Topical Application of Bisphosphonates to Enhance Alveolar New Bone Formation

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Si me caí, es porque estaba caminando.

Y caminar vale la pena aunque te caigas.

(Eduardo Galeno)

A Oscar y a mis Aitas, María y Juan, porque me sujetan fuerte para que no me caiga y me ayudan a levantarme cuando me caigo.

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Research Environment

- Universitat Internacional de Catalunya (UIC)
- Universidad de Murcia (UM)
- Universidad Católica San Antonio de Murcia (UCAM)

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Summary

This PhD thesis is a compendium of three publications, which set out to broaden our knowledge and understanding of the topical application of bisphosphonates alone or mixed with a bone graft material in alveolar bone defects, to evaluate their potential capacity to preserve or enhance alveolar bone formation.

In recent years, research has focused on improving bone substitutes in order to achieve faster and better regeneration through morphologic and biochemical modification. Bisphosphonates are a group of drugs that reduce bone resorption by inhibiting the formation, recruitment and activity of mature osteoclasts, and by promoting their apoptosis. In addition, some bisphosphonates enhance osteoblast differentiation and activity. Moreover, it has been demonstrated that the topical application of a bisphosphonate can minimize bone resorption following muco-periosteal flap surgery or in cases of peri-implantitis, improve the osteoconductive and regenerative capacity of a biomaterial, prevent the surface resorption of onlay bone grafts, or reduce post-extraction dimensional changes.

Mandibular second premolars (P2) and first molars (M1) were extracted from six Fox-Hound dogs. P2 were categorized as small defects (SD) and M1 as large defects (LD). Four random groups were created: SC (small control defects filled with MP3®), ST (small test defects MP3® + pamidronate), LC (large control defects with MP3®), and LT (large test defects MP3® + pamidronate). After four and eight weeks healing, percentages of new bone formation (NB), residual graft material (RG) and connective tissue (CT) underwent histological and histomorphometric analysis. To complement the information already obtained in histological analysis, the samples were also evaluated by scanning electron microscopy (SEM), and energy dispersive X-ray spectroscopy (EDX) to identify the chemical elements present in the biomaterial and surrounding tissues, in order to reach a better understand the biomaterial's degradation process. The

study was complemented with a systemic literature review of articles published between January 2000 and December 2016 evaluating the *in vivo* effects of topical applications of bisphosphonates on bone regeneration/preservation in alveolar defects. A total of 154 abstracts were identified, of which 18 potentially relevant articles were selected; a final total of 9 papers were included for analysis.

In the present pilot study, histomorphometric and histologic analysis found that after 4 and 8 weeks healing, the test groups (ST and LT) treated topically with pamidronate presented more new bone formation, compared with SC and LC respectively. Residual graft material was significantly greater in both control groups (SC and LC) compared with test (ST and LT) groups. Percentages of connective tissue were higher in large defects (LC and LT) compared with small defects (SC and ST). SEM analysis revealed more mineralized bone in test groups (ST and LT) compared with control groups, demonstrated by higher percentages of Ca obtained in EDX spectroscopy.

Within the limitations of this experimental study, the findings suggest that porcine xenografts (MP3®) modified with pamidronate favour new bone formation and increased porcine xenograft substitution/replacement after 4 and 8 weeks healing. These results are in accordance with the conclusions obtained from the systematic review. Although it was difficult to compare the findings of the studies reviewed due to the heterogeneity of the articles, the topical application of bisphosphonate solution would appear to favour new bone formation in alveolar defects, and boosts the regenerative capacities of biomaterials resulting in increased bone density.

Index

1. List of Papers

This thesis is based on three previously published papers, as follows:

Paper I

Xenografts supplemented with pamindronate placed in post-extraction sockets to avoid crestal bone resorption. Experimental study in Foxhound dogs. Lozano-Carrascal N, Delgado-Ruiz RA, Gargallo-Albiol J, Maté-Sánchez JE, Hernandez Alfaro F, Calvo-Guirado JL.

Clin Oral Implants Res. 2016 Feb; 27(2): 149-55. doi: 10.1111/clr.12550

IMPACT FACTOR (2015): 3.464

ISI JCR Ranking (2015): 7/91 (Dentistry, Oral Surgery & Medicine)

Paper II

Scanning electron microscopy study of new bone formation following small and large defects preserved with xenografts supplemented with pamidronate – A pilot study in Foxhound dogs at 4 and 8 weeks. Lozano-Carrascal N, Satorres-Nieto M, Delgado-Ruiz R, Maté-Sánchez de Val JE, Gehrke SA, Gargallo-Albiol J, Calvo-Guirado JL.

Ann Anat. 2017 Jan; 209: 61-68. doi: 10.1016/j.aanat.2016.09.009

IMPACT FACTOR (2015): 1.308

ISI JCR Ranking (2015): 9/21 (Anatomy & Morphology)

Paper III

Do topical applications of bisphosphonates improve bone formation in oral implantology? A systematic review. Naroa Lozano-Carrascal, Oscar Salomó-Coll, Federico Hernández-Alfaro, Sergio Alexandre Gehrke, Jordi Gargallo-Albiol, José Luis Calvo-Guirado.

Accepted in Medicina Oral Patología Oral Cirugía Bucal

IMPACT FACTOR (2015): 1.087

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2. Background

Bone Tissue

Bone is a highly vascularized and innervated, mineralized conjunctive tissue, which is structured in lamellae of calcified osteoid matrix. The arrangement of these lamellae determines whether the bone is cortical or trabecular. Both cortical and trabecular bone are composed of specialized cells, organic matrix and mineral phase (1).

Bone Cells

Bone cells are found within the bone tissue itself or in the conjunctive stroma of bone marrow, which is rich in mesenchymal stem cells. The cells harboured in bone tissue include osteocytes, osteoblasts, osteoclasts, but predominantly bone-lining cells (1).

Osteoblasts

Osteoblasts originate from the mesenchymal stem cells of the bone marrow, endosteum, periosteum, and perivascular pericytes. Osteoblast functions involve: 1) synthesizing collagen and non-collagen proteins of the organic bone matrix; 2) directing the arrangement of extracellular matrix fibrils; 3) contributing to the mineralization of the osteoid material, due to alkaline phosphatase; 4) mediating the resorption carried out by osteoclasts, through the synthesis of specific cytokines; and 5) synthesizing growth factors (1,2).

Osteocytes

Once the matrix is mineralized, some osteoblasts remain trapped within, becoming osteocytes. Osteocytes participate in the synthesis and mineralization of the osteoid matrix, but it is believed that their principal function is to control bone remodeling (1,3).

Osteoclasts

Osteoclasts are the cells responsible for bone resorption. Osteoclasts have two special features in the membrane: a ruffled border, where resorption takes place, and a clear area rich in microfilaments, with integrins that serve as an anchor for the matrix. To this end, osteoclasts move towards the area to be resorbed and then immediately adhere to the mineralized bone surface (1).

Organic matrix

The organic matrix or osteoid material makes up a third of the bone mass, and it is mainly made up of proteins, especially collagen (90%). It is considered a reservoir of calcium, phosphorous, and proteins that participate in the regulation of cell differentiation and in the integrity and function of bone tissue (4).

Mineral phase

The mineral component of bone represents 65% of the bone mass. It is made up of calcium, phosphate and carbonate (in proportions of 10:6:1) and in smaller quantities of magnesium, sodium, potassium, manganese and fluoride (1).

Bone remodeling

Bone is a dynamic tissue, in a constant process of resorption and formation. This balanced phenomenon (the amount of resorbed bone is similar to the newly formed) is known as the remodeling process. Under normal conditions, this allows the renovation of about 5% of cortical bone and 20% of trabecular bone mass per year. Microscopically, bone remodeling takes place in small areas of the cortical and the trabecular surface, known as basic multicellular units (BMU). Bone remodeling can be divided into the following phases: quiescent, activation, resorption, formation and mineralization (5,6).

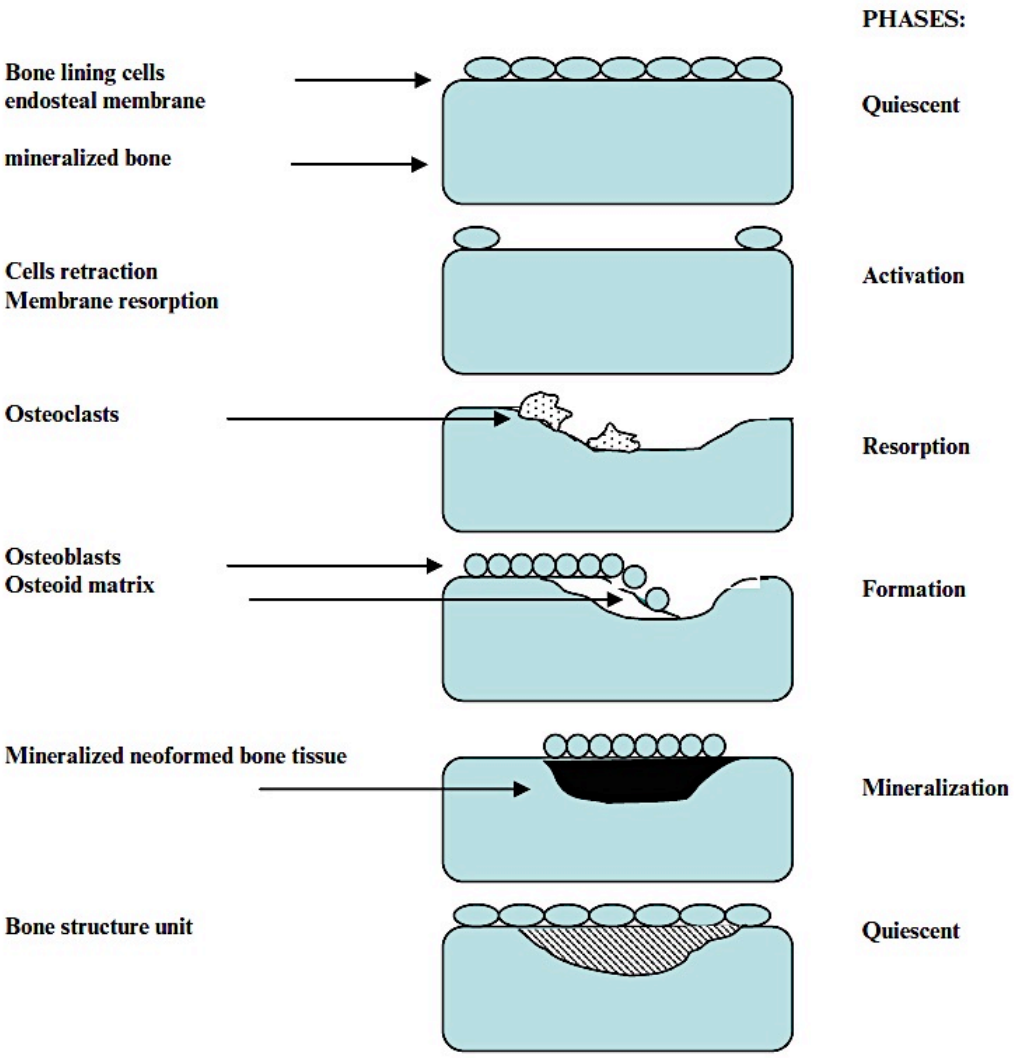

Fig 1. Bone remodeling phases (Fernádez-Tresguerres et al. 2006) (5).

Bone remodeling is regulated by: a) genetic factors: which determine maximum bone mass; between 60 and 80% of this is genetically determined; b) mechanical factors: physical activity is essential for the correct development of bone; c) vascular factors: vascular neoformation is the first event in bone regeneration, oxygen supply is fundamental to this event; d) hormonal factors: the most important hormones in bone physiology are thyroid hormones, parathyroid hormones (PTH), calcitonin, calcitriol, androgens, oestrogens, progesterone, insulin, glucocorticoids and growth hormone; e) nutritional

factors; and f) local factors: principally growth factors, cytokines and bone matrix proteins (5,7).

Socket Healing

After dental extraction, the process of socket healing involves a series of events that starts with the formation of a coagulum. This coagulum is progressively replaced by a provisional matrix, which, thanks to calcium and phosphate deposits, transforms into woven bone and finally, with tissue maturation, evolves into lamellar and bone marrow. The bone resorption-apposition process has been studied in different animal models, resulting in the loss of bone volume in both horizontal and vertical dimensions (8,9).

Approximately 25-50% of the volume of the socket is reabsorbed during the first year. Resorption is faster during the first 3 to 8 weeks, is higher in horizontal than in vertical dimensions, more accentuated at the buccal plate bone compared with lingual (due to the greater proportion of bundle bone present in the buccal plate) (10-13). In a recent review, *Tan et al. 2012* (14), estimated horizontal bone loss as 29-63% and vertical bone loss as 12-22% during the six first months after dental extraction. *Vander Weijden et al. 2009* (15), in another systematic review, showed that the healing of undisturbed post extraction sockets resulted in a higher reduction in width (3.87 mm) compared with the reduction in height (1.67 mm).

To overcome the natural bone resorption process that takes place following dental extraction, different techniques and methods have been proposed, socket preservation being the most widely studied. In socket preservation techniques, a bone grafting material or scaffold is placed to preserve the ridge volume within the envelope existing at the time of extraction (16,17); *Ten Heggeler et al. 2011* (18), showed that socket preservation procedures reduced (but did not halt) both the height and width of ridge reduction, with reductions ranging from +1.3 to –2.64 mm and from –1.2 to –2.64 mm respectively

depending on the biomaterials used. A meta-analysis performed by *Vignoletti et al. 2012* (19), showed the potential benefit of socket preservation therapies, which resulted in significantly greater reduction of bone ridge height in control groups compared with test groups (a mean difference in height of -1.47 mm); and a significant reduction in bone width for control groups compared with test groups (mean difference in width of -1.83 mm). Nevertheless, research has not provided clear guidelines as to the optimal choice of biomaterial type or surgical procedure.

The materials and techniques available are extremely heterogeneous, but none have been demonstrated to be superior to the others. Moreover, these techniques only limit bone resorption; none of them achieve complete preservation of the initial bone volume (20), and the influence of each technique on bone quality, implant success and peri-implant tissue stability remains unknown (21). In techniques of ridge preservation involving a bone substitute and membranes, clinical success will depend on the bone substitute's ability to support primary bone formation, regenerate mature bone, and on its chemical and physical properties (22).

Bone Grafts

An ideal bone substitute should meet the following criteria: a) it must be safe and biocompatible; b) provide conductivity for attachment and proliferation of committed cells or their progenitors for the production of new extracellular matrix; c) be able to incorporate inductive factors to direct and enhance new tissue growth; d) act as a scaffold for bone ingrowth from the recipient site; e) stimulate bone ingrowth from the recipient site; f) provide mechanical integrity to support loads at the implant site; g) undergo a controlled, predictable, reproducible rate of degradation into non-toxic species that are easily metabolized or excreted; and h) be cost-effective and easily processed into irregular 3D shapes of sufficient size to fill clinically relevant defects (23). Bones

substitutes should maintain biological support during healing and gradually become replaced by newly formed bone (24).

Several factors govern the successful incorporation of grafted bone including: a) the type of bone graft used; b) the site of implantation; c) the vascularity of the graft and the host-graft interface; d) the immune-genetics between the donor and the host; e) preservation techniques; f) local and systemic factors; and g) the mechanical properties that depend on the type of graft, its size and shape. The latter is crucial for the formation of bone inside the material; its geometry should favour the ingrowth of blood vessels. This means that the material needs not only to be porous but also to have interconnected macropores to potentiate vascular ingrowth between the graft particles (25,26).

The following properties are desirable in any bone graft material: **Osteogenesis** is the synthesis of new bone by cells derived from either the graft or the host. When correctly handled, cells from cortical and cancellous grafts can survive the transfer to the host site and form new bone that is critical in the initial phase of bone repair. **Osteoinduction** is the process by which mesenchymal stem cells (MSCs) at and around the host site are recruited to differentiate into chondroblasts and osteoblasts. Recruitment and differentiation are modulated by graft matrix-derived growth factors whose activity is triggered when bone mineral is removed. **Osteoconduction** is the process by which an ordered, spatial, three-dimensional ingrowth of capillaries, perivascular tissue, and MSCs takes place from the host site through the implanted graft. This scaffold permits the formation of new bone following a predictable pattern determined by the biology of the graft and the mechanical environment of the host-graft interface (25).

Autografts

Autogenous bone grafts can be harvested from intraoral or extraoral donor sites in the same individual. They are composed of approximately 30% organic and

70% inorganic compounds. Of the organic compounds, 90-95% are collagen (type I) and the rest non-collagenous proteins such as osteocalcin, calcitonin, osteopontin, and sialoprotein. The inorganic component is composed of calcium phosphate, predominantly in the form of crystalline hydroxyapatite (HA) (24,26).

Autografts continue to be the gold standard for regenerative procedures, due to: a) osteogenic properties (marrow-derived osteoblastic cells, as well as pre-osteoblastic precursor cells); b) osteoinductive properties (non-collagenous bone matrix proteins, including growth factors); and c) osteoconductive properties (bone mineral and collagen). Autografts are the only graft materials with a well-documented osteoinductive potential, and so, constitute the most commonly used and favoured graft material (25). However, donor site morbidity, unpredictable resorption, the limited quantities available, and the need to create additional surgical sites are drawbacks that have intensified the search for suitable alternatives (23,26).

Bone substitute materials have increased in popularity as adjuncts to or replacements for autografts in bone augmentation procedures that aim to overcome the limitations deriving from the use of autografts. Bone substitute materials can be categorized as three groups: a) allogenic, from another individual within the same species; b) xenogenic, from another species; or c) alloplastic, synthetically produced (27).

Allografts and xenografts are alternatives in terms of availability and well demonstrated osseointegrative and osteoconductive properties, and donor site morbidity is not an issue. But they suffer some disadvantages compared with autografts, namely a lack of osteogenic and osteoinductive properties (28).

Allografts

Allografts consist of bone obtained from a donor and used in another individual within the same species. Allografts are usually stored in bone banks, and may

be used as fresh frozen bone (FFB), freeze-dried bone allograft (FDBA), or demineralized freeze-dried bone allograft (DFDBA). FFB is rarely used in GBR procedures because of a high risk of immunological rejection and disease transmission. FDBA and DFDBA have been shown to be biocompatible and to contain osteoinductive molecules such as BMPs. However, it is debatable whether the concentration and activity of these molecules is of clinical relevance and resorption is reported to take place, as with autografts. Allografts are widely used in the United States, whereas local regulations in Europe restrict the collection of human bone, which has limited their use by clinicians (23-26).

Xenografts

Xenografts, or xenogenic bone substitutes, consist of bone mineral derived from animals or bone-like minerals produced by calcifying corals or algae from which the organic component has been removed. The organic component is removed by heat treatment, by a chemical extraction method, or by a combination of the two to eliminate the risk of immunological reactions and disease transmission. Xenografts derived from natural bone sources have been extensively investigated in numerous experimental and clinical studies (24,26,27).

Two of the most frequently used xenografts are derived from bovine and porcine bone, because of their close similarity to human cancellous bone. Bone substitutes of bovine origin (made up of natural bone mineral with extensive interconnected pores) (29), is derived from trabecular bone; porcine bone substitute is derived from both trabecular bone (80%) and cortical bone (20%) (30).

