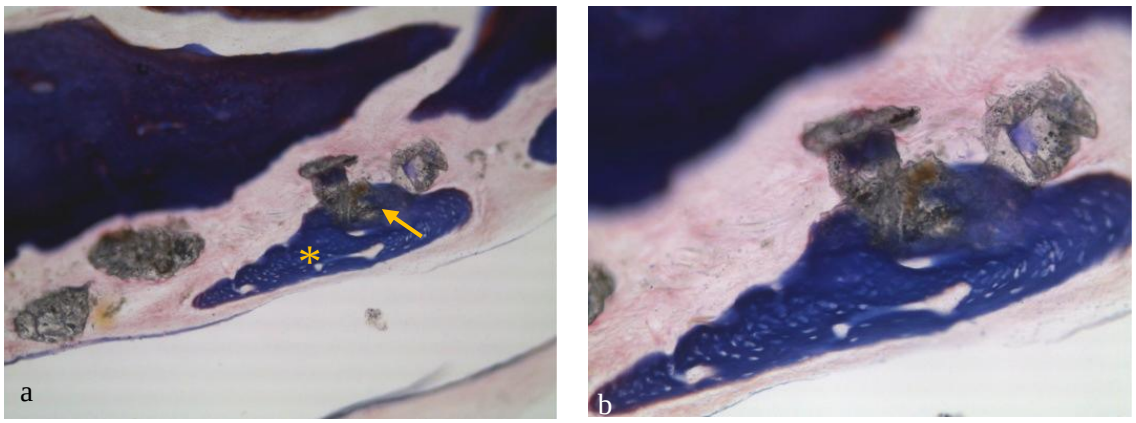


Fig. 25. Granule (arrow) distant from the bone surface surrounded by a calcification front of newly formed bone (asterisk), from the specimen # 5 test defect (Solochrome Cyanine R stain; a: 10x; b: 20x)

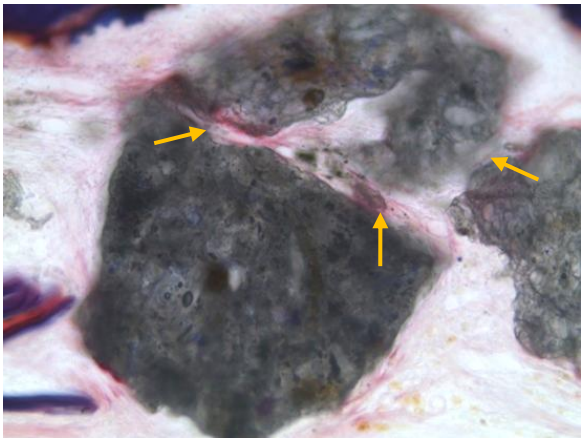


Fig. 26. Thin pink-orange bands of osteoid (arrows) can be seen connecting adjacent granules. (Solochrome Cyanine R stain; 20x)

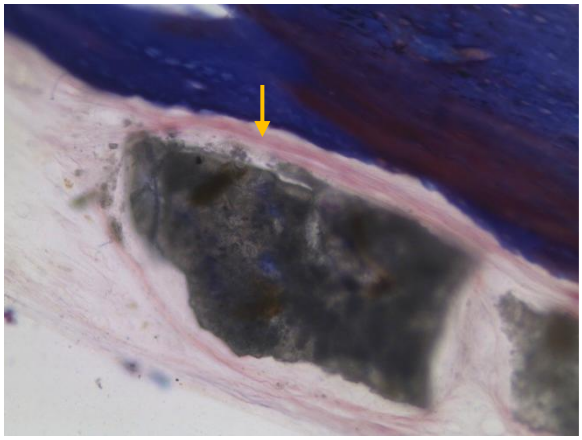


Fig. 27. Collagen fibers (arrow) precursors of osteogenesis surround the larger Osteopatite® granules, from the specimen # 1 test defect (Solochrome Cyanine R stain; 20x)

Although in many cases the MES membranes were lost, moved or partially reabsorbed, it is clear that the membranes had some positive influence in the bone growth. Not only have they contributed to fix a bigger concentration of granules in the defects (Figure 28), but also promoted osseous regeneration themselves in the control defects (Figure 29).

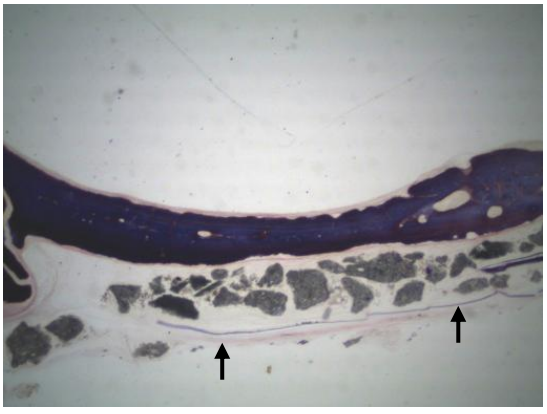


Fig. 28. A group of Osteopatite® granules underneath the MES membrane (arrows) (Solochrome Cyanine R stain; 2,5x)