Alloplastic grafts

Synthetic grafts, such as calcium phosphate, offer another alternative offering advantages of availability, sterility, osteoconductive properties and reduced morbidity. So far it has not been possible to mimic the surface of natural bone

mineral; long-term complications include stress shielding, loosening and mechanical or chemical breakdown of the material (23,24).

Bisphosphonates

Another approach to minimizing bone loss would be to inhibit osteoclast action and consequently bone resorption. Bisphosphonates are a group of drugs commonly used for the treatment of various bone diseases, including osteoporosis, malignant hypercalcemia, multiple myeloma or Paget's disease (31,32). The nuclear structure of bisphosphonates consists of two phosphate groups joined by a single carbon atom (a P-C-P structure). The two covalently bonded groups attached to the terminal carbon, usually referred to as R1 and R2, allow for a wide range of possible chemical structures. Bisphosphonates form a three-dimensional structure capable of chelating divalent metal ions such as Ca^{2+}, Mg^{2+}, Fe^{2+} (33).

Two groups of bisphosphonates are available, with different mechanisms of action: amino and non-amino-bisphosphonates. Non-amino-bisphosphonates, such as clodronate and etidronate, inhibit bone resorption primarily by inducing osteoclast apoptosis through the formation of intracellular metabolites in osteoclasts. Amino-bisphosphonates, such as pamidronate, alendronate or zoledronate, offer greater potency through the addition of a primary amino-nitrogenated base ($-NH_2$) at the terminus of the R2 alkyl chain (to form $C_2H_5NH_2$) (Fig. 2) (33,34). These act by inhibiting farnesyl diphosphate (FPP) synthase, a key enzyme in the mevalonate pathway (35).

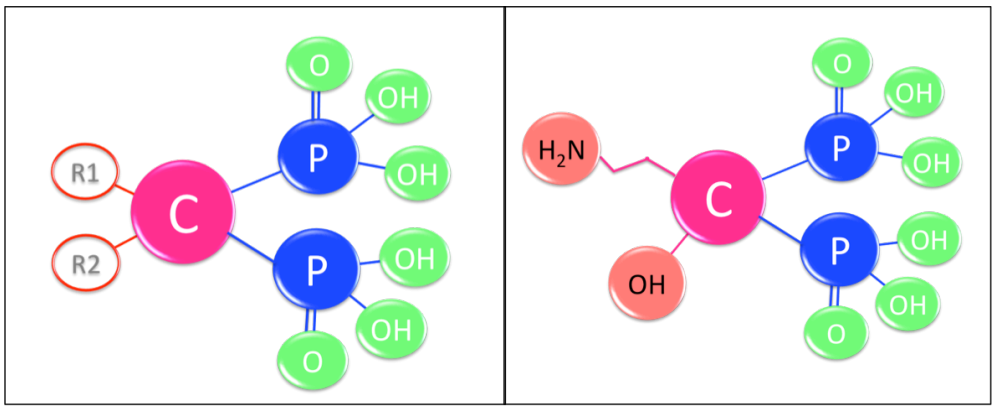

Fig 2. The general structure of a bisphosphonate and a pamidronate.

As a consequence of their high affinity for Ca^{2+} ions, bisphosphonates are rapidly cleared from circulation and target hydroxyapatite bone mineral surfaces *in vivo* at sites of active bone remodeling. Several experimental studies have demonstrated that these drugs reduce bone resorption by inhibiting the activity of mature osteoclasts and promoting their apoptosis (36,37). They also inhibit the formation and recruitment of new osteoclasts, suppressing their multinucleated cells during the osteoclast differentiation process (38-41). In addition, recent experimental studies have demonstrated that some bisphosphonates enhance osteoblast differentiation and activity. For example, alendronate and clodronate seem to act directly on these cells, stimulating differentiation, proliferation, and bone formation/mineralization (42-45).

Bisphosphonates for prevention of alveolar bone resorption

Traditionally, bisphosphonates have been administrated both intravenously and orally. *Reddy et al. 1995* (46) observed that systemic administration of bisphosphonates prevented the alveolar bone destruction associated with periodontal disease in Beagle dogs. But several experimental studies have reported non-desirable secondary effects such as: increased bacterial adhesion and biofilm formation (47); areas of necrotic bone in post-extraction sockets and no evidence of bone formation in augmented sites (48); greater susceptibility to

osteonecrosis of the jaw (ONJ) in areas with increased bone turn-over (49-53); and less bone remodeling around endosseous implants (54).

Due to the potential risks involved in intravenous administration of bisphosphonates, other methods have been proposed. It has been demonstrated that the topical application of bisphosphonates can minimize bone resorption following muco-periosteal flap surgery (55-58), inhibit the progression of alveolar bone resorption in cases of peri-implantitis (59), improve the osseointegration of dental implants (60-63), improve the osteoconductive and regenerative capacity of a biomaterial (64), prevent the surface resorption of onlay bone grafts (65), or reduce post-extraction dimensional changes (66). Furthermore, several researchers have attempted to combine bisphosphonates with other molecules to achieve a more sustained release into the surrounding bone (67-69).

But few studies have assessed the behaviour of xenografts modified with bisphosphonates or bioactive elements to minimize crestal bone resorption and to improve the regenerative capacity of the biomaterial. For this reason, the present study set out to compare *in vivo* the effects of a porcine bone graft alone and modified with pamidronate to repair small and large defects in post-extraction sockets, after 4 and 8 weeks healing, and to perform a systematic literature review to evaluate the potential capacity of the topical application of bisphosphonates to preserve/enhance alveolar bone in oral implantology.

3. Objectives

Main Objective:

The aim of the present study was to asses *in vivo* the effects of a porcine bone graft modified with pamidronate to repair small and large defects in post-extraction sockets, after 4 and 8 weeks healing, and to perform a systematic review of the current literature, to evaluate the potential capacity of the topical application of bisphosphonates to preserve/enhance alveolar bone in oral implantology.

Objectives of the Papers:

Paper I

The aim of this paper was to compare *in vivo* the effects of porcine bone graft (MP3®) alone and modified with pamidronate to repair small and large defects in post-extraction sockets, and to determine the percentage of new bone, residual graft and connective tissue, after 4 and 8 weeks healing by histologic and histomorphometric analysis.

Paper II

The aim of this paper was to evaluate the feasibility of SEM and EDX microanalysis for evaluating the bone healing of small and large defects filled with pamidronate combined with collagenized porcine material, at four and eight weeks.

Paper III

The aim of this systemic literature review was to evaluate the potential capacity of the topical application of bisphosphonates to preserve/enhance alveolar bone in oral implantology.

4. Materials and Methods

Papers I and II (Experimental Study)

The study used six male Fox-Hound dogs of 1.5±0.5 years of age, weighing 12-13 kg each. The study protocol was designed following Spanish and European guidelines (2007/526/CE) for animal experiments. The experiment was approved by the Ethics Committee for Animal Research of the University of Murcia (Spain) and the Universitat Internacional de Catalunya (Spain) (study code: CIR-ELB-2014-01). The study design fulfilled regulations established by the European Union Council Directive of February 1st, 2013, and Royal Decree 53/2013 (BOE no. 34, s. I, p. 11370).

Surgical Procedure

The animals were pre-anesthetized with acepromazine maleate (0.12% - 0.25 mg/kg), buprenorphine (0.01 mg/kg), and medetomidine (35 μg/kg). The mixture was injected intramuscularly in the femoral quadriceps. Then, an intravenous catheter was inserted (diameter 22 or 20 gauge) into the cephalic vein, and propofol was infused at the rate of 0.4 mg/kg/min at a slow constant infusion rate. Conventional dental infiltration anaesthesia articaine 4%/1:100.000 (articaine 40 mg, 0,01 mg epinephrine) was administered at the surgical sites. These procedures were carried out under the supervision of a veterinary surgeon.

Tooth Extraction and Grafting Procedures

In both quadrants of the lower jaws, second premolars (P2) and first molars (M_1) were used as experimental sites. The alveoli corresponding to P2 were classed as small defects (SD) and M1 as large defects (LD).

Teeth were sectioned with a carbide tungsten drill; the roots were removed with forceps, without damaging the remaining bony walls. Sulcular marginal incisions were made along the vestibular and lingual areas adjoining the alveoli, separating tissues in order to make the crestal hard tissue walls visible (Fig.3). Prior to graft placement, the external dimensions of the post-extraction sockets (diameter) were measured using a calliper and recorded. Extraction socket mean bucco-lingual alveolar ridge measurements were as follows: 3.8±0.21 for P2 and 5.6±0.07 mm for M1.

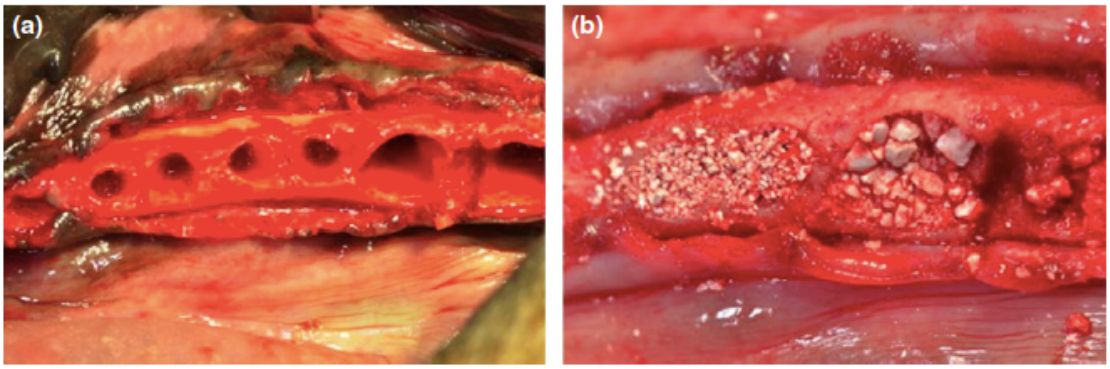

Fig 3. Overview of dog`s mandible: (a) small and large defects after dental extractions; (b) small and large defects filled with MP3® with and without pamidronate.

Afterwards, following a randomized (www.randomization.org) and split-mouth design, the alveoli (SD and LD) corresponding to the right hemi-mandible were used as controls (C) and were filled with MP3® (OsteoBiol, Tecnoss Dental, Turin, Italy) porcine collagenated bone, after rehydration with sterile saline. Left hemi-mandible defects (SD and LD) were filled with MP3® prehydrated with pamidronate (Novartis Pharma, Basel, Switzerland) as test sites (T). Pamidronate solution was prepared by dissolving 90 mg pamidronic acid in 10 ml saline (9 mg/ml) and mixed with 2.0 cc (approximately 4 gr) of 600-1000 µm particles of porcine bone. In this way, four treatment groups were created:

- SC: small defects filled with MP3® alone.
- ST: small defects filled with MP3® modified with pamidronate.
- LC: large defects filled with MP3® alone.

- LT: large defects filled with MP3® modified with pamidronate.

Membranes were not used.

Tissue flaps were repositioned without tension free adaptation using interrupted and horizontal mattress sutures for wound closure, Monofil® 4/0 (Ancladén SL, Barcelona, Spain). During the first week after surgery, the animals were medicated with Amoxicillin (500 mg twice daily) and Ibuprofen (600mg three times a day) administered systemically. Sutures were removed after two weeks. The dogs received a soft diet and a plaque control regime that included tooth cleaning with toothbrush and dentifrice, and administration of 0.2% chlorhexidine solution three times a week until the end of the experiment (four weeks or eight weeks).

The animals were sacrificed at four (three animals) and eight weeks (three animals) by means of an overdose of Pentothal Natrium® (Abbott Laboratories, Chicago, IL, U.S.A.) perfused via the carotid arteries.

Sample Processing

The soft tissues of each mandible were dissected leaving the bone surfaces exposed. Each mandible was block-sectioned and tissues fixed with 4% formalin. The samples were dehydrated in a graded ethanol series. The blocks were infiltrated with Technovit 7200® resin (Heraeus Kulzer, Hanau, Germany) and polymerized with ultraviolet light. Blocks containing the entire graft area (bone, grafted particles, and connective tissue) were divided into two halves: one for histologic and histomorphometric analysis, the other for scanning electron microscopy **(**SEM) and energy dispersive X-ray spectroscopy (EDX) analysis.

Optical Microscopy

The polymerized blocks were then sectioned in buccolingual direction using a precision saw. Three slices were obtained per site, and reduced by micro

grinding and polishing using an Exakt® grinding unit (Exakt, Norderstedt, Germany) to an even thickness of approximately 15-30μm. The slides were stained with Hematoxylin-Eosin; the entire circumference of each section (containing bone, grafted particles and connective tissue) was traced manually to create individual regions of interest.

Histomorphometric Analysis

Percentages of new bone, residual graft material, and connective tissue were calculated in relation to the total measurement area (socket walls). The central portion of each core was selected in order to avoid any potential bias. In this way, both the coronal (remaining native host bone) and the apical portions were excluded from analysis (using a safe margin of 1.5-2 mm). Histomorphometric measurement of the samples was conducted using Image J software®, developed by the National Institutes of Health (U.S.A.). Descriptive evaluation and morphometric measurements were performed under a Nikon Eclipse 80i® microscope (Teknooptik AB, Huddinge, Sweden) equipped with the EasyImage 2000® system (Teknooptik AB) (Fig. 4).

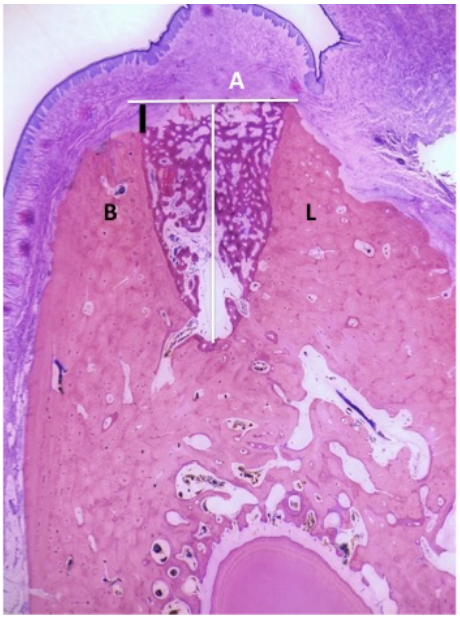

Fig.4. Overview of a bucco-lingual section of a small defect. Each section was stained with hematoxylin eosin. Original magnification ×16.

Scanning Electron Microscopy (SEM)

The surface structure of the bone graft was evaluated *in vitro* by SEM study and *in vivo* by histological analysis. For the SEM study, test and control bone grafts were put into liquid nitrogen for approximately 2 minutes and then split longitudinally, and the other halves were cut in the middle with a diamond-coated water-cooled band saw. The freshly formed and cleaned surfaces were coated with carbon film (BalTec CED 030®; BalTec, Balzers, Liechtenstein) for analysis by SEM (JSM-6100 JEOL®, Tokyo, Japan) (Fig. 5).

Fig. 5. Photograph of the Scanning Electron Microscope (SEM) used in the study (JSM-6100 JEOL®).

EDX Analysis

Samples were examined using energy-dispersive X-ray spectroscopy (EDX) at a working distance of 19 mm, an acceleration voltage of 15 kV and ×15 magnifications, with an Oxford Instruments INCA 300 EDX System® (Oxfordshire, UK), evaluating the element composition of the graft material and bone in the medullar area. Regions of interest were delimited by the inner cortical walls and reached into the medullar core. Element mapping was performed to identify and quantify all chemical elements and observe the

chemical degradation process and changes in the tissues, using point analysis and elemental mapping to determine mineral distribution. EDX spectra were collected at discrete points in each biopsy. Elemental composition (atomic %) of the graft material and bone were calculated from the spectra.

Statistical Analysis

Statistical analysis was performed using SPSS 21.0® (SPSS Inc., IBM Corporation, Chicago, IL, USA) statistical software. Data obtained were expressed as mean ± standard deviation (SD). The mean differences between groups were analysed using the Friedman Test (non-parametric repeated measures analysis of variance) considering the data from one dog as dependent. The Friedman test is designed for a sample, or two-dimensional analysis of variance with one observation per cell. The Friedman test's null hypothesis was that k-related variables came from the same population. For each case, the k variables were assigned the rank 1 to k. The statistic was based on these ranks. Statistical significance was set as $p < 0.05$. In cases of statistically significant differences, the Bonferroni Method for multiple comparisons was applied.

Paper III (Systematic Review)

Focused Question

Based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, a specific answerable question was formulated according to Participants, Interventions, Control, Outcomes (PICO) recommendations: "Does the topical application of bisphosphonate solution improve bone preservation/regeneration in alveolar bone?"

The PICO framework was as follows:

- (P) Participants: samples that underwent treatment with topical applications of bisphosphonate solution.
- (I) Type of intervention: the intervention of interest was the effect of the topical application of bisphosphonates on bone regeneration/preservation in alveolar defects.
- (C) Control intervention: bone regeneration/preservation without topical application of bisphosphonates.
- (O) Outcome measures: bone resorption, new bone formation and/or bone volume/tissue volume, radiographic/histologic changes with and without topical application of bisphosphonates.

A preliminary search for previous systematic reviews and meta-analyses was conducted. Then, a search was made in the MEDLINE and Cochrane Oral Health Group databases for scientific articles published between January 2000 and December 2016, applying the following search terms: “alveolar bone,” “bone regeneration,” “socket preservation,” “bone preservation,” “bisphosphonates,” “pamidronate,” “alendronate,” “zoledronic acid.”

Eligibility Criteria

Eligibility criteria for inclusion in the review were as follows: (a) original studies (clinical and experimental); (b) inclusion of a control group (bone remodeling without topical application of bisphosphonates); (c) intervention: effect of topical application of bisphosphonates on bone preservation/regeneration; (d) studies published in the English language. Only articles published between January 2000 and December 2016 were included. Letters to the editor, historic reviews, commentaries, case reports and *in vitro* studies were excluded.

Search Strategy

A literature search was conducted among the PubMed/Medline (National Library of Medicine, Washington, DC), EMBASE, Scopus, Web of knowledge, and Google-Scholar databases for articles published from January 2000 up to and including December 2016, using different combinations (and Boolean Operators: AND, OR, NOT) of the following search terms/key words: “topical bisphosphonates,” “bone preservation,” “bone regeneration,” “bone substitutes,” “bone graft,” “bone defects,” “bone remodeling,” “alveolar bone.” The titles and abstracts of studies identified in the search were screened by the authors (N.L.C and O.S.C.) and checked for agreement. The full texts of studies screened by title and abstract and considered to be of interest were read and evaluated independently, applying the eligibility criteria. References to any other published articles were also screened to identify potentially relevant original or review articles. Following the electronic search, a further manual search was performed in the websites of the leading scientific journals on dentistry and implant dentistry: Clinical Oral Implants Research, Clinical Oral Investigations, Clinical Implant Dentistry and Related Research, European Journal of Oral Implantology, European Journal of Prosthodontics and Restorative Dentistry, Journal of Oral Maxillofacial Surgery, Journal of Oral Surgery, Journal of Clinical Periodontology, Journal of Periodontology, Implant Dentistry, International Journal of Periodontics and Restorative Dentistry, The International Journal of Oral & Maxillofacial Implants, and European Journal of Inflammation. Again, the eligibility criteria were applied independently and any disagreement between the reviewers was resolved through discussion.

Study Selection and Data Collection Process

Two reviewers (N.L.C. and O.S.C) carried out the selection process, screening the articles’ titles and abstracts. The full texts of all studies of possible relevance were then obtained, and eligibility assessment and data extraction were performed independently in an unblinded standardized manner by the two

authors. The data extracted included eligibility criteria, baseline characteristics, interventions, outcomes, and methodological quality. When the reviewers did not agree, a third reviewer and statistical researcher (J.L.C-G.) scored the abstracts to decide whether the article should be included or excluded. Afterwards, the full text of all the selected manuscripts were read and carefully evaluated.

Data Items

The information extracted from each article included: (1) type of article; (2) specimen and sample; (3) type of bisphosphonate; (4) dose of bisphosphonate; (5) scenario; (6) results. Any disagreements on data extraction were resolved by discussion between the two reviewers.

Quality Assessment

The methodological quality of the studies was assessed focusing on the following issues: bibliography, randomization method, examiner blinding, study population characteristics, baseline and outcome evaluations.

Two reviewers assessed the quality of each study independently. Disagreements on validity assessment were resolved by consensus and discussion; when consensus could not be reached, a third reviewer was consulted.

A study was classed as at a low risk of bias when the study population was selected randomly, when inclusion/exclusion criteria were defined, losses to follow-up reported, measurements validated, and the statistical analysis reported. If one of these five criteria were lacking, the study was classed as having a moderate potential risk of bias. If the study was lacking two or more of these criteria, it was considered as suffering a high potential risk of bias.

5. Results

Paper I

In all experimental sites, healing was uneventful. After 4 and 8 weeks healing, keratinized mucosa was observed covering the edentulous zones without dehiscences or exposure of bone or graft particles.

Histological description at 4 weeks

Under optical microscopy, in both groups after four weeks healing, a hard tissue bridge sealing the coronal part of the extraction socket was observed. The bridge showed a small amount of continuous trabecular bone with some areas of mature bone. This marginal bridge was mainly made of woven bone with areas of lamellar bone. Compared with the lingual region, the buccal soft tissue component exhibited a concave appearance with an invagination towards the extraction socket. Amounts of new bone formation could also be observed along the laterals walls of the sockets.

The apical portion of the control sockets grafted with MP3® harboured areas of newly formed bone, while the central and marginal regions were occupied by granulation/provisional matrix that included a large number of MP3® particles. The newly formed ridges of woven bone were lined by osteoblasts and enclosed primary spongiosa. Some of the MP3® particles were obviously engaged in the process of de novo bone formation, and were partially reabsorbed at 4 weeks (Fig.6).

In the test sockets with MP3® modified with pamindronate, abundant lining cells were observed around neoformed bone that included MP3® particles. The resorption of the biomaterial was evident; the xenograft had been substituted by trabecular bone, and so the amount of residual graft was reduced (Fig. 6). No inflammatory response was observed in the socket areas. There were no signs

of pamindronate particles after 4 weeks healing.

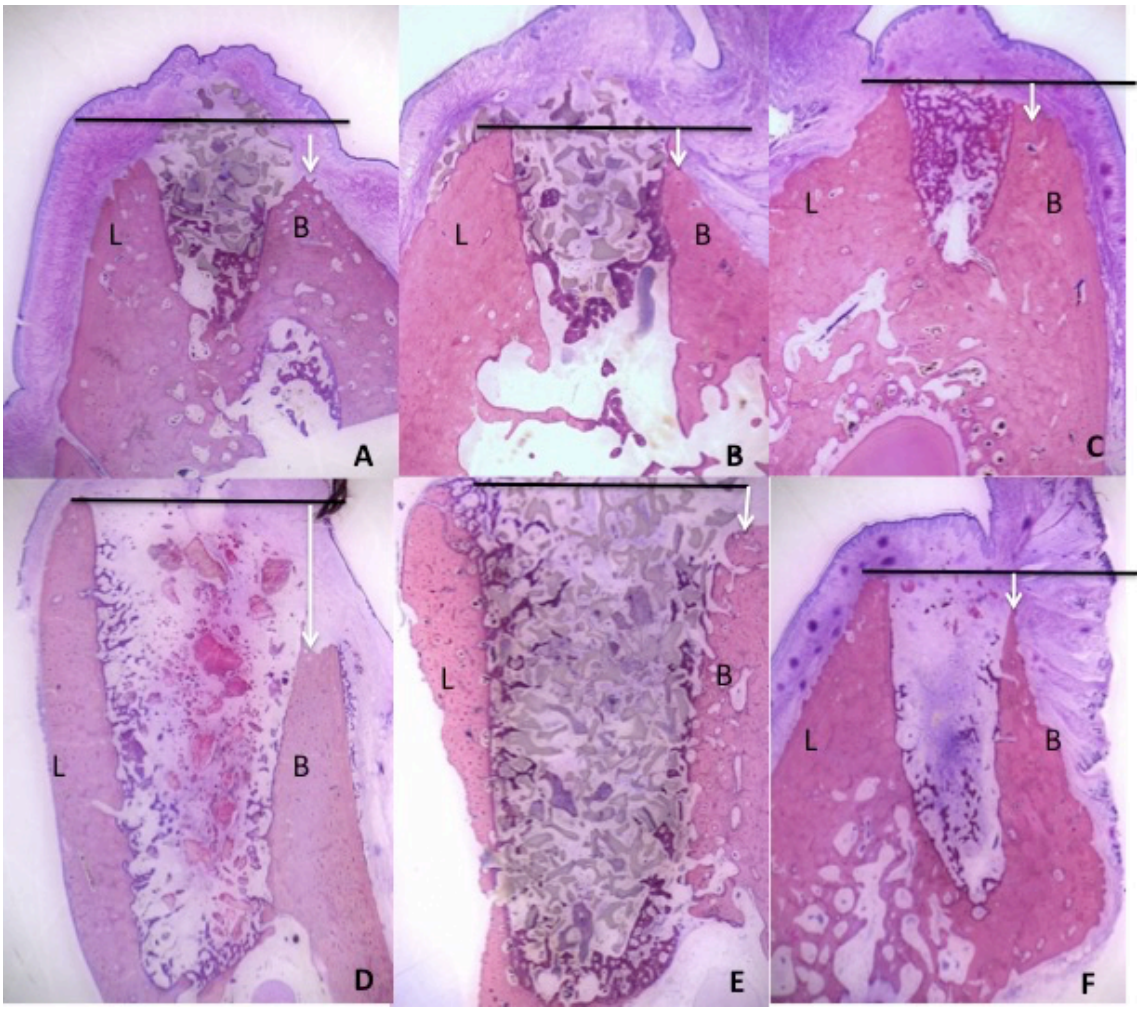

Fig. 6. Overview of a bucco-lingual section representing 4 weeks healing: (a) small defect filled with MP3®; (b) MP3® with pamidronate particles in small defects; (c) small control defect; (d) MP3® in large defects group; (e) MP3® with pamidronate; (f) the width of the ridge is well maintained. Hematoxylin eosin stain, original magnification ×16.

Histological description at 8 weeks

No histological differences could be observed between control and test groups after 8 weeks healing. The apical half of the extraction socket was occupied by newly formed bone; this woven bone extended from the apical and lateral walls of the extraction site and completely surrounded MP3® particles in a phase of resorption and substitution. Porcine bone was partially resorbed (Fig. 7).

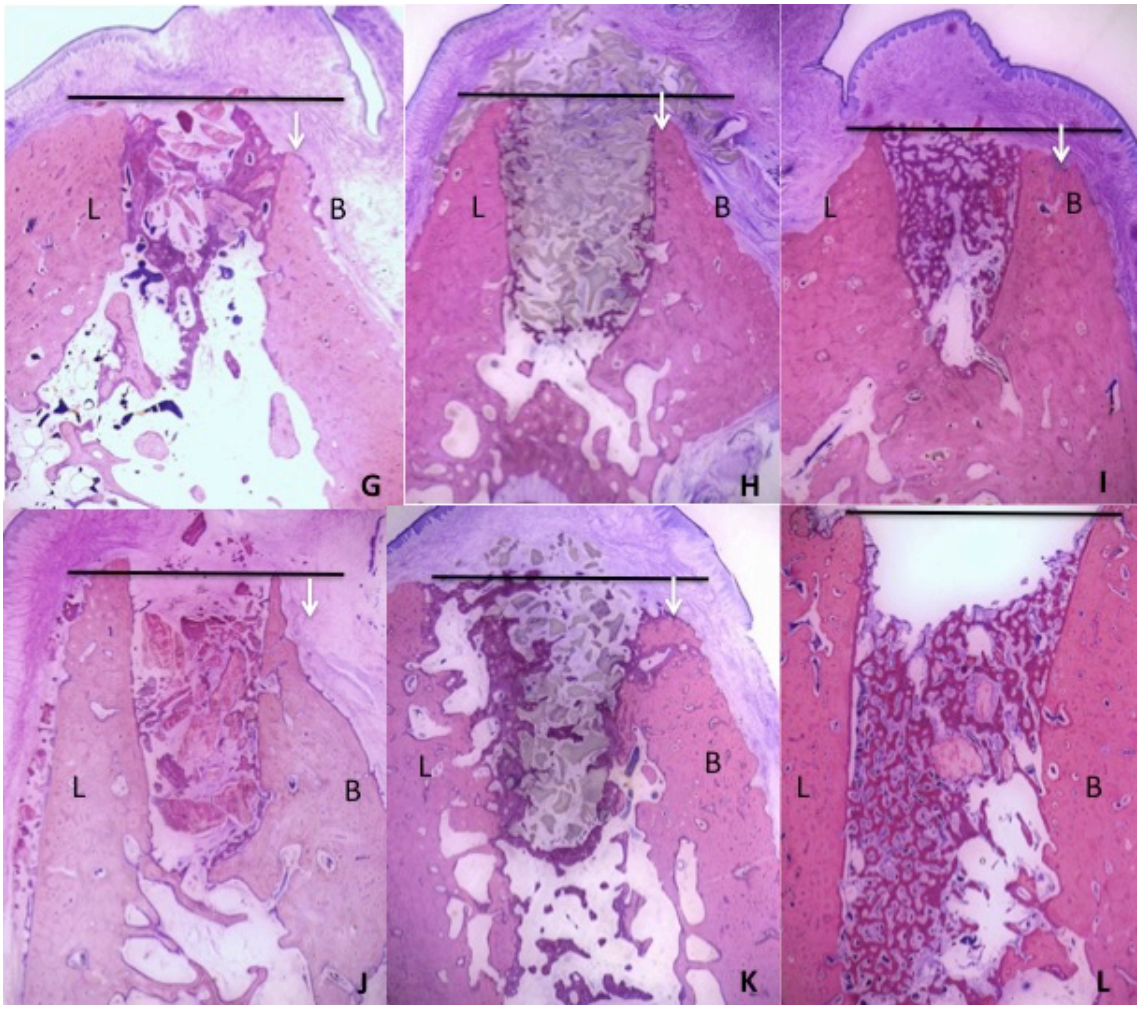

Fig. 7. Overview of bucco-lingual sections after 8 weeks healing: (g) MP3® particles; (h) MP3® with pamidronate particles in small defects; (i) control group woven bone width in small defect; (j) MP3® in large defect group; (k) hard tissue formation, partly mineralized MP3® with pamindronate in large defects; (l) control group large defects. Hematoxylin eosin stain, original magnification ×16.

Histomorphometry at 4 weeks

After 4 weeks healing, the percentage of new bone formation was greater in ST (37.4±0.41) > SC (34.8±0.84) > LT (32.1±0.22) > LC (18.3±0.64); multiple comparisons showed that ST was significantly higher and LT was significantly lower compared with all groups (p =0.001).

The amount of residual graft was higher in SC (38.6±0.28) > LC (26.5±0.92) >

ST (22.3±0.76) > LT (17.8±1.12), multiple comparisons showed that the SC group obtained significantly higher amounts of residual graft compared with the other groups (p =0.005).

Percentages of connective tissue in LC (55.2±0.32) and LT (50.1±0.14) were significantly higher compared with small defects ST (40.3±0.12) and SC (26.6±0.26) (p =0.031) (Table 1).

Table 1. New bone, Residual graft and Connective Tissue percentages after 4 weeks healing. Data expressed as mean ± standard deviation and median. Non-parametric Friedman Test for multiple related samples.

	New bone (%)		Residual graft (%)		Connective tissue (%)	
4 Weeks	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
SC[a]	34.8 ± 0.84	34.80	38.6 ± 0.28*	38.59	26.6 ± 0.26	26.56
ST[b]	37.4 ± 0.41	37.69	22.3 ± 0.76	22.32	40.3 ± 0.12	40.26
LC[c]	18.3 ± 0.64*	18.3	26.5 ± 0.92	26.54	55.2 ± 0.32*	55.20
LT[d]	32.1 ± 0.22	32.09	17.8 ± 1.12	17.76	50.1 ± 0.14	50.08
P value	0.001		0.005		0.031	

* is differences between values achieving statistical significance $P > 0.05$.

Histomorphometry at 8 weeks

After 8 weeks healing, significantly more new bone formation was observed in test groups ST (52.6±0.84) and LT (38.3±0.81), compared with control groups SC (41.7±0.71) and LC (24.3±0.35) (p =0.030).

The percentage of residual graft was significantly higher in control groups SC (35.2±0.54) and LC (24.1±0.84), compared with test groups ST (18.7±0.62) and LT (15.9±0.45) (p =0.017).

Lastly, connective tissue was significantly higher in large defects LC (51.6±0.64) and LT (45.8±0.72) compared with small defects SC (23.1±0.76) and ST (28.7±0.41) (p=0.002) (Table 2) (Figures 8-11).

Table 2. New bone, Residual graft and Connective Tissue percentages after 8 weeks healing. Data expressed as mean ± Standard Deviation and median. Non-parametric Friedman Test for multiple related samples.

	New bone (%)		Residual graft (%)		Connective tissue (%)	
8 Weeks	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
SC[a]	41.7 ± 0.71	41.75	35.2 ± 0.54*	35.25	23.1 ± 0.76	22.85
ST[b]	52.6 ± 0.84*	52.57	18.7 ± 0.62	18.65	28.7 ± 0.41	28.70
LC[c]	24.3 ± 0.35	24.32	24.1 ± 0.84	24.10	51.6 ± 0.64*	51.59
LT[d]	38.3 ± 0.81	38.28	15.9 ± 0.45	15.94	45.8 ± 0.72	45.76
P value	0.030		0.017		0.002	

* is differences between values achieving statistical significance $P > 0.05$.

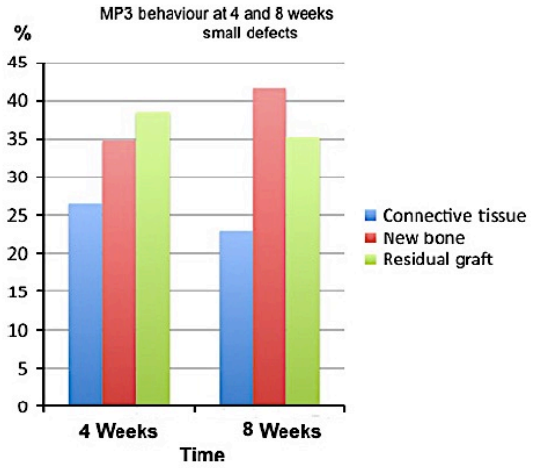

Fig. 8. SC group behaviour at 4 and 8 weeks.

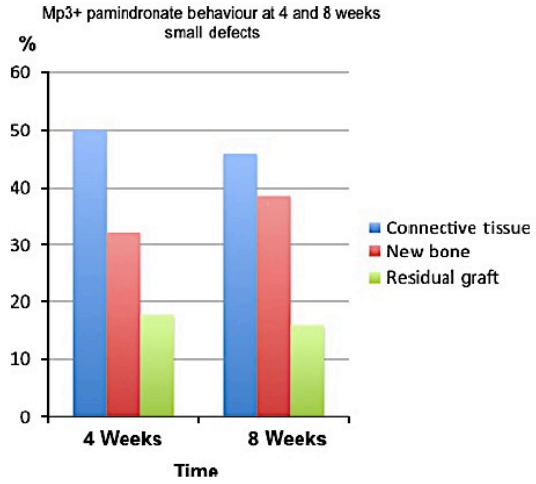

Fig. 9. ST group behaviour at 4 and 8 weeks

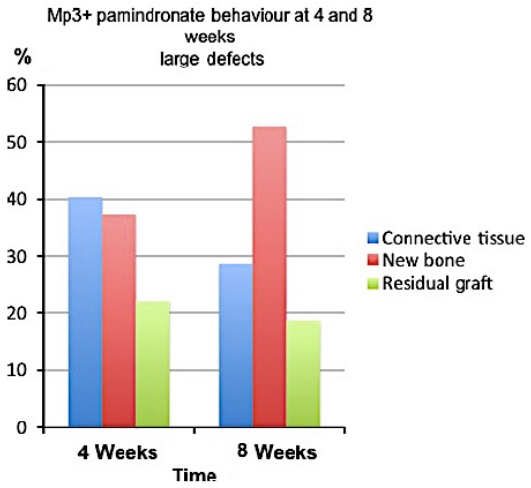

Fig. 10. LC group behaviour at 4 and 8 weeks.

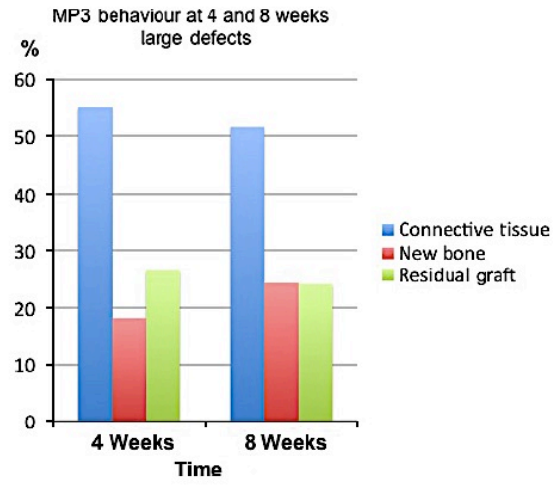

Fig. 11. LT group behaviour at 4 and 8 weeks

Paper II

Scanning Electron Microscopy description

ST and LT Groups (four weeks)

SEM analysis revealed the presence of a hard tissue bridge sealing the coronal part of the extraction socket. While the most coronal third of the socket was occupied by immature bone with pores, the central third showed bone with a density similar to adjacent cortical bone, due to more active resorption of the biomaterial that had been substituted by bone trabeculae. In the apical third, a lack of new bone formation was observed in large defects, due to deficient xenograft compaction during the filling procedure (Fig.12). MP3® particles could be distinguished by their whiter and denser appearance. Resorption of the biomaterial was evident, with abundant osteocytes involved in new bone formation (Fig. 13).