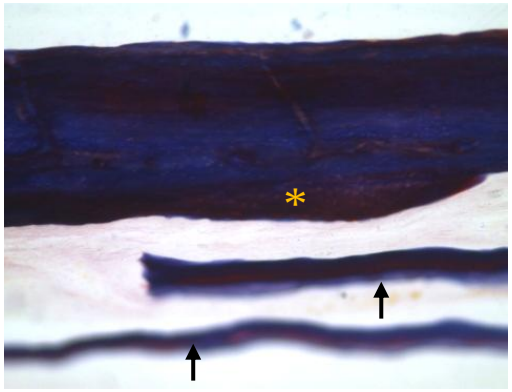
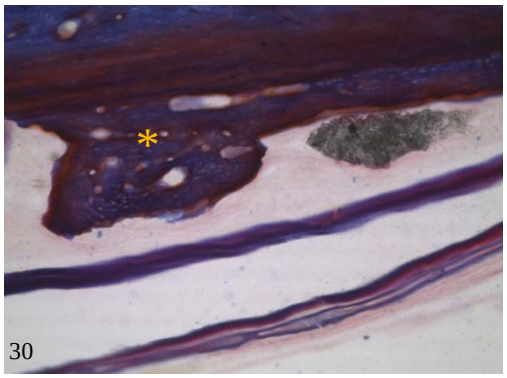
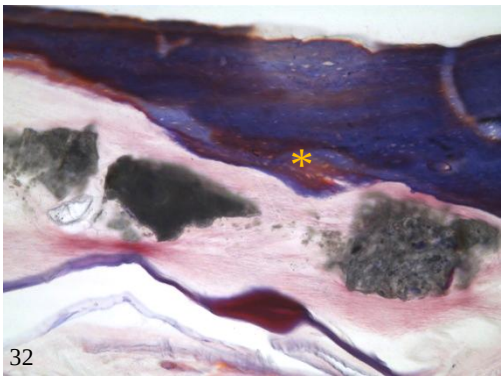
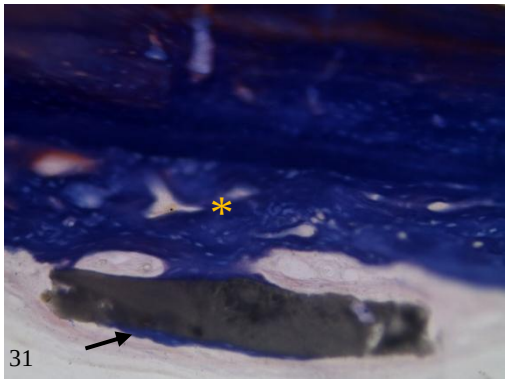


Fig. 29. Bone formation (purple – asterisk) underneath the MES membrane (arrows) without the granule's influence. (Solochrome Cyanine R stain; 10x)







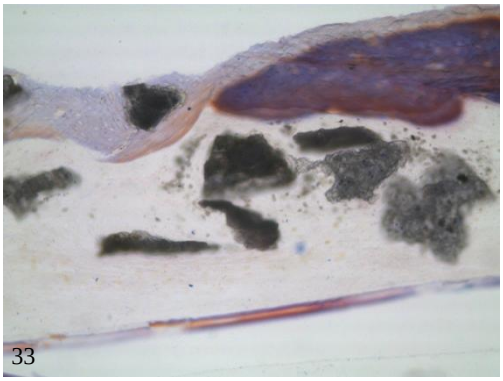
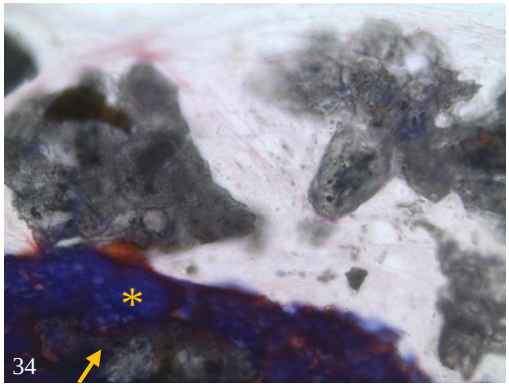
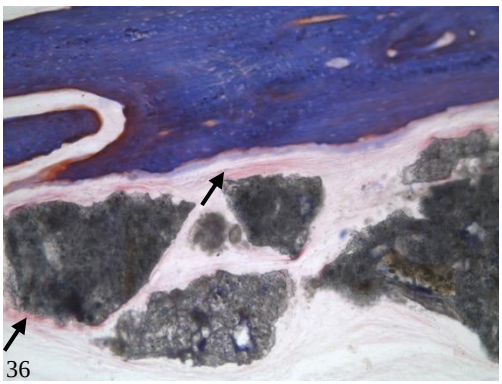
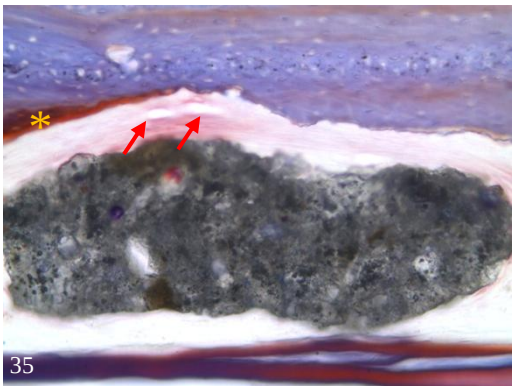