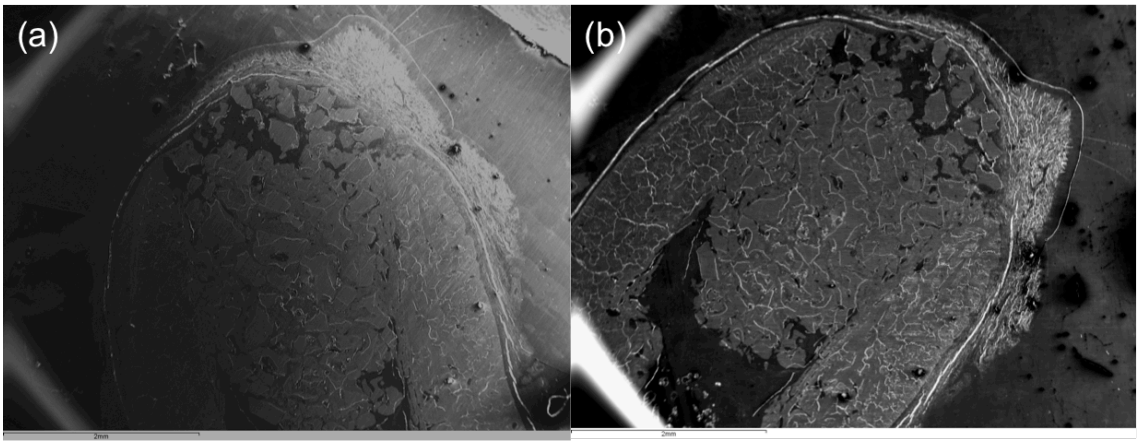

Fig. 12. SEM image of sockets filled with MP3® modified with pamidronate after 4 weeks healing: (a) ST (small test defects), (b) LT (large test defects).

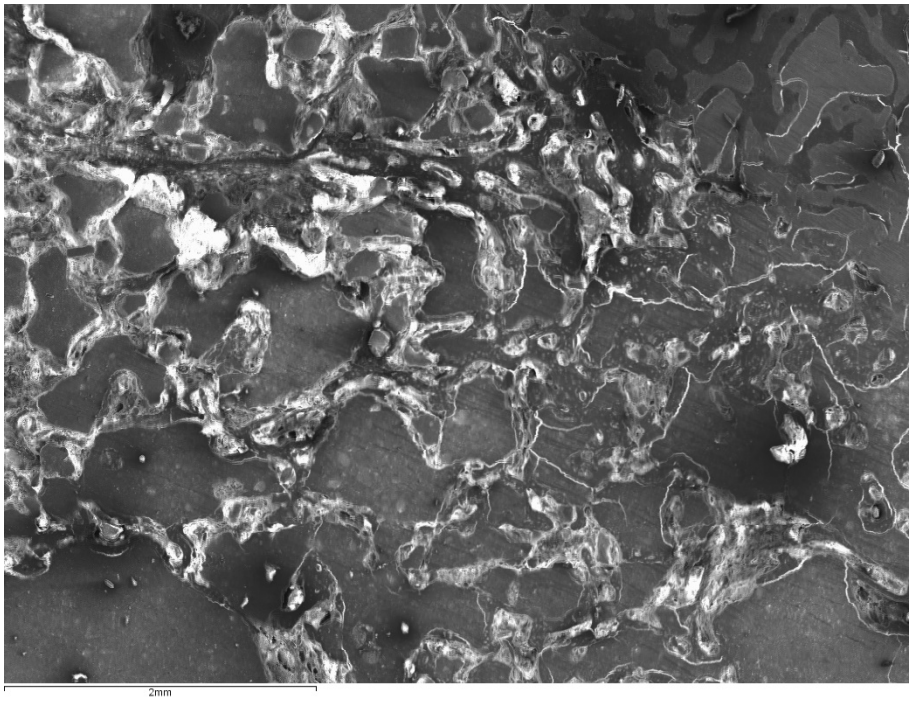

Fig. 13. SEM image of bone formation inside a socket filled with MP3® modified with pamidronate after 4 weeks healing.

SC and LC Groups (four weeks)

SEM images monitored the graft incorporation process; at four weeks, spaces between the graft material and new bone were observed in both small and large defects. Cortical closure was incomplete. Bone marrow was organized with slight cortical formation, accompanied by immature bone with less density than the adjacent cortical bone (Fig. 14).

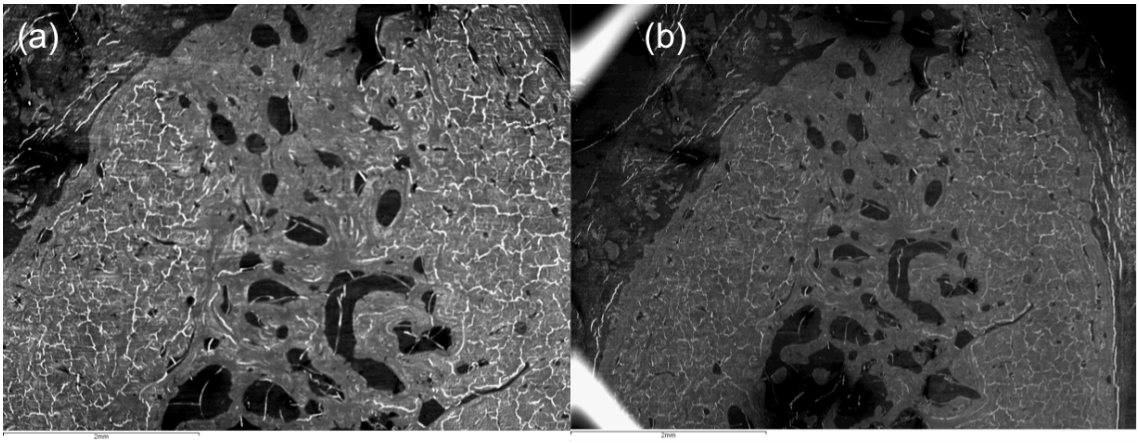

Fig. 14. SEM image of sockets filled with MP3® only after 4 weeks healing: (a) SC (small control defects); (b) LC (large control defects).

ST and LT Groups (eight weeks)

After eight weeks healing, sockets filled with MP3® modified with pamidronate showed internal bone formation inside the bone marrow and complete cortical closure. Mineralized bone of a similar density to the adjacent cortical bone was observed in the coronal third (Figs. 15 and 16).

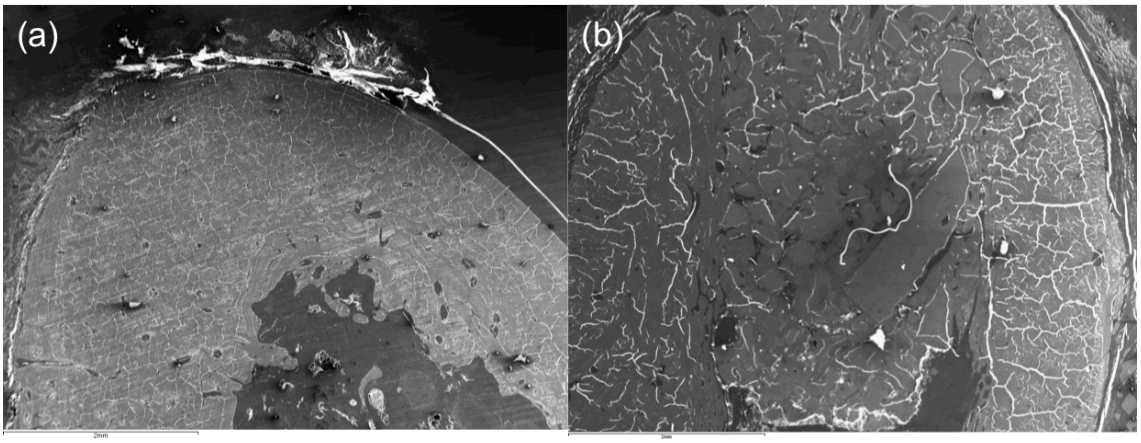

Fig. 15. SEM image of sockets filled with MP3® modified with pamidronate after 8 weeks healing: (a) ST (small test defects); (b) LT (large test defects).

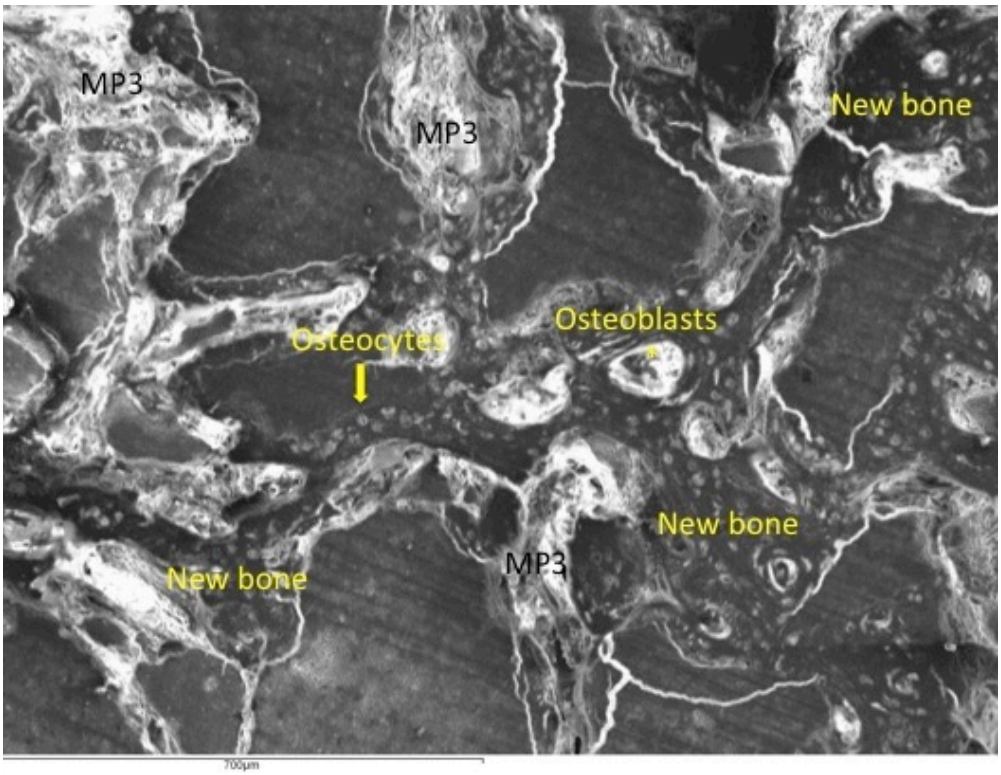

Fig. 16. SEM image of bone formation inside a socket filled with MP3® modified with pamidronate after 8 weeks healing.

SC and LC Groups (eight weeks)

In SEM analysis of small and large control defects filled with MP3® alone after 8 weeks healing, bone appeared to be similar to that described for test sockets filled with MP3® modified with pamidronate after four weeks. Trabecular bone density was similar to adjacent mineralized cortical bone, especially in the coronal third; complete cortical closure was observed (Fig. 17).

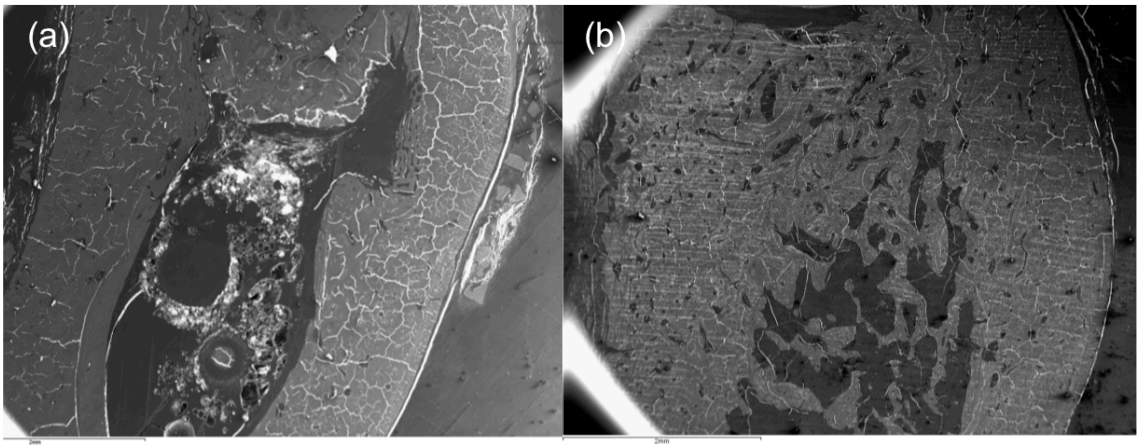

Fig. 17. SEM image of sockets filled with MP3® alone after 8 weeks healing: (a) SC (small control defects); (b) LC (large control defects).

No osseous malformations or structural changes in bone development were observed over the study period.

EDX analysis

EDX analysis evaluated the following chemical elements: carbon (C), oxygen (O), phosphorous (P), calcium (Ca), and silicon (Si). The Ca/P ratio (Ca/P) was also calculated. Mean and standard deviation (SD) for all groups at four and eight weeks (based on samples located inside the length of cortical bone formation) are expressed in Tables 3 and 4, respectively.

Table 3. EDX chemical elements analysis after 4 weeks healing. Data expressed as percentages ± Standard Deviation. Non-parametric Friedman Test for multiple related samples.

4 Weeks	C (%)	O (%)	P (%)	Ca (%)	Si (%)	Ca/P (%)
SC[a]	15.67 ± 0.33[c,d]	12.63 ± 0.20	3.64 ± 0.15	15.07 ± 0.25	51.81 ± 0.65	4.15 ± 0.23
ST[b]	16.72 ± 1.6[c,d]	12.17 ± 0.48	3.89 ± 0.15	16.1 ± 0.35[a,c]	51.95 ± 0.60	4.89 ± 0.22
LC[c]	12.91 ± 0.25	12.09 ± 0.38	3.83 ± 0.65	15.02 ± 0.36	51.11 ± 0.72	4.27 ± 0.67
LT[d]	12.48 ± 0.67	11.93 ± 0.46	4.01 ± 0.29	16.45 ± 0.39[a,c]	49.92 ± 0.91[a,b,c]	4.13 ± 0.39
p value	0.0002	0.0856	0.5880	0.0009	0.0164	0.6608

The level of significance was set at $p < 0.05$.

Table 4. EDX chemical elements analysis after 8 weeks healing. Data expressed as percentages ± Standard Deviation. Non-parametric Friedman Test for multiple related samples.

8 Weeks	C (%)	O (%)	P (%)	Ca (%)	Si (%)	Ca/P (%)
SC[a]	12.30 ± 0.47	12.47 ± 0.22	4.2 ± 0.71	16.89 ± 0.35[c,d]	49.95 ± 0.9[c,d]	4.11 ± 0.71
ST[b]	12.07 ± 0.58	12.35 ± 0.38	4.01 ± 0.29	16.5 ± 0.17[c,d]	49.99 ± 0.39[c,d]	4.13 ± 0.33
LC[c]	11.41 ± 1.11	12.54 ± 0.49	4.2 ± 0.61	15.17 ± 0.42	38.01 ± 0.36	3.67 ±0.57
LT[d]	11.85 ± 0.41	12.22 ± 0.38	3.88 ±0.38	15.15 ± 0.32	38.37 ± 0.23	3.94 ± 0.44
p value	0.2875	0.7281	0.8358	0.0004	0.0000	0.6805

The level of significance was set at $p < 0.05$.

After four weeks healing (Fig. 18), multiple comparisons showed significantly higher percentages of C in SC (15.67±0.33%) and ST (16.72±1.6%) compared with LC (12.91±0.25%) and LT (12.84±0.67%) (p=0.0002). As for Ca measurement, multiple comparison revealed significantly higher amounts of Ca in ST (16.1±0.35%) and LT (16.45±0.39%) compared with SC (15.07±0.25%) and LC (15.02±0.36%) (p= 0.0009). For Si, the LT (49.92±0.91%) group presented a significantly lower percentage compared with other groups (p=0.0164). No statistical differences were found regarding the other elements or the Ca/P ratio, which was slightly higher in the ST (4.89±0.22%) group.

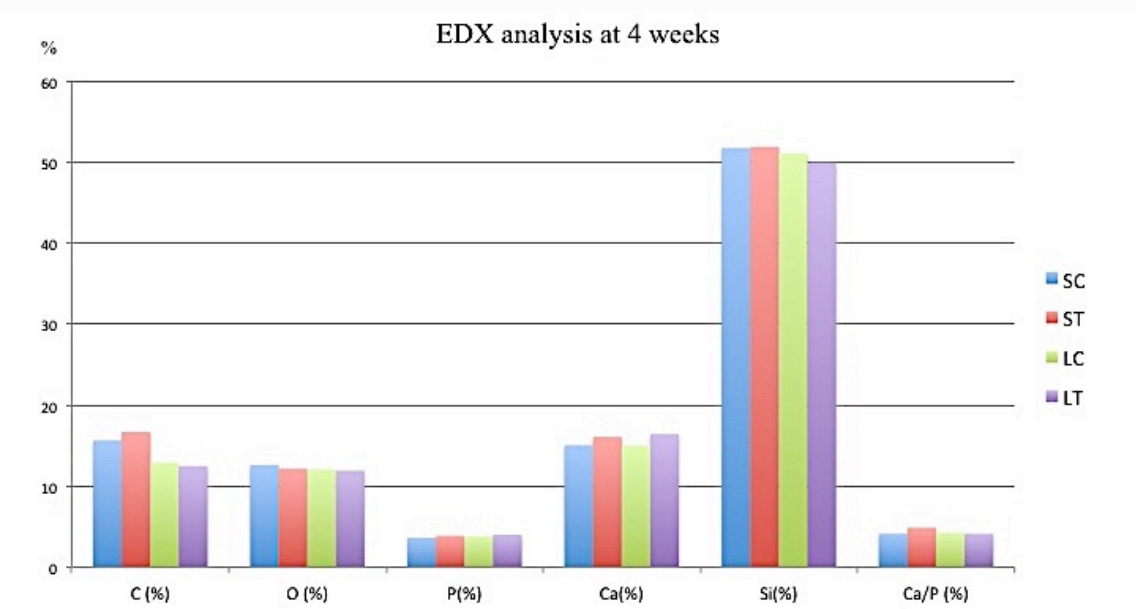

Fig. 18. EDX chemical elements analysis based on samples located inside the sockets for all groups after 4 weeks healing.

After eight weeks healing (Fig. 19), multiple comparisons showed significantly higher amounts of Ca in groups SC (16.89±0.35%) and ST (16.5±0.17%) compared with LC (15.17±0.42%) and LT (15.15±0.32%) (p=0.0004). Significantly more Si was also observed in SC (49.95±0.9%) and ST (49.99±0.39%), compared with the large defect groups LC (38.01±0.36%) and LT (38.37±0.23%) (p=0.0000). No statistically significant differences were found in C, O, or P elements. The Ca/P ratio was slightly higher for groups SC (4.11±0.71%) and ST (4.13±0.33%) (p>0.05).

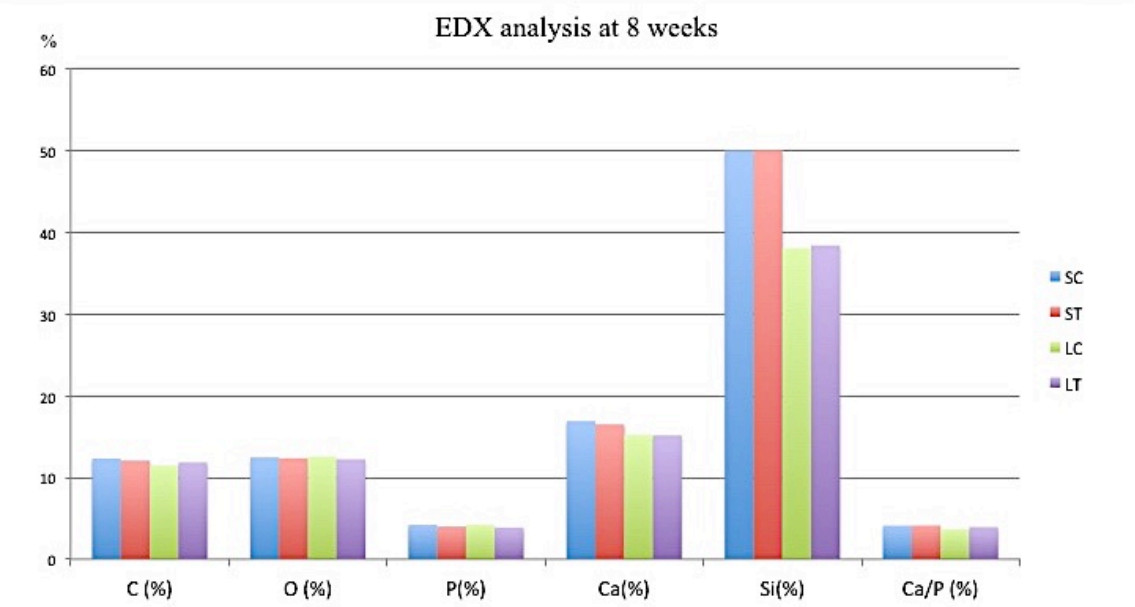

Fig. 19. EDX chemical elements analysis based on samples located inside the sockets for all groups after 8 weeks healing.

Paper III

The initial electronic search identified 154 studies. After screening abstracts and key words, 18 potentially relevant articles were selected (agreement between reviewers 88.67%; kappa = 0.65). After reading the complete manuscripts, nine studies were excluded due to inadequate study design, absence of a control group, or because the data reported was insufficient. The manual search and cross-referencing did not locate any further articles, so the final selection included nine articles (Fig.20).

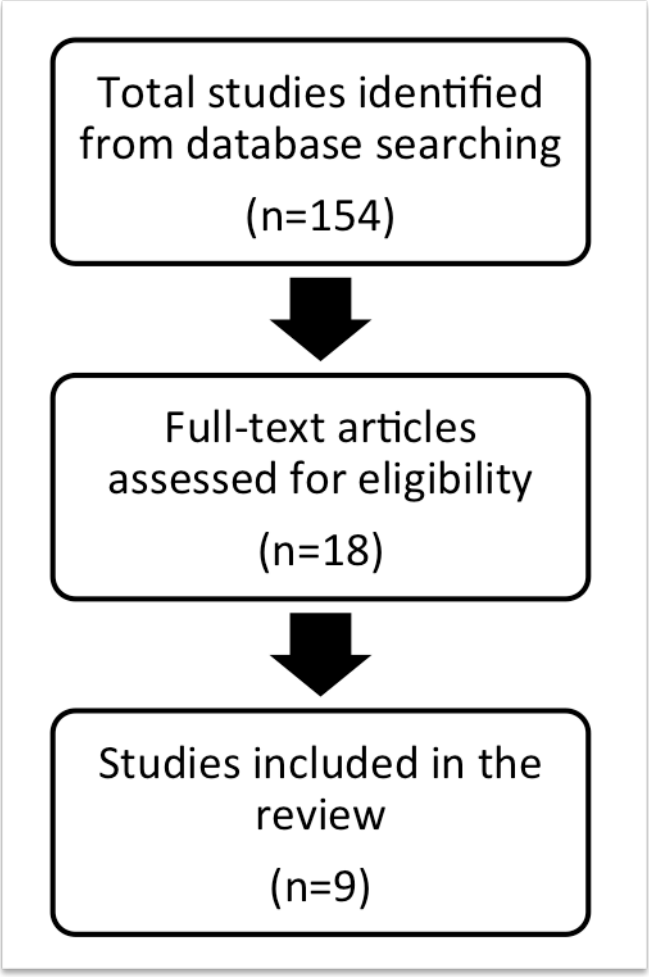

Fig. 20: Flow chart of studies included in the review.

Selected study characteristics

The articles that met the inclusion criteria detailed above were all animal research studies:

- Participants: the studies included involved a total of 94 rats, 8 sheep, 15 rabbits, 8 domestic pigs, and 8 Beagle dogs.
- Evaluation period: all studies had an evaluation period of at least of four weeks.
- Intervention: data from each article were analysed and information about the study type, animals, type of bisphosphonate applied, its dose, scenario, and outcomes were extracted (Table 5). In four out of the nine studies, the bisphosphonate used was alendronate (#1, #3, #6 and #7),

in four the bisphosphonate was pamidronate disodium (#4, #5, #8 and #9), and one study applied clodronate (#2). Different bone fillers were used: allografts (#1); autografts (#2, #3); xenografts; and alloplastic materials.

- Outcomes: the outcomes reported varied greatly.

Table 5. Summary of the articles included in this systematic review (authors, year of publication, type of article, type of bisphosphonate, dose, scenario and mean results).

#	Authors & Year	Type of Article	Sample	Bisphosphonate	Dose	Scenario	Results
#1	Aspenberg & Ästrand et al. 2002	Animal research	10 Sprague Dawley rats	Alendronate Fosamax®	1mg./ml.	Tibia	Lower resorption of the graft on bisphosphonate (BP) groups
#2	Jeppsson et al. 2003	Animal research	42 Sprague Dawley rats	Clodronate Bonefos®	60mg/ml.	Tibia	BP reduced bone graft resorption
#3	Tägil et al. 2004	Animal research	16 Sprague Dawley rats	Alendronate Fosamax®	1mg./ml.	Tibia	Alendronate improved new bone formation and increased bone density
#4	Houshmand et al. 2007	Animal research	8 Sheeps	Pamidronate Disodium Aredia ™	0,1mg./ml.	Mandible	Increased new bone formation on BP groups
#5	Choi et al. 2007	Animal research	15 New Zealand White Rabbits	Pamidronate Disodium	2 mg./ml. 3 mg./ml.	Frontal Bone	Pamidronate inhibited bone healing
#6	Srisubut et al. 2007	Animal research	26 Sprague Dawley rats	Alendronate Fosamax®	20mg/ml.	Mandible	Alendronate improved new bone formation
#7	Möller et al. 2014	Animal research	8 Domestic Pigs	Alendronate Fosamax®	1mg./ml.	Mandible	BP reduced bone block graft resorption
#8	Fischer et al. 2015	Animal research	2 American Fox Hound Dogs	Pamidronate Disodium Aredia ™	90mg/ml.	Mandible	BP delayed post-extraction socket healing and reduced dimensional changes
#9	Lozano-Carrascal et al. 2016	Animal research	6 Beagle Dogs	Pamidronate Disodium Aredia ™	9mg/ml.	Mandible	BP improved new bone formation and increase xenograft substitution

Quality assessment

Quality assessment of the studies analysed is shown in Figure 21. The estimated risk of bias was considered to be moderate in four cases (#1, #2, #5, and #9) and high in five (#3, #4, #6, #7, and #8). None of the studies were considered to have a high level of evidence with an estimated low risk of bias.

#	Authors & Year	Randomization	Blindness	Drop/Out	Validated Measurements	Statistical Analysis
#1	Aspenberg & Åstrand et al. 2002	🟢	🟢	🟠	🟢	🟢
#2	Jeppsson et al. 2003	🟢	🟢	🟠	🟢	🟢
#3	Tägil et al. 2004	🔴	🔴	🟢	🟢	🟢
#4	Houshmand et al. 2007	🟢	🔴	🟠	🟢	🟢
#5	Choi et al. 2007	🟢	🔴	🟢	🟢	🟢
#6	Srisubut et al. 2007	🔴	🔴	🟠	🟢	🟢
#7	Möller et al. 2014	🔴	🔴	🟠	🟢	🟢
#8	Fischer et al. 2015	🟢	🔴	🟠	🟢	🟢
#9	Lozano-Carrascal et al. 2016	🟢	🔴	🟢	🟢	🟢

🟢 Yes
🔴 No
🟠 Not reported

Fig. 21: Quality and potential risk of bias assessment of the included studies.

Individual study results

It was difficult to compare the findings between studies due to the heterogeneity of study designs, the lack of consistency in the methodologies used for data collection and analysis, and the lack of concurrence between outcome definitions. Details of each of the studies reviewed, the treatment performed, and the results obtained (Table 5) are as follows:

Aspenberg & Åstrand (70): this study evaluated the effect of the immersion of cancellous bone allografts in a bisphosphonate solution before implantation in a rat model, in a bone conduction chamber. In the experimental group, grafts were immersed in an alendronate solution (1 mg alendronate / 1 ml water) for 10 minutes, and then rinsed 3 times for 3 minutes in saline, to remove any unbound alendronate. In the control group, the grafts underwent the same treatment with saline only. In the control group chambers after 6 weeks healing,

the grafts were entirely resorbed; only 22% of the space was filled by newly formed bone. In alendronate-treated specimens, grafts seemed intact, and 70% of the space was filled by graft and newly formed bone. The authors concluded that local graft treatment with a bisphosphonate appears to be risk-free, and may prevent mechanical graft failure due to resorption.

Jeppsson et al. (71): in this study, 10 out of 42 rats received bilateral chambers containing bone grafts from the rats' proximal tibiae. On the experimental sides, the grafts were soaked in a clodronate solution (60 mg/ml) for 10 minutes. On the control sides, grafts were treated with saline. After 6 weeks healing, the bisphosphonate-treated side showed increased bone density and higher graft resistance.

Tägil et al. (72): the authors extracted pairs of frozen cylindrical osteochondral grafts from rats' patellar grooves; these were placed in chambers made in the proximal tibiae of 16 rats. One graft from each pair was submerged in an alendronate solution (1mg/ml) for 10 minutes. The other graft was immersed in water. After 6 weeks healing, histological examination found denser trabecular bone (42%) in alendronate-treated rats, versus 20% in untreated control samples. The authors concluded that the topical application of alendronate reduced the risk of collapse of osteochondral grafts during revascularization and bone remodeling.

Houshmand et al. (64): this study evaluated the capability of pamidronate disodium to enhance bone regeneration of bovine-derived hydroxyapatite placed in infrabony defects in eight sheep. Three defects were prepared: (negative-control group) unfilled; (positive-control group) filled with bovine-derived hydroxyapatite (Bio-Oss^{TM}) alone; (case group) bovine-derived hydroxyapatite (Bio-Oss^{TM}) mixed with pamidronate disodium (1 mg of pamidronate disodium was dissolved in 10 ml of sterile distilled water and mixed with 1 gr of bovine-derived hydroxyapatite). After 6 weeks healing, the cavities of the case group showed significantly higher amounts of bone formation, and

fewer osteoclasts and xenograft particles embedded in the regenerated bone. The authors concluded that adding pamidronate disodium to a demineralized bovine-derived hydroxyapatite improved the osteoconductive and regenerative capacity of the biomaterial.

Choi et al. (73): these authors mixed a high-dose topical application of pamidronate with L-lactide-co-glycolide (PLGA) as carrier material. The study included 15 rabbit calvarial bone defects. Four defect groups were created in each rabbit calvaria: (1) untreated bone defect; (2) PLGA only; (3) 2 mg of pamidronate with PLGA; and (4) 3 mg of pamidronate with PLGA. In radiographic analysis, radiopacity was lower in the pamidronate groups at 1, 2, 4, 6 and 8 weeks after surgery. In histological analysis, after 2-8 weeks healing, the amount of newly formed bone was lower in pamidronate groups, and signs of avascular necrosis were observed. The authors concluded that pamidronate inhibited bone healing, which the authors explained was due to the blocking of angiogenesis, and/or inhibition of the osteoclast activity necessary for bone healing.

Srisubut et al. (74): created 5 mm diameter bone defects in the mandible angle of 26 rats. In the experimental group, bioactive glass was mixed with an alendronate solution (20 mg alendronate / 1 ml saline) and placed in the defects; in the control group, the bioactive glass was soaked with physiological saline. Four weeks after surgery, no statistically significant differences were found in the number of osteoclasts or the lesion sizes between the two groups. The experimental group showed a significantly higher amount and percentage of new bone formation.

Moller et al. (65): experimented with topical applications of alendronate aqueous solution (1mg/ml) to prevent the surface resorption of onlay bone grafts in eight adult pigs: (1) in combination with a collagen membrane (Bio-Gide®); (2) mixed with bovine bone mineral (Bio-Oss®); (3) applied directly to autologous bone grafts. The same materials without bisphosphonates were used as controls on

the contralateral side. After 3 months healing, significantly lower loss of graft height was seen on the test side for Bio-Gide® + alendronate, Bio-Oss® + alendronate, and bone graft + alendronate versus Bio-Gide®, Bio-Oss® and bone graft alone, respectively. In five cases, necrosis of the overlaying periosteal tissues with alendronate was observed macroscopically. The authors concluded that the use of bisphosphonate-treated membranes or bovine bone mineral reduced bone graft resorption; however, the risk of periosteal necrosis demands better adaptation of the dose.

Fischer et al. (66): placed collagenated porcine bone substitute (Osteobiol Gen-Oss®; CPB) rehydrated with 90 mg/ml pamidronate (test), or with sterile saline (control) in post-extraction sockets in two American foxhound dogs. After 4 months healing, they observed limited amounts of bone at test sites. The combination appeared to delay extraction socket healing and to obstruct the resorption of the porcine bone substitute. In contrast, it seemed to reduce post-extraction dimensional changes in terms of horizontal bone width, as horizontal bone loss was nearly three times higher at control sites, compared with sites treated with pamidronate.

Lozano-Carrascal et al. (75): this study used six Fox-Hound dogs. Small (SD) and large defects (LD) were created in both quadrants of the lower jaw. Using a randomized design. The alveoli corresponding to the right hemi-mandible were used as controls (C) and were filled with MP3® porcine collagenated bone (OsteoBiol™) after rehydration with sterile saline. The left hemi-mandible defects were filled with MP3® prehydrated with pamidronate solution (9 mg/ml). After 4 and 8 weeks healing, histomorphometric analysis revealed greater new bone formation and lower residual graft particles for both SD and LD test groups, compared with SD and LD control groups, respectively. The authors concluded that porcine xenografts modified with pamidronate favour new bone formation and increased porcine xenograft substitution/replacement.

6. General Discussion

The biological effects of bisphosphonates are many and varied. Recent data drawn from *in vivo* and *in vitro* studies have demonstrated that they act not only by inhibiting bone resorption mediated by osteoclasts but also have the capacity to stimulate osteoblast differentiation and activity, and therefore enhance new bone formation (42,43). But these properties depend on the means of administration, concentration, and the active principle used (34).

Topical application of an amino-bisphosphonate solution to post-extraction sockets mixed with a bone graft would be appear to be a risk-free procedure, according to the present pilot study and to most of the articles analysed in the present review. With this means of administration, the bisphosphonates act on the early phases of bone healing and are mainly absorbed by the adjacent bone, so that only a small part of the total amount is released into circulation.

After 4 weeks healing, histomorphometric analysis of the test groups showed significantly more new bone formation in ST and LT groups compared with SC and LC respectively, and less residual graft material in ST and LT. The test sockets presented abundant lining cells around neoformed bone that included MP3® particles. Resorption of the biomaterial was evident; the xenograft had been substituted by trabecular bone, reducing the amount of residual graft material. Stimulation of cell production and differentiation in the cortical area accelerated osteoid matrix formation. This led to new bone formation and increased the length of cortical bone closure, demonstrated by higher percentages of Ca –indicative of the degree of bone mineralization– observed in EDX analysis. Moreover, SEM images revealed a bone density similar to the adjacent cortical bone.

After eight weeks healing, EDX analysis of Ca content revealed higher amounts of immature bone in large defect groups LC and LT. For small defects (groups SC and ST), Ca/P ratios were slightly higher, in accordance with

histomorphometric analysis, which revealed significantly greater new bone formation in ST sockets. In SEM description of control small and large defects (SC and LC), bone formation appeared to be similar to test groups ST and LT at four weeks. In both control and test groups, trabecular bone density was similar to the adjacent mineralized cortical bone, especially in the coronal third, and both groups achieved complete cortical closure. As with SEM observation, no histological differences could be observed between control and test groups at 8 weeks. Woven bone completely surrounded MP3®, and porcine bone was partially resorbed.

Corroborating the idea that bisphosphonates improve the regenerative capacity of biomaterials, *Houshmand et al. 2007* (64) demonstrated that adding a low dose of pamidronate disodium to a demineralized bovine-derived hydroxyapatite, improved the osteoconductive and regenerative capacity of the biomaterial. As in the present study, the authors did not observe differences in inflammatory responses. More recently, an experimental study performed by *Moller et al. 2014* (65) assayed alendronate aqueous solution (1mg/ml) applied topically to prevent the surface resorption of onlay bone grafts in eight adult pigs: (1) in combination with a collagen membrane (Bio-Gide®); (2) mixed with bovine bone mineral (Bio-Oss®); (3) or applied directly to autologous bone grafts. The same materials without bisphosphonates were used as controls on the contralateral sides. After 3 months healing, a significantly lower loss of graft height was seen on the test side for Bio-Gide® + alendronate, Bio-Oss® + alendronate, and bone graft + alendronate versus Bio-Gide®, Bio-Oss® and bone graft alone, respectively. The authors concluded that the topical pre-treatment of a graft with a bisphosphonate solution could prevent mechanical graft failure caused by resorption. Moreover, once the graft surface has been covered by newly formed bone, this seems to protect against bone resorption, increasing new bone formation and bone density (70,71).

Although most of the studies reviewed confirmed the positive effects of bisphosphonates on new bone formation, even at high doses (74), others

observed delayed bone healing and lower amounts of newly formed bone, with some signs of avascular necrosis. *Choi et al. 2007* (73) observed that a high topical application of pamidronate, mixed with L-lactide-co-glycolide (PLGA) as carrier material, inhibited bone healing in rabbit calvarial bone defects. This might be explained by: blocking of angiogenesis, and/or inhibition of osteoclast activity, which could be necessary for bone healing. *Fischer at al. 2015* (65), placed pamidronate adsorbed on a collagenated porcine bone substitute in post-extraction sockets in two American Foxhound dogs. They observed that this combination appeared to delay extraction socket healing and to obstruct the resorption of the porcine bone substitute, but also seemed to reduce post-extraction dimensional changes in terms of horizontal bone width, after four months healing. These results disagree with the present study, which revealed that low doses of pamidronate mixed with porcine-derived xenografts promote the calcification and formation of new bone with faster resorption and substitution of the biomaterial particles, especially during the earlier phases of socket healing. This discrepancy between results might be explained by methodological differences, especially in terms of the active principle, dosage, and follow-up duration.

Multiple experimental and clinical studies have monitored the behavior of bovine and porcine xenografts by means of histologic and histomorphometric analyses (76-79). Histology only supplies a description of the tissues surrounding the graft material, while histomorphometric analysis quantifies the percentage of new bone, residual graft and connective tissue, but may fail to disclose the graft's resorptive changes. In the present study, SEM and EDX analyses were carried out to complement the information already obtained from histological analysis. EDX analysis provides information of the chemical elements present in the biomaterial and surronding tissues, disclosing the graft's resorptive changes, and may reveal changes in the healing process (80,81).