Fig. 30, 31, 32, 33. Histological images from specimen # 1. In the test group (left images) there are osseointegrated granules (arrow), while in the control group (right images) none were found. Although bone growth (asterisks) occurred in both cases, in the test group that growth is more pronounced (Solochrome Cyanine R stain; 30: 10x; 31: 20x; 32: 10x; 33: 10x)







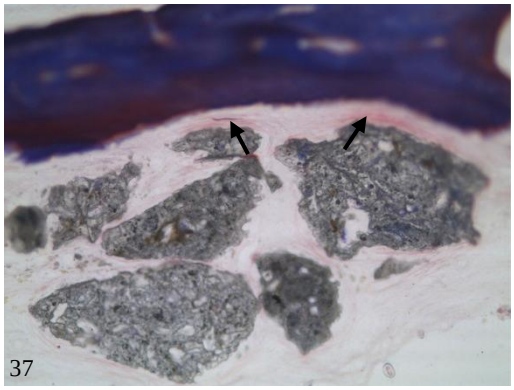
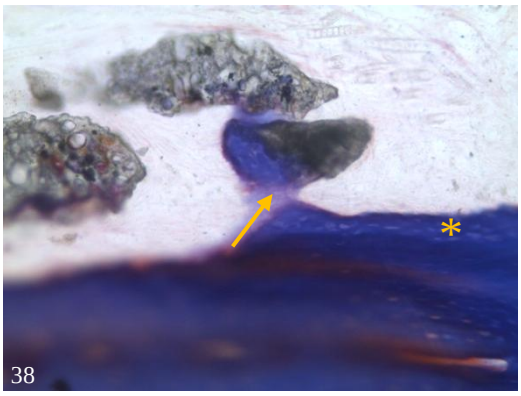
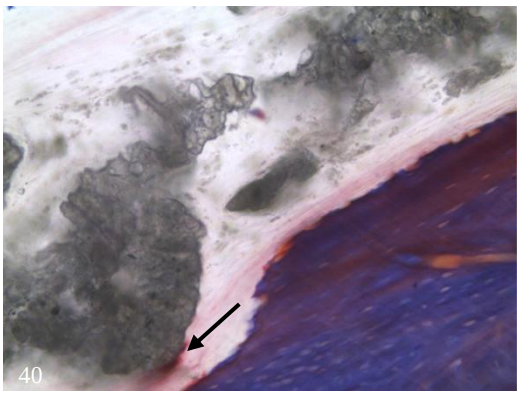
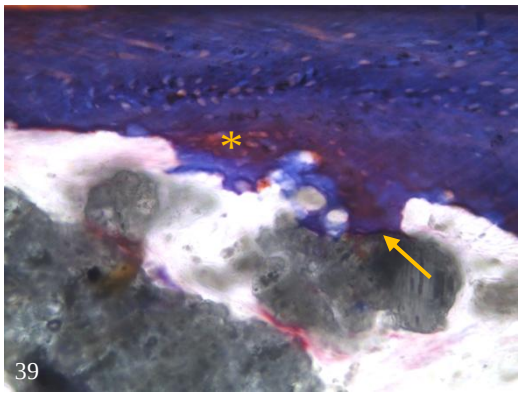


Fig. 34, 35, 36, 37. Histological images from specimen # 2. In the test group (left images) there are osseointegrated granules (orange arrow), while in the control group (right images) none were found. There is bone growth (asterisks) in the test group while the control group only shows collagen fibers (black arrows). In figure 35 the collagen fibers are readily differentiating (red arrows) (Solochrome Cyanine R stain; 34: 20x; 35: 20x; 36: 10x; 37: 10x)







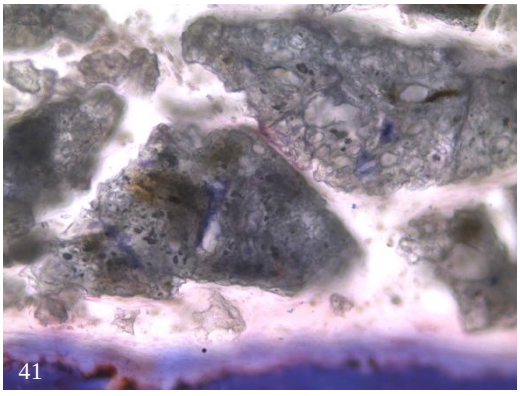
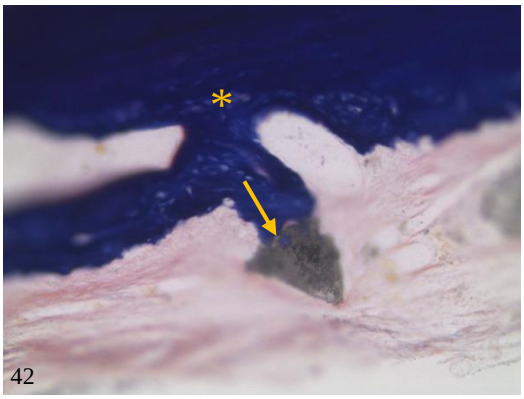
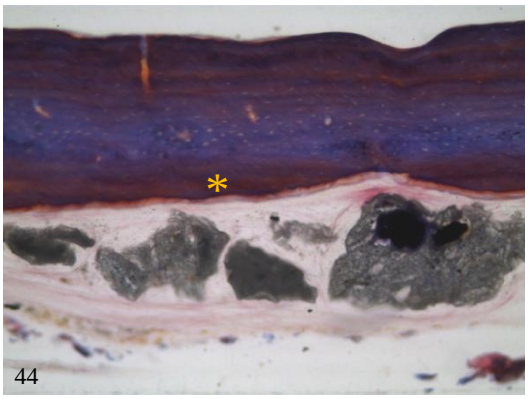
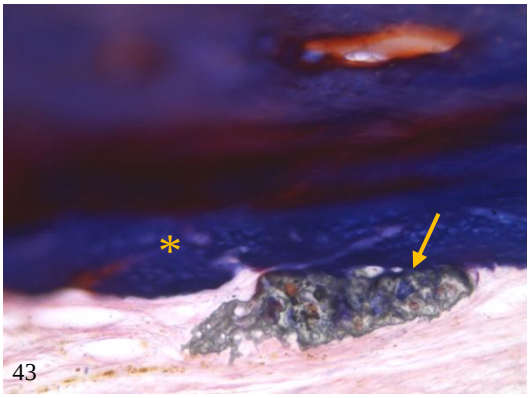


Fig. 38, 39, 40, 41. Histological images from specimen # 3. In the test group (left images) there are osseointegrated granules (orange arrows), while in the control group (right images) none were found. There is bone growth (asterisk) in both groups, despite in the control group most of it is collagen fibers (black arrows) (Solochrome Cyanine R stain; 38: 20x; 39: 20x; 40: 20x; 41: 10x)







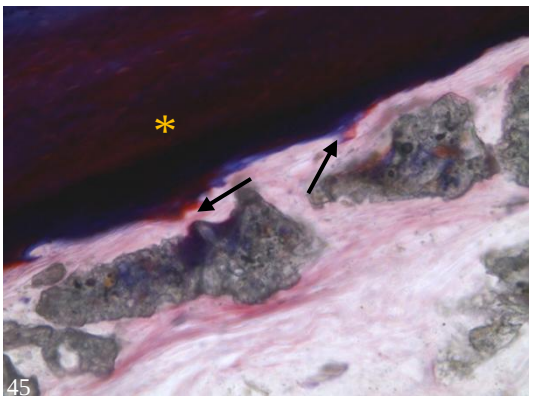
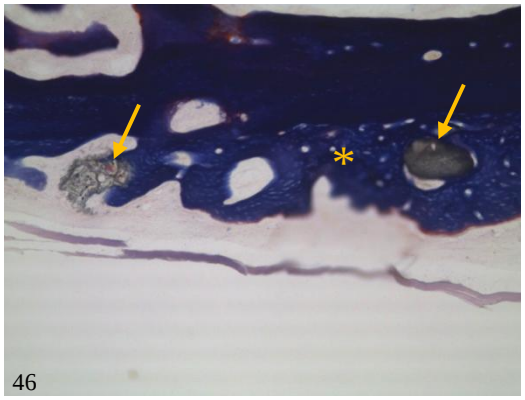
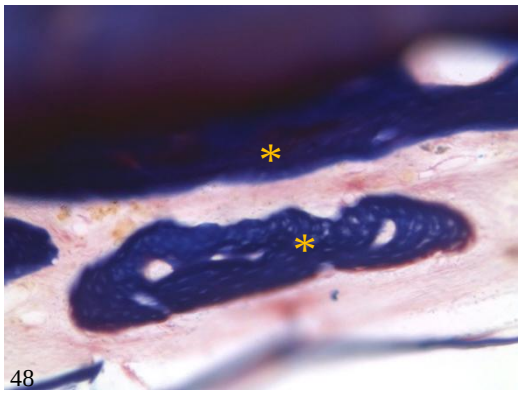
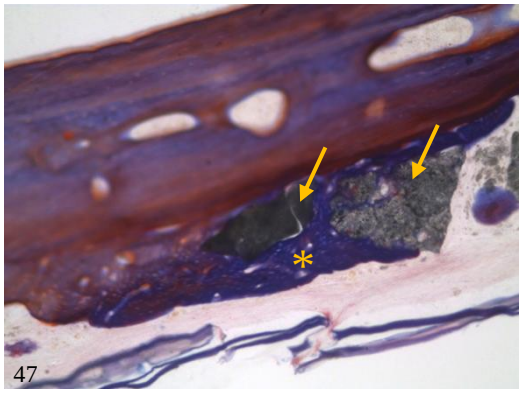