Few studies have used EDX analysis as a tool for understanding a biomaterial's degradation process, and most of them have focused on bone response after

maxillary sinus floor elevation (81,82), or have used EDX to assess bone remodeling around titanium implants (83). In the present study, in the early phase (four weeks), significantly higher amounts of Ca were observed in the test groups, indicating a faster calcification process. Moreover, the Ca/P ratio was slightly higher for the ST group (4.89±0.22). The present study demostrated the effectiveness of EDX analysis for monitoring changes in the biomaterial's Ca/P ratio during the healing process.

Bisphosphonates have been shown to reduce post-extraction dimensional changes (65), to increase new bone formation (72,74,75), and to boost the action of biomaterials, stimulating bone regeneration (64,65,70,71). These outcomes have great clinical relevance in situations in which it is necessary to enhance new bone formation. But in spite of these positive observations, they should be treated with caution given the heterogeneity of the studies, with wide variations in methodology, surgical procedure, and/or healing periods.

The systematic review showed that the estimated risk of bias was considered to be moderate in four studies (#1, #2, #5 and #9) and high in five (#3, #4, #6, #7 and #8). None of the studies were considered to present the highest level of evidence and so a low estimated risk of bias. Although all the studies were performed with validated measurement and statistical analysis, only six articles were randomized. Two out of the six (#1 and #2) were randomized and blind, but failed to report any dropouts. Only one article (#9) explained the randomization method. Three studies (#3, #6 and #7) were not randomized. Only two studies (#4 and #5) were carried out with positive and negative control groups, the rest were performed with test and control groups. All the articles explained the type and dose of bisphosphonate used, but only one article (#5) (64) reported the amount of bone graft material mixed with bisphosphonate solution in detail.

No human studies were found in the literature search and there is a lack of information regarding the long-term longevity of regenerated defects. From the

results obtained it is impossible to determine which type of defect, surgical technique, type of bisphosphonate, dose, bone graft, or healing period provides positive outcomes in the long-term. Furthermore, there is little data regarding the possible influence of these treatments on the success/survival rates of implant therapies. In this context, it would be unwise to recommend any particular technique until more research has been published. Future studies must offer well-designed trials that are randomized and blinded, reproducible, with validated evaluation methods, and complete details of the materials and methods used.

7. Conclusions

Experimental Study

Within the limits of this animal experimental study, it may be concluded that:

- Porcine xenografts modified with pamidronate solution stimulated new bone formation and increased porcine xenograft replacement after 4 and 8 weeks healing.
- SEM description and EDX elemental microanalysis were shown to be useful techniques for assessing bone remodeling of small and large defects. Both techniques identified increased bone formation in test groups (pamidronate combined with collagenized porcine bone) after four and eight weeks healing.

Systematic Review

Despite the heterogeneity of methodologies and the high risk of bias among the animal research studies included in the review, the topical application of bisphosphonate solution would appear to:

- Reduce alveolar bone resorption and increase new bone formation in alveolar bone defects, alone or combined with a xenograft.

- Boost the regenerative capacities of biomaterials, favouring particle substitution, and increasing bone density.

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Paper I

Xenografts Supplemented with Pamindronate placed in post-extraction sockets to avoid crestal bone resorption. Experimental study in Fox hound dogs.

Clinical Oral Implants Research, 2016

CLINICAL ORAL IMPLANTS RESEARCH

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Key words: biomaterials, bisphosphonates, bone grafting, bone substitutes, pamindronate, socket

Abstract

Objectives: The aim of the study was to compare the effects of porcine xenografts (MP3®) with or without pamindronate for the healing of small and large defects of postextraction sockets.

Materials and methods: Six beagle dogs were used in the study; second premolars and first molars of the mandible were extracted, small defects (SD) and large defects (LD) were identified. Each defect was measured and randomly filled as follows: SC (small control defects filled with MP3® alone), ST (small test defects filled with MP3® modified with pamindronate), LC (large control defects filled with MP3® alone), LT (large test defects filled with MP3® modified with pamindronate). After 4 and 8 weeks, the animals were euthanized and the percentages of new bone formation (NB), residual graft (RG) and connective tissue (CT) were analysed by histology and histomorphometry of undecalcified samples.

Results: After 4 weeks, NB formation was higher for ST compared to all groups and for LT compared to LC ($P < 0.05$); RG was significantly higher in both control groups compared to tests ($P < 0.05$); and CT was higher in large defects (LC and LT) compared to small defects. After 8 weeks, NB formation was higher for test groups (ST and LT) compared to controls ($P < 0.05$); RG was significantly higher in both control groups compared to tests ($P < 0.05$); and CT was higher in large defects (LC and LT) compared to small defects ($P < 0.05$).

Conclusions: Within the limitations of this experimental study, the findings suggest that porcine xenografts modified with pamindronate favours the new bone formation and increased the porcine xenograft substitution/replacement after 4 and 8 weeks of healing.

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It has been demonstrated that the dimensions of alveolar ridges in the anterior maxilla may be reduced by 23% in the first 3 months after tooth extraction, and this percentage could be increased in an additional 11% in the following 5 years (Artzi et al. 2000). The socket healing processes after tooth extraction has been studied in different animal models (Amler et al. 1960; Cardaropoli et al. 2003) and is explained by the bone resorption–apposition, which results in the lost of bone volume in horizontal and vertical dimensions.

Approximately between 25 and 50% of the volume of the socket is reabsorbed during the first year, the resorption is faster during the first 3–8 weeks, is higher in the horizontal than in vertical, been more accentuated at the buccal plate bone compared to lingual due to the greater proportion of bundle bone present on lingual (Pietrokovski & Massler 1967; Wang & Al-Shammari 2002; Schropp et al. 2003; Araújo & Lindhe 2005); in a recent review, Tan et al. (2012) estimated in 29–63% the horizontal bone lost and 12–22% the vertical during the six-first months after the tooth extraction. To overcome the natural resorptive process of the bone after the tooth extraction, different techniques and methods have been evaluated: socket preservation techniques are able to reduce the bone loss compared to natural healing (Vittorini-Orgeas et al. 2013); moreover, immediate implant placement was considered, however, was demonstrated that the resorption and dimensions of the ridge continued even with

the immediate insertion of implants (Botticelli et al. 2004; Araújo et al. 2005, 2006).

In spite of the techniques of ridge preservation using a bone substitute and membranes, the clinical success of bone substitute materials depends on their ability to support primary bone formation, regenerate mature bone and their chemical and physical properties (Schwarz et al. 2007).

Allografts and xenografts are alternatives in terms of availability and well-demonstrated osseo-integrative and osteoconductive properties but have some disadvantages compared with autografts, the lack of osteogenic and osteoconductive properties (Scheller et al. 2009; Pérez-Sánchez et al. 2010). Two of the most frequently used biomaterials are derived from bovine and porcine bone. Bone substitutes of bovine origin (made up of natural bone mineral with extensive interconnected pores) (Kim & Kim 2008) are derived from trabecular bone; the porcine bone substitute is derived from both trabecular bone (80%) and cortical bone (20%) (Calvo-Guirado et al. 2010).

Another approach to avoid the bone loss after tooth extraction may be to inhibit osteoclast action and consequently the bone resorption which results of the bone remodelling. Bisphosphonates are a group of drugs that inhibit bone resorption, they act on osteoclasts inhibiting chemotaxis, cutting osteoclast lifespan, slowing their activity and inducing apoptosis. (Montoya-Carralero et al. 2010). It has been demonstrated in series of experimental studies that topical application of bisphosphonates such as alendronate can minimize the bone readsorption that occurs as a result of raising a mucoperiosteal flap (Yaffe et al. 1995, 1997, 2000, 2003).

Late, Houshmand et al. (2007) demonstrated that adding a bisphosphonate, such as pamindronate, to a demineralized bovine-derived hydroxyapatite improves the osteoconductive and regenerative capacity of the biomaterial. In contrast, recent findings by Moller et al. (2014) claim that pamindronate applied topically to the surface of bovine hydroxyapatite was able to prevent the surface resorption of onlay bone grafts. Thus, the capacity of topical bisphosphonates to avoid bone reasorption or their effects when are applied directly to a bone substitute (i.e. synthetic, xenograft) after a dental extraction still are unclear. Fischer et al. (2014) placed pamindronate adsorbed on a collagenated porcine bone substitute in particulate form and observed that this combination appeared to delay extraction socket healing, but may also reduce postextraction dimensional changes in terms of horizontal bone width and pamindronate appears to obstruct resorption of the porcine bone substitute.

For this reason, the aim of this study was to compare *in vivo* the effects of a porcine bone graft alone and modified with pamindronate to repair small and large defects in postextraction sockets, and to determine the percentage of new bone, residual graft and connective tissue, after 4 and 8 weeks of healing by histologic and histomorphometric analysis.

Material and methods

Six male beagle dogs of 1.5 ± 0.5 years of age and weighing 12–13 kg each one were used in the study. The experiment protocol was designed in accordance with the Spanish and European guidelines for animal experiments. The experiment was approved by the Ethic Committee for Animal Research of the University of Murcia (Spain), following the European Union Council Directive of February 1st 2013 (R.D.53/2013).

Surgical procedure

The animals were pre-anesthetized with acepromazine (0.12%–0.25 mg/kg), buprenorphine (0.01 mg/kg) and medetomidine (35 μg/kg). The mixture was injected intramuscularly in the femoral quadriceps. Then, an intravenous catheter was inserted (diameter 22 or 20 gauge) into the cephalic vein, and propofol was infused at the rate of 0.4 mg/kg/min at a slow constant infusion rate. Conventional dental infiltration anaesthesia (articaine 40 mg, 1% epinephrine) was administered at the surgical sites. These procedures were carried out under the supervision of a veterinary surgeon.

Teeth extraction and grafting procedures

In both quadrants of the lower jaws, second premolars (PM_2) and first molars (M_1) were used as experimental sites. The alveoli corresponding to PM_2 and PM_3 were qualified as small defects (SD) and M_1 as large defects (LD), respectively.

Teeth were sectioned with a carbide tungsten drill; the roots were removed with forceps, without damaging the remaining bony walls. Sulcular marginal incisions were made along the vestibular and lingual areas adjoining the alveoli, separating tissues to make crestal hard tissue walls visible (Fig. 1).

Prior to graft placement, the external dimensions of the postextraction sockets (diameter) were measured using a calliper and recorded. The extraction sockets mean alveolar ridge measurements were as follows: 3.8 ± 0.21 (P2), 4.0 ± 0.5 mm (P3), 4.1 ± 1 mm (P4), 5.6 ± 0.07 mm (M1).

Posteriorly with a randomized design (www.randomization.org), the alveoli (SD and LD) corresponding to the right hemi-mandible were used as a controls (C) and were filled with MP3® (OsteoBiol, Tecnoss Dental, Turin, Italy) porcine collagenated bone, after rehydration with sterile saline, meanwhile the left hemi-mandible defects (SD and LD) were filled with MP3® prehydrated with pamindronate (Novartis Pharma, Basel, Switzerland) test (T). Pamindronate solution was prepared by dissolving 90 mg pamidronic acid in 1 ml saline.

The dimension of a bone defect was considered critical more than 8 mm (Sohn et al. 2010; Borie et al. 2011; Pelegrine et al. 2014).

Summarizing, four treatment groups were created:

SC: small defects filled with MP3® alone.
ST: small defects filled with MP3® modified with pamindronate.
LC: large defects filled with MP3® alone.
LT: large defects filled with MP3® modified with pamindronate.

Membranes were not used.

Tissue flaps were repositioned without tension-free adaptation using interrupted and horizontal mattress sutures for wound closure, Monofil® 4/0 (Ancladén SL, Barcelona, Spain). During the first week after surgery, the animals were medicated with Amoxicyllin (500 mg twice daily) and Ibuprofen

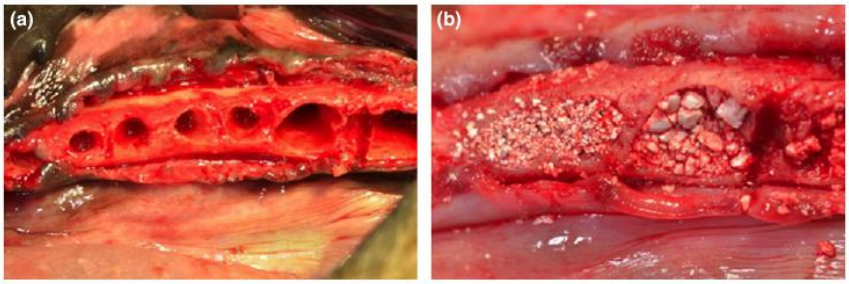


Fig. 1. Overview of dog's mandible: (a) small and large defects after teeth extraction, (b) small and large defects filled with MP3® with and without pamindronate.

(600 mg three times a day) administered systemically. Sutures were removed after 2 weeks. The dogs received a soft diet and a plaque control regimen that included tooth cleaning with the use of toothbrush and dentifrice, and administration of 0.2% chlorhexidine solution three times a week until the end of the experiment (4 and 8 weeks).

The animals were sacrificed at four (three animals) and 8 weeks (three animals) by means of an overdose of Pentothal Natrium® (Laboratorios Abbott, Chicago, IL, USA) perfused via the carotid arteries.

Sample processing

The soft tissues of each mandible were dissected to leave exposed the bone surfaces. Each mandible was block-sectioned and tissues fixed with 4% formalin. The samples were dehydrated in a graded ethanol series. The blocks were infiltrated with Technovit 7200® resin (Heraeus Kulzer, Hanau, Germany) and polymerized with ultraviolet light.

The polymerized blocks were then sectioned in buccolingual direction. Three slices were obtained per site and reduced by micro grinding and polishing using an Exakt grinding unit (Exakt, Norderstedt, Germany) to an even thickness of approximately 15–30 μm. The slides were stained with haematoxylin–eosin; the entire circumference of each section (containing bone, grafted particles and connective tissue) was traced manually to create individual regions of interest.

Histomorphometric analysis

The percentages of new bone, residual graft material and connective tissue were calculated in relation to the total measurement area (socket walls). The central portion of each core was selected to avoid any potential bias. In this way, both the coronal (remaining native host bone) and the apical portions were excluded from analysis (using a safe margin of 1.5–2 mm). Histomorphometric measurement of the samples was conducted using Image J software, developed by the NIH of the United States. Descriptive evaluation and morphometric measurements were performed under a Nikon Eclipse 80i microscope (Teknooptik AB, Huddinge, Sweden) equipped with the EasyImage 2000 system (Teknooptik AB) using ×1 to ×4 lenses (Fig. 2).

Statistical analysis

The Friedman test is the nonparametric repeated measures design for a sample or two-dimensional analysis of variance with one observation per cell equivalent. Friedman tests the null hypothesis that k-related variables come from the same population. For each case, the k variables are assigned the rank 1 to k. The statistic is based on these ranks. The significance was set as $P < 0.05$.

Fig. 2. Overview of a mesio-distal section of small defect. Each section was stained with hematoxylin–eosin. Original magnification ×16.

Results

In all experimental sites, healing was uneventful. After 4 and 8 weeks of healing, a keratinized mucosa was observed covering the edentulous zones without dehiscences or expositions of bone or graft particles. Histomorphometric and histological results of new bone formation, residual graft and connective tissue, after 4 and 8 weeks of healing, are describe below.

Histological description 4 weeks

Under optical microscopy, in both groups at 4 weeks of healing, the presence of a hard tissue bridge that sealed the coronal part of extraction socket was observed. The bridge was a continued small amount of trabecular bone with some areas of mature bone. This marginal bridge was mainly made of woven bone with areas of lamellar bone. Compared with the lingual region, the buccal soft tissue component exhibited a concave appearance with an invagination towards the extraction socket. Amounts of new bone formation could also be observed along the laterals walls of the sockets.

The apical portion of the sockets grafted with MP3®, harboured areas of newly formed bone while the central and marginal regions were occupied by granulation/provisional matrix that included a large number of MP3® particles. The newly formed ridges of woven bone were lined by osteoblasts and enclosed a primary spongiosa. Some of the MP3® particles were obviously engaged in the process of the novo bone formation, partially reabsorbed at 4 weeks (Fig. 3).

In the sockets with MP3® modified with pamindronate, there were abundant lining cells around neoformed bone that included MP3® particles. The resorption of the biomaterial was evident, the xenograft had been substituted by osseous trabecular, and in this way, the amount of residual graft was reduced (Fig. 1b). No inflammatory response of the sockets was observed. There were no signs of pamindronate particles at 4 weeks of healing (Graphs 1–4).

Histomorphometry 4 weeks

After 4 weeks of healing, the percentage of new bone formation was greater in ST (37.4 ± 0.41) >SC (34.8 ± 0.84) >LT (32.1 ± 0.22) >LC (18.3 ± 0.64), multiple comparison showed that ST was significantly higher compared to all groups ($P < 0.05$), and the LT was significantly lower compared to all the groups ($P < 0.05$).

The amount of residual graft was higher in SC (38.6 ± 0.28) >LC (26.5 ± 0.92) >ST (22.3 ± 0.76) > LT (17.8 ± 1.12), multiple comparison showed that SC group resulted in significant higher amounts of residual graft compared to the other groups ($P < 0.05$).

The percentages of connective tissue in large defects LC (55.2 ± 0.32) and LT (50.1 ± 0.14) were significantly higher compared to small defects ST (40.3 ± 0.12) and SC (26.6 ± 0.26) ($P < 0.05$) (Table 1).

Histological description 8 weeks

No histological differences could be observed between control and test groups at 8 weeks. The apical half of the extraction socket was occupied by newly formed bone this woven bone extended from the apical and lateral walls of the extraction site and surrounded completely MP3® particles in a phase of resorption and substitution. Porcine bone was partially resorbed at this period of time (Fig. 4) (Table 2).