Fig. 42, 43, 44, 45. Histological images from specimen # 4. In the test group (left images) there are osseointegrated granules (orange arrows), while in the control group (right images) none were found. There is bone growth (asterisk) in both groups. In the control group a few granules are about to start osseointegrating (black arrows) (Solochrome Cyanine R stain; 42: 20x; 43: 20x; 44: 10x; 45: 20x)







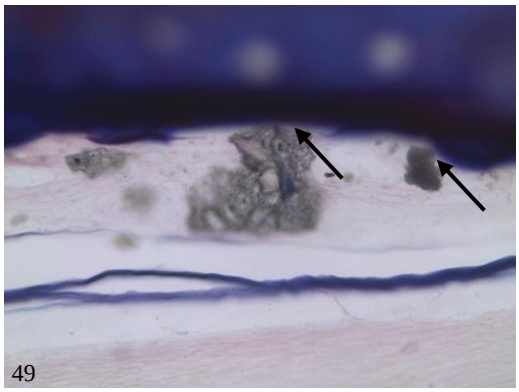
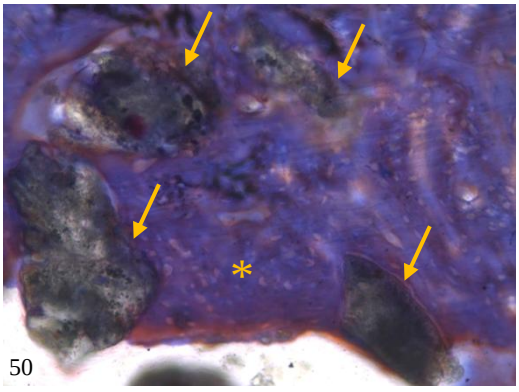
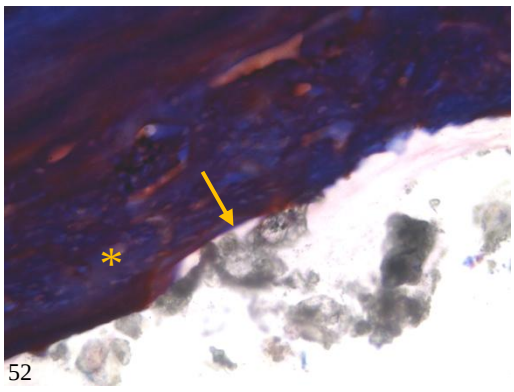
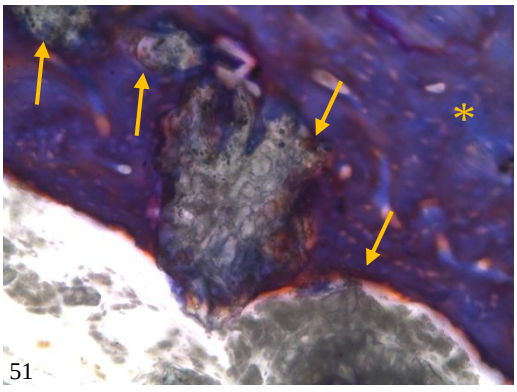


Fig. 46, 47, 48, 49. Histological images from specimen # 5. In the test group (left images) there are osseointegrated granules (orange arrows), while in the control group (right images) none were found. There is bone growth (asterisk) in both groups. In the control group (49) a few granules are about to start osseointegrating (black arrows) (Solochrome Cyanine R stain; 46: 10x; 47: 10x; 48 20x; 49: 20x)







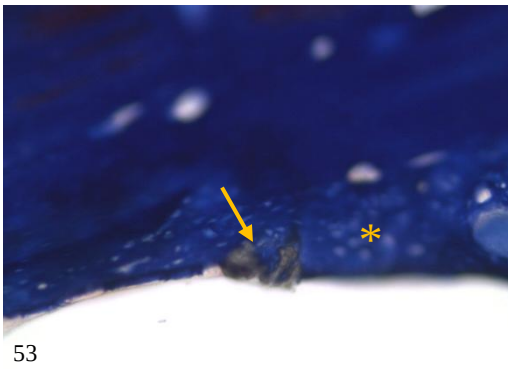


Fig. 50, 51, 52, 53. Histological images from specimen # 6. There are osseointegrated granules in both groups, despite in the test group (left images) there are more and larger osseointegrated granules (orange arrows), than in the control group (right images). There is bone growth (asterisk) in both groups. (Solochrome Cyanine R stain; 46: 10x; 47: 10x; 48 20x; 49: 20x)

Figures 30 to 53 show some features found in the test defects and in the control defects for each specimen. Generally, there is new bone formation equivalent in all animals and usually in all defect types. In all of the 6 animals at least two granules osseointegrated, as well as in all test defects. Despite this is more common in the test defects, some granules occasionally osseointegrated in the controls.

It was not noticed the presence of any inflammatory cells in any of the samples.

Discussion

This preliminary study attempted to evaluate the topical effect of omeprazole powder on the prevention of bone resorption by the osteoclasts. This experiment was based in a paired sample design, in which in the same specimen there is a test and a control, making the results in each one directly comparable. In general, the test defects seem to present slightly better performances than the controls, however the results should be considered inconclusive, as the differences do not seem to be significant.

3 mm critical size defects were made and filled with granules of Osteopatite®, which function as support structures for bone growth. The fact that it is a modified hydroxyapatite makes it chemically more similar to the natural inorganic component of bone, increasing its biological activity when compared to other types of hydroxyapatite granules [26]. These are also microporous enabling the ionic exchange from within the granules and modifying the pH, making it more alkaline, which favors the precipitation of Ca^{2+} from the medium, resulting in the formation of a natural hydroxyapatite film on the surface of the granules [21]. This fact is verified in the histological images where blue spots from the calcification front can be seen on the granules surface. Furthermore, Osteopatite® granules do not undergo significant phenomena of degradation and resorption, which comprise beneficial features [21,27].

During the surgical procedures there were some difficulties in the placement and condensation of the Osteopatite® granules to fill the defects because of their size when compared to the defects diameter. Corroborating this clinical observation, it seems from the histological analysis that the granules size (-20/+100 mesh) is too large for the diameter of the defects, because the majority of the osseointegrated granules are those of smaller dimensions. Besides, it is usually the largest granules that are displaced from the defects and are spread in the calvaria. Still, some experimental studies showed that if the granules are too small, there may be the risk of the granule's extensive phagocytosis by macrophages, especially for those which granulometry is inferior to 100 mesh [28]. Moreover, some of the defects surgically prepared were flatter, which may have influenced the displacement of the graft material.

When compared to the amount of studies published on the other osteoclast PPIs cited previously, studies on omeprazole effect on bone resorption are scarce. Nevertheless, according to the literature, omeprazole would be expected to inhibit the osteoclasts function, either by systemic or local administration [19,29,30]. In the literature, there are not many clear references on the minimum or maximum quantity of omeprazole that should be administered, which is aggravated by the heterogeneity of the few published studies on this issue.

One of the first studies made in this area was by Mattsson and Väänänen *et al.* (1991) [30], *in vitro*, where H^+/K^+ATPases were purified from the gastric mucosa and V-ATPases were prepared both from kidney medulla and from osteoclast-containing medullar bone. The aim was to evaluate and compare the potency of inhibition of omeprazole against bafilomycin A1 for both samples. As a result, omeprazole was much more potent in inhibiting H^+/K^+ATPases (IC_{50} approximately 0,25 µM) than V-ATPases (IC_{50} approximately 200 µM). On the other hand, bafilomycin A1 was much more effective in inhibiting the V-ATPases (IC_{50} approximately 2 nM) than H^+/K^+ATPases (IC_{50} approximately 50 µM).

In a study by Mizunashi *et al*. (1993) [19], the systemic effect of 20 mg per day of omeprazole in patients, who had a history of gastric ulcer, was evaluated through the urinary excretion of hydroxyproline and calcium, which decreased after omeprazole treatment in the study group, and increasing serum intact PTH, alkaline phosphatase, osteocalcin and tartrate-resistant acid phosphatase in the same group, when compared to the control groups. These parameters variations may indict a reduction in the bone resorption. In the same study it is stated that the peak plasma concentrations of omeprazole obtained during clinical use are approximately 1-2 μM and that omeprazole inhibits the osteoclast V-ATPase *in vitro* only at high concentrations (≥100 μM) at pH 7,4.

In a recent study by Sheraly *et al*. (2009) [29], pantoprazole or omeprazole were added to the chemical composition of calcium phosphate cements, which were implanted in rodent femora. In the first stage of the study, pantoprazole 0,5 mg/ml produced a delay in local osteoclast resorption, whilst this effect was not as evident using omeprazole at an equivalent dose (0,4 mg/ml). In a second stage higher doses of omeprazole (40 mg/ml, 100 times more concentrated) were used, which delayed the cement resorption. An important explanation for this phenomenon may be the fact that some studies have stated that omeprazole is generally 3 times more instable in solution than pantoprazole, which makes it degrade faster and loose its therapeutic effect. Omeprazole stability is temperature (if stored at 4º C it is 3 times more stable than at 22º C), pH (at a pH of 5,1 the half-life of pantoprazole was 4,7 hours while that of omeprazole was 1,4 hours) and time dependent (the longer it remains in solution, the more likely it will protonate, become activated and degraded) [29]. Still, omeprazole conversion to its active form is slow at neutral pH, but the rate of transformation is rapidly increased as the pH is lowered. Inside the bone resorption lacunae pH varies between 3 and 4, which may potentiate the omeprazole effect [19].

Summarizing, the only certain conclusion one can take from the literature is that in order to avoid osteoclast bone resorption, omeprazole must be present locally in large quantities. Therefore, we decided to try 80 mg of omeprazole powder, to make sure that it would be enough quantity. Nevertheless, during the surgical procedures it was quite difficult to condensate enough granules plus all of the volume of omeprazole in the defects. Further experiments will be needed to determine the most appropriated quantity of omeprazole. Another interesting trial could be using smaller Osteopatite® granules (inferior to 100 mesh). Despite the fact that there is a risk of these being phagocytosed by macrophages [28], it is also true that the V-ATPases are present in the macrophages' membrane as well [3,5,6,11], therefore their function could also be inhibited by the omeprazole, which would stop them from destroying the granules [29].