Histomorphometry 8 weeks

At 8 weeks of healing, significant more new bone was also observed in test groups ST (52.6 ± 0.84) and LT (38.3 ± 0.81), compared to controls SC (41.7 ± 0.71) and LC (24.3 ± 0.35) ($P < 0.05$). The percentage of residual bone was significantly higher in control groups SC (35.2 ± 0.54) and LC (24.1 ± 0.84), compared to tests ST (18.7 ± 0.62) and LT (15.9 ± 0.45) ($P < 0.05$), finally the connective tissue was significantly higher in large defects LC (51.6 ± 0.64) and LT

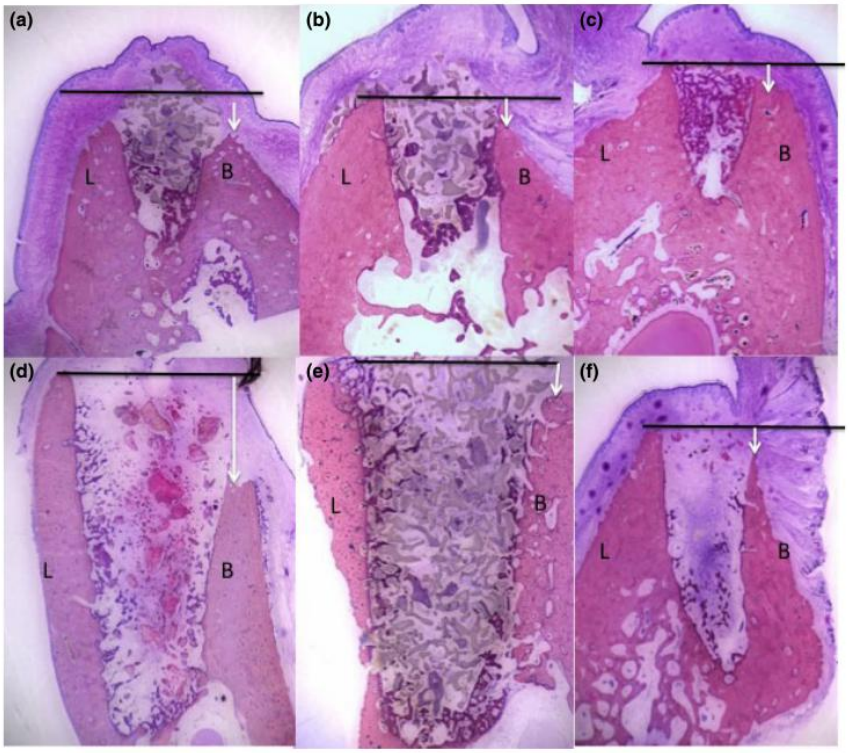
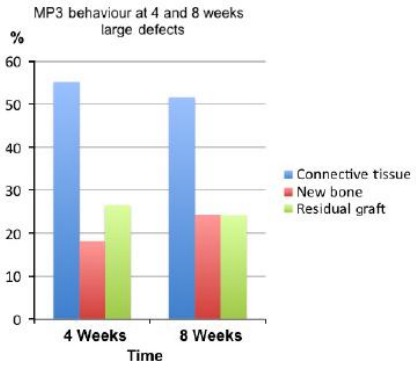
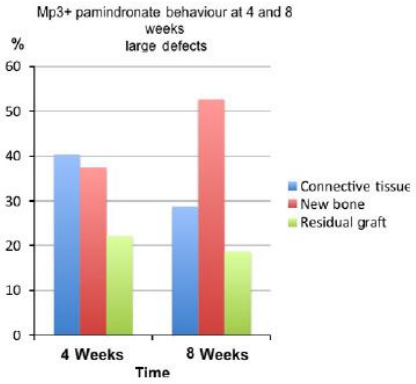


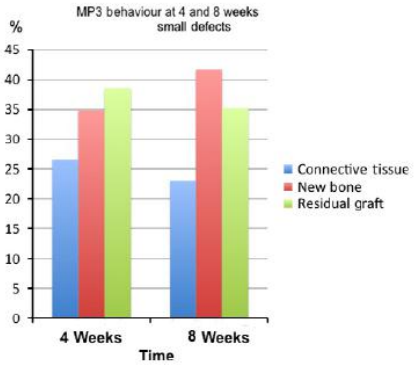
Fig. 3. Overview of a mesio-distal section representing 4 weeks of healing: (a) small defect filled with MP3, (b) MP3® with pamindronate particles in small defects, (c) control defects at 4 weeks with, (d) MP3® in large defects group, (e) MP3® with pamindronate, (f) the width of the ridge is well maintained, Hematoxylin–eosin stain; original magnification ×16.



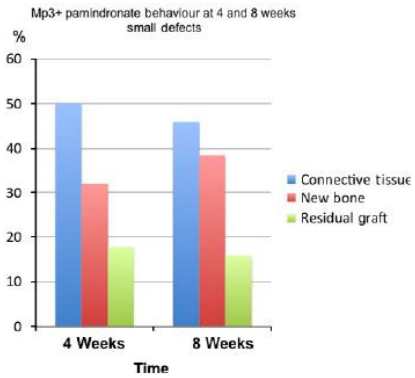
Graph 3. LC group behaviour at 4 and 8 weeks.



Graph 4. LT group behaviour at 4 and 8 weeks.



Graph 1. SC group behaviour at 4 and 8 weeks.



Graph 2. ST group behaviour at 4 and 8 weeks.

(45.8 ± 0.72) compared to small defects SC (23.1 ± 0.76) and ST (28.7 ± 0.41).

Discussion

The present study compares porcine xenografts alone vs. porcine xenografts modified with pamindronate to test the local effects of pamindronate on new bone formation, the xenograft resorption rate through the evaluation of the residual graft and the connective tissue.

The experiment demonstrated that the early healing of an extraction socket that had been grafted with porcine bone alone and modified pamindronate involved the formation of a coagulum that was replaced by provisional matrix in which woven bone was established. The histomorphometric results obtained in the present study are in the accordance with multiples studies; one of them is the research carried out by Barone et al. (2008), compared extraction alone and socket preservation with cortico-cancellous porcine bone and a collagen membrane. After 7 months of healing, the histologic analysis showed higher bone volume (35.5 ± 10.4%) in preserved sockets, statistically significant; the quantity of residual biomaterial was 29.2 ± 10.1 and 36.6 ± 12.6% for connective tissue in grafted sites. The results of the present work are in agreement with the study realized by Crespi et al. (2011), which compared sockets filled with magnesium-enriched hydroxyapatite (MHA), corticocancellous porcine bone (Tecnoss®) and unfilled sockets, the results of the study demonstrated a slightly higher vital bone for the group of sockets filled with porcine bone (38.0 ± 16.2%) at 4 months of healing.

Ramírez-Fernández et al. (2011) performed an experimental animal study in rabbits to compare corticocancellous porcine bone (Osteobiol® MP3) and bovine bone (Endobon®, RegenerOss®, Biomet 3i. Palm Beach Gardens, FL, USA). They observed greater new bone formation and less residual graft, statistically significant, with porcine at 2, 3 and 4 months of healing. They explain that Osteobiol® MP3 is an open porosity structure biomaterial similar to cancellous bone, this

Table 1. **New bone, residual graft and connective tissue percentages after 4 weeks healing. Data expressed as mean ± Standard Deviation and median. Nonparametric Friedman Test for multiple-related samples**

	New bone (%)		Residual graft (%)		Connective tissue (%)	
4 Weeks	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
SC[a]	34.8 ± 0.84	34.80	38.6 ± 0.28*	38.59	26.6 ± 0.26	26.56
ST[b]	37.4 ± 0.41	37.69	22.3 ± 0.76	22.32	40.3 ± 0.12	40.26
LC[c]	18.3 ± 0.64*	18.3	26.5 ± 0.92	26.54	55.2 ± 0.32*	55.20
LT[d]	32.1 ± 0.22	32.09	17.8 ± 1.12	17.76	50.1 ± 0.14	50.08
P value	0.001		0.005		0.031	

* is differences between values achieving statistical significance $P > 0.05$.

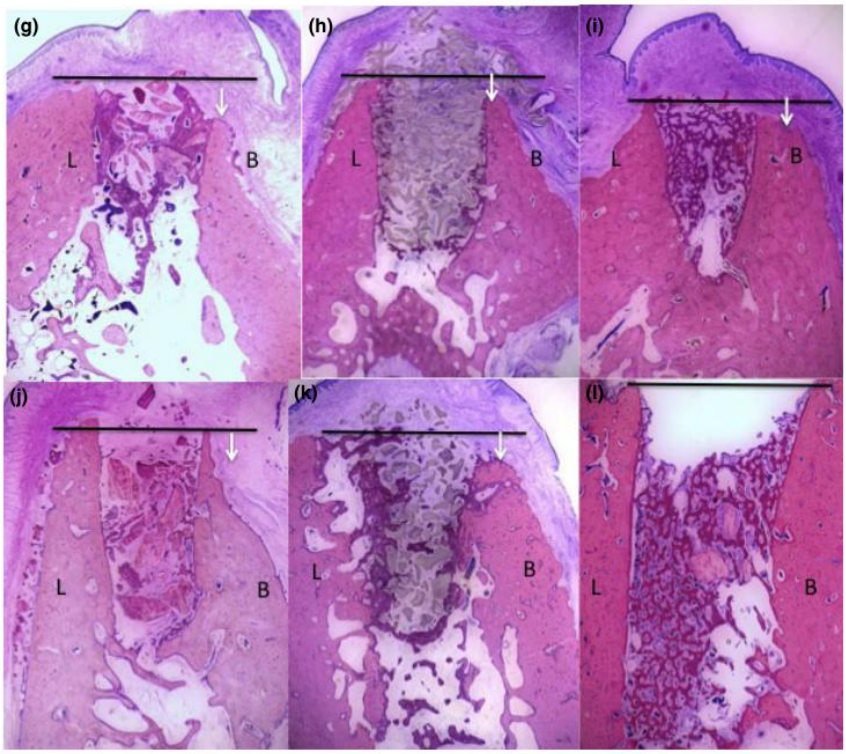


Fig. 4. Overview of a mesio-distal section representing 8 weeks of healing: (g) MP3® particles, (h) MP3® with pamindronate particles small defects, (i) control with woven bone in small defect, (j) MP3® particles large defects, (k) hard tissue formation, partly mineralized MP3® with pamindronate in large defects, (l) control in large defects. Hematoxylin–eosin stain; original magnification ×16.

Table 2. **New bone, Residual graft and Connective Tissue percentages after 4 weeks healing. Data expressed as mean ± standard deviation and median. Nonparametric Friedman Test for multiple-related samples**

	New bone (%)		Residual graft (%)		Connective tissue (%)	
8 Weeks	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median
SC[a]	41.7 ± 0.71	41.75	35.2 ± 0.54*	35.25	23.1 ± 0.76	22.85
ST[b]	52.6 ± 0.84*	52.57	18.7 ± 0.62	18.65	28.7 ± 0.41	28.70
LC[c]	24.3 ± 0.35	24.32	24.1 ± 0.84	24.10	51.6 ± 0.64*	51.59
LT[d]	38.3 ± 0.81	38.28	15.9 ± 0.45	15.94	45.8 ± 0.72	45.76
P value	0.030		0.017		0.002	

* is differences between values achieving statistical significance $P > 0.05$.

fact will promote the infiltration by bone tissue, bone marrow and blood vessels.

Slightly greater new bone formation and less amount of residual graft were shown in the test group of the present study, but we have to take care about the different observation time frames used in each study, and the reduced sample of the present study. Similar results could be found in the literature, with other types of xenografts. In the study carried out by Araújo & Lindhe 2011 in beagle dogs, it compares the dynamics of chips of autologous bone and Bio-Oss® Collagen (Geistlich Pharma AG, Wohlusen, Switzerland) bovine bone, after 3 months in the sites treated with Bio-Oss® Collagen found more residual graft (24.6 ± 3.7%), less mineralized bone (43.5%) and a substantial amount of remaining provisional connective tissue (14%). Araújo & Lindhe (2009) also made a comparative study of alveolar bone formation at sites grafted with Bio-Oss® Collagen vs. ungrafted alveoli. Bone formation was greater in alveoli grafted with the bovine bone material, achieving 62.5% of lamellar bone after 6 months, and only 15.4% of immature woven bone and 5% of residual graft. Bone formation would appear to be greater for Araújo and Lindhe's study, but of course a clear comparison is not possible given the different time frames used in each study.

Another study that assess the behaviour of Bio-Oss® Collagen in socket preservation was made by Heberer et al. (2008) in patients, found mean overall new bone formation of 28% at 6 weeks of healing, the percentage of residual particles was 11% and connective tissue fills the 54% of the grafted sites. In contrast with this results, the same author 3 years later (Heberer et al. 2011) found statistically significant lower bone formation in human alveoli grafted with Bio-Oss® Collagen in comparison with ungrafted alveoli (24.4% vs. 44.2%) after 12 weeks of healing. They justify the diversity of results with the idea of the healing of postextraction socket is dependent on yet unidentified individual factors, with contribute to individual healing patterns (Trombelli et al. 2008). Also, the Bio-Oss® collagen has demonstrated to be a good scaffold for bone formation during healing, but needs time for bone formation and delayed the healing of the preserved sockets (Jensen et al. 2006; Araújo & Lindhe 2009). And the study performed by Cardaropoli et al. (2012) did not found statistically significant differences in the mineralized fraction after 4 months of healing of sockets grafted with Bio-Oss® Collagen and ungrafted sockets (44.8 ± 11.45% vs. 43.82 ± 12.33%).

The present study demonstrated that the pamindronate promotes the new bone formation with a faster resorption and substitution of the biomaterial particles, in the earlier phases of the alveoli healing. So it is not unusual to found higher values of residual graft in the literature. Carmagnola et al. (2003) after 8 months of healing found 21.1%

of residual graft in sockets preserved with Bio-Oss®. Cardaropoli et al. (2005) after 3 months of healing in a dog model found 27.2% of residual particles of Bio-Oss® Collagen. Cardaropoli & Cardaropoli (2008), the volume of residual graft in sockets filled with corticocancellous porcine bone Osteobiol® Gen Os (Tecnoss®) was 24.5 ± 11.65%, after 4 months of healing. The volume of residual graft in all cases was significantly lower, than the limit (40%) set for obtaining successful implant placement (Zitzmann et al. 2001; Carmagnola et al. 2003).

If compare our results with similar studies performed with alloplastic grafts Araújo et al. (2010), grafted dog sockets with β-tricalcium phosphate after tooth extraction, observed a new bone formation of 37.7 ± 1.7% after 4 weeks, 90% was woven bone. According with this results, Calvo-Guirado et al. (2012) observed more new bone formation in critical size defects in rabbits filled with Ossceram®, biphasic β-tricalcium phosphate (Bredent Medical GmbH & Co. KG, Senden, Germany), than unfilled defects. Lindhe et al. (2013) using an alloplastic graft (BPCAP; α-TCP core coated with nanocrystalline biomimetic hydroxyapatite) embedded in porcine collagen to fill fresh extraction sockets in a dog model observed 38.4% of new bone formation and 21.1% of residual graft at 8 weeks. Crespi et al. (2009) demonstrated statistically significant more vital bone (45.0 ± 6,5% vs. 40.0 ± 2,7%) and less residual graft (13.9 ± 3.4% vs. 20.2 ± 3.2%) in human sockets preserved with calcium sulphate than in the sockets preserved with magnesium-enriched hydroxyapatite, after 3 months of healing. In agreement with these results, the study of Aimetti et al. (2009) found a percentage of vital bone was 58,8% with calcium sulphate at 3 months also. Serino et al. (2008) conducted a study to compare human alveolar sockets filled with a bioabsorbable polylactide-polyglycolide acid sponge with natural healing. After 3 months of healing, the mean percentage of mineralize bone was higher for polylactide–polyglycolide sponge (59.9%) than for natural healing (48.8%).

The different percentages reported in the literature, related to the new bone formation, amount of connective tissue in the alveoli or residual quantity of different biomaterials, is possible to be likely related to differences in studies designs, surgical procedures and/or healing periods.

Within the limitations of this experimental study, the findings suggest that porcine bone with and without pamindronate is a viable biomaterial to regenerate bone defects compared with lack of negative control (graft free sites).

Conclusions

Within the limits of this animal experiment, it may be concluded that implant macrogeometry of the alveoli does not have any influence related to the quantity and quality of bone formation. Furthermore, porcine xenografts modified with pamindronate solution (90 mg pamindronic acid) stimulate the new bone formation and increased the porcine xenograft replacement after 4 and 8 weeks of healing.

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Paper II

Scanning electron microscopy study of new bone formation following small and large defects preserved with xenografts supplemented with pamidronte – A pilot study in Foxhound dogs at 4 and 8 weeks.

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RESEARCH ARTICLE

Scanning electron microscopy study of new bone formation following small and large defects preserved with xenografts supplemented with pamidronate—A pilot study in Fox-Hound dogs at 4 and 8 weeks

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ABSTRACT

The aim of the present study was to evaluate the feasibility of SEM and EDX microanalysis on evaluating the effect of porcine xenografts (MP3®) supplemented with pamidronate during socket healing. Mandibular second premolars (P2) and first molars (M1) were extracted from six Beagle dogs. P2 were categorized as small defects (SD) and M1 as large defects (LD). Four random groups were created: SC (small control defects with MP3®), ST (small test defects MP3® + pamidronate), LC (large control defects with MP3®), and LT (large test defects MP3® + pamidronate). At four and eight weeks of healing the samples were evaluated fisically through scanning electron microscopy (SEM), and chemical element mapping was carried out by Energy dispersive X-ray spectroscopy (EDX). After four weeks of healing, SEM and EDX analysis revealed more mineralized bone in ST and LT groups compared with control groups ($p < 0.05$). After eight weeks, Ca/P ratios were slightly higher for small defects (groups SC and ST); in SEM description, in both control and test groups, trabecular bone density was similar to the adjacent mineralized cortical bone. Within the limitations of this experimental study, SEM description and EDX elemental microanalysis have demonstrated to be useful techniques to assess bone remodelling of small and large defects. Both techniques show increased bone formation in test groups (MP3® modified with pamidronate) after four and eight weeks of healing.

1. Introduction

After dental extraction, the socket healing process involves a series of events that results in the loss of bone volume in both horizontal and vertical dimensions (Amler et al., 1960; Cardaropoli et al., 2003). Various techniques and methods have been proposed to overcome the natural resorptive process (Pietrokovski and Massler, 1967; Schropp et al., 2003; Araújo and Lindhe, 2005), and thus reduce the bone loss that accompanies natural healing (Heinemann et al., 2012; Vignoletti et al., 2012; Vittorini-Orgeas et al., 2013).

The scientific evidence does not provide clear guidelines regarding the type of biomaterial. The clinical success of bone substitute materials depends on their ability to support primary bone formation, regenerate mature bone and their chemical and physical properties (Pérez-Sánchez et al., 2010; Heinemann et al., 2015). In recent years, research has focused on improving bone substitutes and implant surfaces to achieve faster and better osseointegration by morphologic or biochemical modification. Biochemical modifications consist of the application of biologic mediators into the biomaterial, to improve bone quality and quantity (Gredes et al., 2015; Salomó-Coll et al., 2015).

One approach to minimizing bone loss could be to inhibit osteoclast action and consequently bone resorption. Bisphospho-

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nates are a group of drugs frequently used for the treatment of various bone diseases, including osteoporosis, malignant hypercalcemia, multiple myeloma, or Paget's disease (Benford et al., 2001; Montoya-Carralero et al., 2010). Experimental studies have reported that bisphosphonates directly inhibit bone resorption, promoting apoptosis of mature osteoclasts and suppressing the osteoclasts' multinucleated cells during the osteoclast differentiation process (Rogers et al., 1996; Rogers, 2003; Roelofs et al., 2006; Nagaoka et al., 2015). Traditionally, bisphosphonates have been administrated intravenously and orally. Reddy et al. (1995) observed that systemic administration of bisphosphonates prevented alveolar bone destruction associated with periodontal disease in Beagle dogs.

At the same time, it has been shown that the topical application of a bisphosphonate can minimize bone resorption following mucoperiosteal flap surgery (Yaffe et al., 1995, 1997, 2000, 2003); inhibit the progression of alveolar bone resorption in peri-implantitis (Shibutani et al., 2001); improve the osseointegration around dental implants (Ganguli et al., 2002; Yoshinari et al., 2002; Kajiwara et al., 2005; Aspenberg, 2014); improve the osteoconductive and regenerative capacity of a biomaterial (Houshmand et al., 2007); prevent the surface resorption of onlay bone grafts (Moller et al., 2014); or reduce post-extraction dimensional changes (Fischer et al., 2015).