Another advantage of using V-ATPases inhibitors, namely omeprazole, is that inhibition of the osteoclastic acidification leads to inhibition of bone resorption and, recently discovered, increasing of the osteoclasts lifespan [10]. Because the osteoclasts are not destroyed and are still present next to the bone matrix, the coupling cycle goes on without inhibiting bone formation. Consequently, there will be a stimulus for bone formation by the osteoblasts along with no bone resorption [1,10].

Citing Sheraly *et al*. (2009) [29] and Rzeszutek *et al*. (2003) [12] once more, in both studies the drugs were solved in a proper solvent, dimethyl sulfoxide (DMSO), until the intended drug concentration was reached and this vehicle was added to the liquid phase of the calcium phosphate. This alternative method to deliver the drugs has the advantage of not being

almost right away absorbed by the organism by entering too soon in the blood circulation. However, as stated by the authors, further experiments are required before we can rule out DMSO effects on drug activity and eventual cell toxicity [29].

More recently other therapeutic targets for PPIs, namely omeprazole, are being studied. Resistance to antitumor agents is a major handicap for cure in cancer patients and some of these mechanisms may be related with increased acidification of extracellular compartments. Luciani *et al.* (2004) [31] made an experiment in which tumor cells derived from human melanomas, adenocarcinomas, and lymphomas were submitted to the action of a variety of gastric PPIs before treatment with cytotoxic agents. The inhibition of the V-ATPase activity induced a marked increase in the cytoplasmic retention of the cytotoxic drugs. In *in vivo* experiments, oral pretreatment with omeprazole was able to induce sensitivity of human solid tumors to cisplatin [31].

Conclusions

Despite the fact that the results from the present preliminary study must be considered inconclusive, there is still some evidence that omeprazole can contribute to inhibition of resorption by the osteoclasts. Although not well explored yet, omeprazole presents some beneficial features against the other V-ATPases inihibitors, especially the fact that it is a very well known and secure drug with very few and rare adverse effects. Accordingly, further studies should be planned, in which new methodological approaches should be tried, such as incorporation of the drug in the graft material composition, use of smaller hydroxyapatite granules, determination of the most appropriated omeprazole doses, using larger animals and assessing the results in different time periods.

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References

[1] Sørensen MG, Henriksen K, Neutzsky-Wulff AV, Dziegiel MH, Karsdal MA. Diphyllin, a novel and naturally potent V-ATPase inhibitor, abrogates acidification of the osteoclastic resorption lacunae and bone resorption. J Bone Miner Res. 2007 Oct;22(10):1640-8.

[2] Niikura K, Takeshita N, Takano M. A vacuolar ATPase inhibitor, FR167356, prevents bone resorption in ovariectomized rats with high potency and specificity: potential for clinical application. J Bone Miner Res. 2005 Sep;20(9):1579-88.

[3] Keeling DJ, Herslöf M, Ryberg B, Sjögren S, Sölvell L. Vacuolar H(+)-ATPases. Targets for drug discovery? Ann N Y Acad Sci. 1997 Nov 3;834:600-8.

[4] Xu J, Cheng T, Feng HT, Pavlos NJ, Zheng MH. Structure and function of V-ATPases in osteoclasts: potential therapeutic targets for the treatment of osteolysis. Histol Histopathol. 2007 Apr;22(4):443-54.

[5] Supanchart C, Kornak U. Ion channels and transporters in osteoclasts. Arch Biochem Biophys. 2008 May 15;473(2):161-5.

[6] Yuan FL, Li X, Lu WG, Li CW, Li JP, Wang Y. The vacuolar ATPase in bone cells: a potential therapeutic target in osteoporosis. Mol Biol Rep. 2010 Feb 25.

[7] Kajiya H, Okamoto F, Li JP, Nakao A, Okabe K. Expression of mouse osteoclast K-Cl Co-transporter-1 and its role during bone resorption. J Bone Miner Res. 2006 Jul;21(7):984-92.

[8] Yao G, Feng H, Cai Y, Qi W, Kong K. Characterization of vacuolar-ATPase and selective inhibition of vacuolar-H(+)-ATPase in osteoclasts. Biochem Biophys Res Commun. 2007 Jun 15;357(4):821-7.

[9] Henriksen K, Sørensen MG, Jensen VK, Dziegiel MH, Nosjean O, Karsdal MA. Ion transporters involved in acidification of the resorption lacuna in osteoclasts. Calcif Tissue Int. 2008 Sep;83(3):230-42.

[10] Karsdal MA, Henriksen K, Sørensen MG, Gram J, Schaller S, Dziegiel MH, et al. Acidification of the osteoclastic resorption compartment provides insight into the coupling of bone formation to bone resorption. Am J Pathol. 2005 Feb;166(2):467-76.

[11] Gagliardi S, Nadler G, Consolandi E, Parini C, Morvan M, Legave MN, et al. 5-(5,6-Dichloro-2-indolyl)-2-methoxy-2,4-pentadienamides: novel and selective inhibitors of the vacuolar H+-ATPase of osteoclasts with bone antiresorptive activity. J Med Chem. 1998 May 7;41(10):1568-73.

[12] Rzeszutek K, Sarraf F, Davies JE. Proton pump inhibitors control osteoclastic resorption of calcium phosphate implants and stimulate increased local reparative bone growth. J Craniofac Surg. 2003 May;14(3):301-7.

[13] Visentin L, Dodds RA, Valente M, Misiano P, Bradbeer JN, Oneta S, et al. A selective inhibitor of the osteoclastic V-H(+)-ATPase prevents bone loss in both thyroparathyroidectomized and ovariectomized rats. J Clin Invest. 2000 Jul;106(2):309-18.

[14] Farina C, Gagliardi S, Nadler G, Morvan M, Parini C, Belfiore P, et al. Novel bone antiresorptive agents that selectively inhibit the osteoclast V-H+-ATPase. Farmaco. 2001 Jan-Feb;56(1-2):113-6.

[15] Farina C, Gagliardi S. Selective inhibition of osteoclast vacuolar H(+)-ATPase. Curr Pharm Des. 2002;8(23):2033-48.

[16] Niikura K, Takeshita N, Chida N. A novel inhibitor of vacuolar ATPase, FR202126, prevents alveolar bone destruction in experimental periodontitis in rats. J Toxicol Sci. 2005 Dec;30(4):297-304.

[17] Shi S, Klotz U. Proton pump inhibitors: an update of their clinical use and pharmacokinetics. Eur J Clin Pharmacol. 2008 Oct;64(10):935-51.

[18] Thomson AB, Sauve MD, Kassam N, Kamitakahara H. Safety of the long-term use of proton pump inhibitors. World J Gastroenterol. 2010 May 21;16(19):2323-30.

[19] Mizunashi K, Furukawa Y, Katano K, Abe K. Effect of omeprazole, an inhibitor of H+,K(+)-ATPase, on bone resorption in humans. Calcif Tissue Int. 1993 Jul;53(1):21-5.

[20] Tuukkanen J, Väänänen HK. Omeprazole, a specific inhibitor of H+-K+-ATPase, inhibits bone resorption in vitro. Calcif Tissue Int. 1986 Feb;38(2):123-5.

[21] Vasconcelos M, Afonso A, Branco R, Cavalheiro J. Guided bone regeneration using osteopatite granules and polytetrafluoroethylene membranes. J Mater Sci Mater Med. 1997 Dec;8(12):815-8.

[22] Tharanathan RN, Kittur FS. Chitin--the undisputed biomolecule of great potential. Crit Rev Food Sci Nutr. 2003;43(1):61-87.

[23] Khor E, Lim LY. Implantable applications of chitin and chitosan. Biomaterials. 2003 Jun;24(13):2339-49.

[24] Mesquita P, Branco R, Afonso A, Vasconcelos M, Cavalheiro J. Mineralized membranes for bone regeneration. Key Engineering Materials. 2004 254-254:1091-1094.

[25] Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. 5th ed. London: Churchill Livingstone; 2002. p. 292.

[26] Cavalheiro J, Branco R, Vasconcelos M: Modified hydroxyapatite granules and plaster as binder: In vitro and clinical tests. In: Bonfield W, Hastings GW, Tanner KE, eds. Bioceramics, vol 4. London: Butterworth-Heinemann, 1991; 205-211.

[27] Afonso A, Santos JD, Vasconcelos M, Branco R, Cavalheiro J. Granules of osteopatite and glass-reinforced hydroxyapatite implanted in rabbit tibiae. J. Mater. Sci.: Mater. Med. 1996; 7: 507-510.

[28] Carvalho GS, Pereira ML, Peres F, Vasconcelos M, Branco R, CavalheiroJ: Reacções in vitro e in vivo a diferentes dimensões de grânulos de hidroxiapatite modificada. Rev. Port, de Est. e Cir. Maxilofac. 1992; 32: 213-219.

[29] Sheraly AR, Lickorish D, Sarraf F, Davies JE. Use of gastrointestinal proton pump inhibitors to regulate osteoclast-mediated resorption of calcium phosphate cements in vivo. Curr Drug Deliv. 2009 Apr;6(2):192-8.

[30] Mattsson JP, Väänänen K, Wallmark B, Lorentzon P. Omeprazole and bafilomycin, two proton pump inhibitors: differentiation of their effects on gastric, kidney and bone H(+)-translocating ATPases. Biochim Biophys Acta. 1991 Jun 18;1065(2):261-8.

[31] Luciani F, Spada M, De Milito A, Molinari A, Rivoltini L, Montinaro A, et al. Effect of proton pump inhibitor pretreatment on resistance of solid tumors to cytotoxic drugs. J Natl Cancer Inst. 2004 Nov 17;96(22):1702-13.

Anexo

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