Scanning electron microscopy, SEM, used to examine bone-to-biomaterial interfaces was first reported by Jasty et al. (1989). SEM imaging technique has been used to examine the implant-to-bone interface in descriptive studies, fracture healing or to evaluate implant surface roughness (Franch et al., 1998; Ottani et al., 2002; Sul et al., 2005; Botzenhart et al., 2015). SEM observation has sufficient resolution to allow exploration of the different biological processes of tissue healing involved in socket preservation techniques. SEM technique also offers the interesting possibility of describe and identify morphological changes on the cellular components of newly formed bone (Wierzchos et al., 2008). EDX analysis provides information on the chemical elements present in the biomaterial and surrounding tissues; included qualitative and quantitative microanalysis (Lindgren et al., 2010; Ramírez-Fernández et al., 2012). This method allows to calculate Ca/P ratio —indicative of the degree of bone mineralization—. The socket healing process starts with the formation of a coagulum, that is progressively replaced by a provisional matrix, which, thanks to calcium and phosphate deposits, transforms into woven bone and finally, with tissue maturation, becomes lamellar bone and bone marrow. Depending on bone maturation different amounts of the elements will be found (Amler et al., 1960; Cardaropoli et al., 2003).

Given the potential benefits of bisphosphonates and the scientific evidence of pamidronate reducing alveolar bone resorption; the aim of the present study was to evaluate the feasibility of SEM and EDX microanalysis on evaluating the bone healing of small and large defects filled with a bisphosphonate (pamidronate) combined with collagenized porcine material, at four and eight weeks.

2. Material and methods

The study used six male Fox-Hound dogs of 1.5 ± 0.5 years of age and weighing 12–13 kg each. The study protocol was designed following Spanish and European guidelines (2007/526/CE) for animal experiments. The experiment was approved by the Ethics Committee for Animal Research of the University of Murcia (Spain). Following the European Union Council Directive of February 1st 2013, Royal Decree 53/2013 (BOE no. 34, s. I, p. 11370).

2.1. Surgical procedure

The animals were pre-anesthetized with acepromazine (0.12%–0.25 mg/kg), buprenorphine (0.01 mg/kg) and medetomidine (35 μg/kg). The mixture was injected intramuscularly in the femoral quadriceps. Then an intravenous catheter was inserted (diameter 22 or 20 gauge) into the cephalic vein, and propofol was infused at the rate of 0.4 mg/kg/min at a slow constant infusion rate. Conventional dental infiltration anaesthesia (articaine 40 mg, 1% epinephrine) was administered at the surgical sites. These procedures were carried out under the supervision of a veterinary surgeon.

2.2. Tooth extraction and grafting procedures

In both quadrants of the lower jaws, second premolars (P2) and first molars (M1) were used as experimental sites. The alveoli corresponding to P2 were qualified as small defects (SD) and M1 as large defects (LD).

Teeth were sectioned with a carbide tungsten drill; the roots were removed with forceps, without damaging the remaining bony walls. Sulcular marginal incisions were made along the vestibular and lingual areas adjoining the alveoli, separating tissues in order to make the crestal hard tissue walls visible (Fig. 1). Prior to graft placement, the external dimensions of the post extraction sockets were measured using a calliper and recorded. The extraction sockets mean bucco-lingual alveolar ridge measurements were as follows: 3.8 ± 0.21 for P2 and 5.6 ± 0.07 mm for M1.

Using a split-mouth design, the alveoli (SD and LD) corresponding to the right hemi-mandible were used as controls (C) and were filled with MP3® (OsteoBiol, Tecnoss Dental, Turin, Italy) porcine collagenated bone, after rehydration with sterile saline. Left hemi-mandible defects (SD and LD) were filled with MP3® prehydrated with pamidronate (Novartis Pharma, Basel, Switzerland) as test sites (T). Pamidronate solution was prepared by dissolving 90 mg pamidronic acid in 10 ml saline (9 mg/ml) and mixed with 2.0 cc (approximately 4 g) of 600–1000 μm particles of porcine bone.

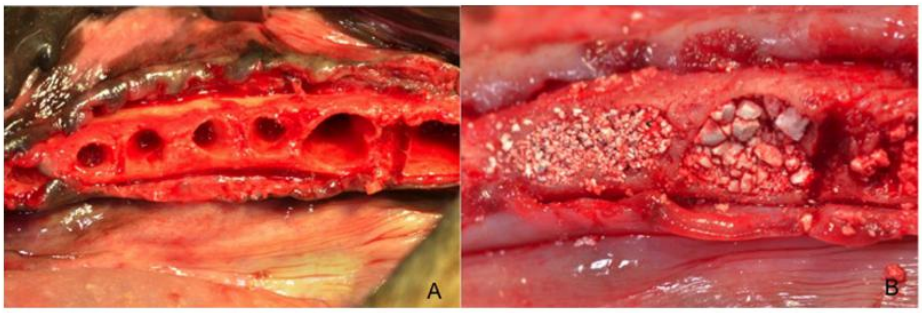


Fig. 1. Overview of a dogís mandible: (a) small and large defects after tooth extraction, (b) small and large defects filled with MP3® with and without pamidronate.

In this way, four treatment groups were created:
SC: small defects filled with MP3® alone.
ST: small defects filled with MP3® modified with pamidronate.
LC: large defects filled with MP3® alone.
LT: large defects filled with MP3® modified with pamidronate.
Membranes were not used.

Tissue flaps were repositioned without tension free adaptation using interrupted and horizontal mattress sutures for wound closure, Monofil® 4/0 (Ancladén SL, Barcelona, Spain). During the first week after surgery, the animals were medicated with amoxicillin (500 mg twice daily) and Ibuprofen (600 mg three times a day) administered systemically. Sutures were removed after two weeks. The dogs received a soft diet and a plaque control regime that included tooth cleaning with the use of toothbrush and dentifrice, and administration of 0.2% chlorhexidine solution three times a week until the end of the experiment (four weeks and eight weeks).

The animals were sacrificed at four (3 animals) and eight weeks (3 animals) by means of an overdose of Pentothal Natrium® (Laboratories Abbott, Chicago, IL, U.S.A.) perfused *via* the carotid arteries with a fixative containing 5% glutaraldehyde and 4% formaldehyde.

2.3. Sample processing

The soft tissues of each mandible were dissected to leave the bone surfaces exposed. Each mandible was block-sectioned and tissues fixed with 4% formalin. The samples were dehydrated in a graded ethanol series. The blocks were infiltrated with Technovit 7200® resin (Heraeus Kulzer, Hanau, Germany) and polymerized with ultraviolet light.

The polymerized blocks were then sectioned in buccolingual direction using a precision saw. Blocks containing the entire graft (bone, grafted particles and connective tissue) were divided into two halves: one for histomorphometric analysis and the other for SEM and EDX analysis.

2.4. Scanning electron microscopy

The surface structure of the bone graft was evaluated *in vitro* by SEM study and *in vivo* by histological analysis. For the SEM study, test and control bone grafts were put into liquid nitrogen for approximately 2 min and then split longitudinally, and the other halves were cut in the middle with a diamond-coated water-cooled band saw. The freshly formed and cleaned surfaces were coated with carbon film (BalTec CED 030; BalTec, Balzers, Liechtenstein) for analysis by SEM (JSM-6100 JEOL®, Tokyo, Japan) (Fig. 2).

2.5. EDX analysis

Samples were examined using Energy dispersive X-ray spectroscopy (EDX) at a working distance of 19 mm, an acceleration voltage of 15 kV and 15× magnification, with an Oxford Instruments INCA 300 EDX System (Oxfordshire, UK), evaluating the element composition of the graft material and bone in the medullar area. Regions of interest were delimited by the inner cortical walls and reached into the medullar core. Element mapping was performed in order to identify and quantify all chemical elements and observe the chemical degradation process and changes in the tissues, using point analysis and elemental mapping to determinate mineral distribution. EDX spectra were collected at discrete points in each biopsy. Elemental composition (atomic%) of the graft material and bone were calculated from the spectra.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 21.0 (SPSS Inc., IBM Corporation, Chicago, IL, USA) statistical software. Data obtained were expressed as mean ± standard deviation (SD). The mean differences between the groups were analysed using the Friedman test (non-parametric repeated measures analysis of variance) considering that the data from one dog are dependent. Significance was set as $p < 0.05$. In cases of statistically significant differences, the Bonferroni method for multiple comparisons was applied.

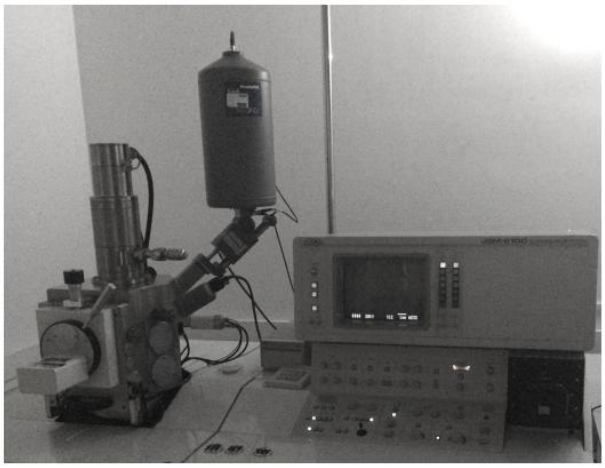


Fig. 2. Photograph of the scanning electron microscope (SEM) used in the study (JSM-6100 JEOL®).

3. Results

3.1. SEM description

3.1.1. ST and LT groups (four weeks)

SEM analysis revealed the presence of a hard tissue bridge that sealed the coronal part of the extraction socket. While the most coronal third of the socket was occupied by immature bone with pores, in the central third a bone with a density similar to adjacent cortical bone was observed, due to more active resorption of the biomaterial that had been substituted by osseous trabeculae. In the apical third, a lack of new bone formation was observed in large defects, due to deficient xenograft compaction during the filling procedure (Fig. 3). MP3 particles could be distinguished by their wither and denser appearance; resorption of the biomaterial was evident, with abundant osteocytes involved in new bone formation (Fig. 4).

3.1.2. SC and LC groups (four weeks)

SEM images monitored the graft incorporation process; at four weeks, spaces between the graft material and new bone were observed in both small and large defects. Cortical closure was incomplete. Bone marrow was organized with slight cortical formation, and immature bone with less density than the adjacent cortical bone (Fig. 5).

3.1.3. ST and LT groups (eight weeks)

After eight weeks healing, sockets filled with MP3® modified with pamidronate showed internal bone formation inside the bone marrow and complete cortical closure. Mineralized bone of a similar density to the adjacent cortical bone was observed in the coronal third (Figs. 6 and 7).

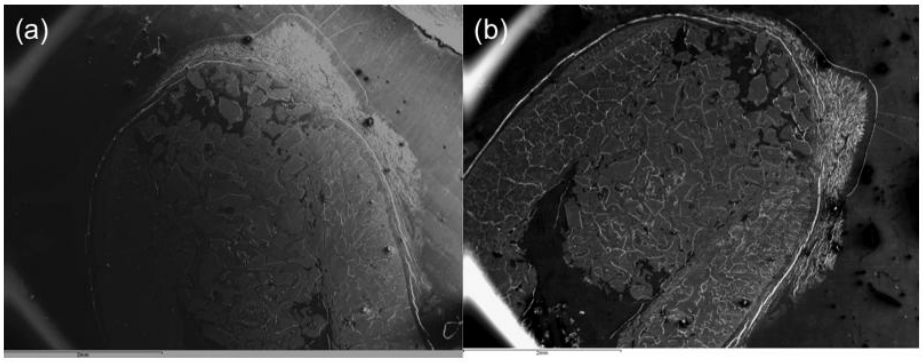


Fig. 3. SEM image of sockets filled with MP3® modified with pamidronate after 4 weeks of healing: (a) ST (small defects test), (b) LT (large defects test).

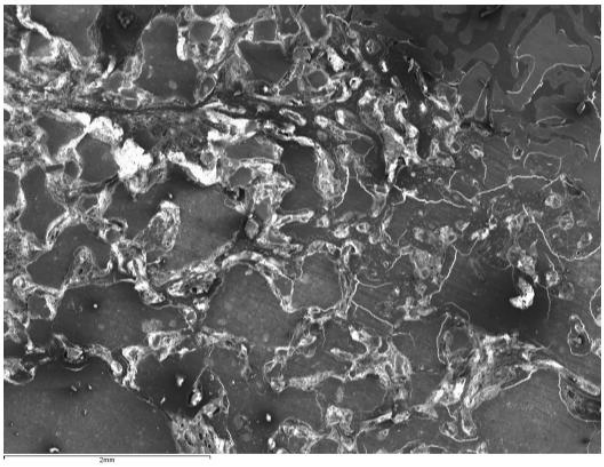


Fig. 4. SEM image of bone formation inside a socket filled with MP3® modified with pamidronate, at 4 weeks.

3.1.4. SC and LC groups (eight weeks)

In SEM analysis of small and large control defects filled with MP3® alone, bone appeared to be similar to that described for test sockets filled with MP3® modified with pamidronate at four weeks. Trabecular bone density was similar to adjacent mineralized cortical bone, especially in the coronal third; complete cortical closure was observed (Fig. 8).

No osseous malformations or structural changes in bone development were observed over the study period.

3.2. EDX analysis

EDX analysis evaluated several chemical elements: carbon (C), oxygen (O), phosphorous (P), calcium (Ca), and silicon (Si). The Ca/P ratio (Ca/P) was also calculated. Mean and standard deviation (SD) for all groups at four and eight weeks (based on samples located inside the length of cortical bone formation) are expressed in Tables 1 and 2respectively.

After four weeks healing (Graph 1), multiple comparisons showed significantly higher percentages of C in SC (15.67 ± 0.33%) and ST (16.72 ± 1.6%) compared with LC (12.91 ± 0.25%) and LT (12.84 ± 0.67%) ($p = 0.0002$). As for Ca measurement, multiple comparisons revealed significantly higher amounts of Ca in ST (16.1 ± 0.35%) and LT (16.45 ± 0.39%) compared with SC (15.07 ± 0.25%) and LC (15.02 ± 0.36%) ($p = 0.0009$). For Si, the LT (49.92 ± 0.91%) group presented a significantly lower percentage compared with other groups ($p = 0.0164$). No statistical differences were found regarding the other elements or the Ca/P ratio, which was slightly higher in the ST (4.89 ± 0.22%) group.

After eight weeks healing (Graph 2), multiple comparisons showed significantly higher amounts of Ca in groups SC (16.89 ± 0.35%) and ST (16.5 ± 0.17%) compared with LC (15.17 ± 0.42%) and LT (15.15 ± 0.32%) ($p = 0.0004$). Significantly more Si was also observed in SC (49.95 ± 0.9%) and ST (49.99 ± 0.39%), compared with the large defect groups LC (38.01 ± 0.36%) and LT (38.37 ± 0.23%) ($p = 0.0000$). No statistically significant differences were found in C, O, or P elements. The Ca/P ratio was slightly higher for groups SC (4.11 ± 0.71%) and ST (4.13 ± 0.33%) ($p > 0.05$).

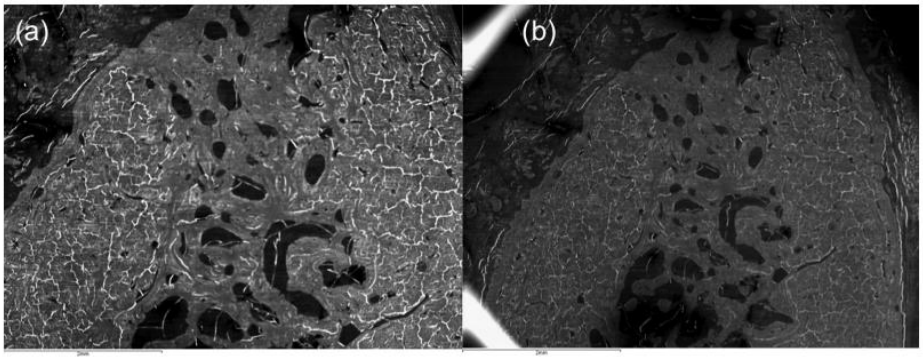


Fig. 5. SEM image of sockets filled with MP3® alone after 4 weeks of healing: (a) SC (small defects control), (b) LC (large defects control).

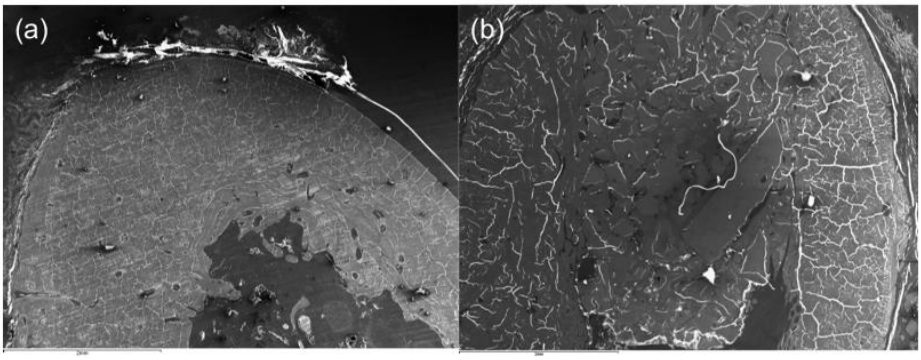


Fig. 6. SEM image of sockets filled with MP3® modified with pamidronate after 8 weeks of healing: (a) ST (small defects test), (b) LT (large defects test).

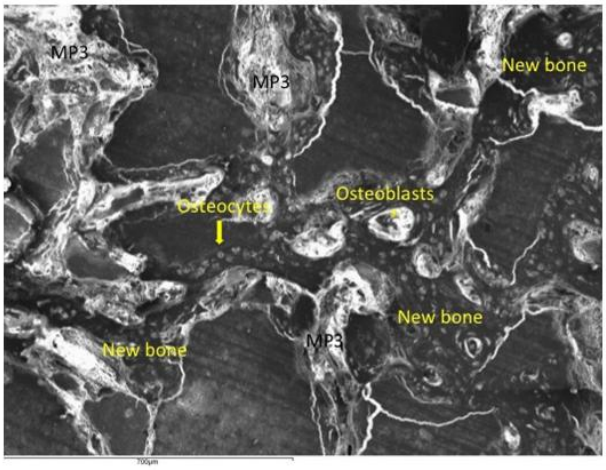


Fig. 7. SEM image of bone formation inside a socket filled with MP3® modified with pamidronate, at 8 weeks.

4. Discussion

The aim of the present pilot study has been to evaluate the feasibility of SEM and EDX microanalysis on evaluating the effectiveness of local applications of pamidronate mixed with MP3®, for filling small and large defects in post-extraction sockets. In addition, comparisons were made between the present histological and histomorphometrical results and those obtained in a previous study by the present team (Lozano-Carrascal et al., 2016), in order to determine whether a topically used pamidronate could act as an accelerant of bone healing after dental extraction.

Multiple experimental and clinical studies have monitored the behaviour of bovine and porcine xenografts by means of histologic and histomorphometric analyses (Carmagnola et al., 2003; Barone et al., 2008; Dominiak et al., 2012; Calvo-Guirado et al., 2015). SEM and EDX analysis were carried out to complement the information already obtained from histological analysis. EDX analysis provides information on the chemical elements present in the biomaterial and surrounding tissues, discloses the graft's resorptive changes, and may reveal changes in healing process. Although these techniques had some limitations, they do not provide an *in vivo* description of the tissues surrounding the graft material, neither quantifying the percentage of new bone, residual graft nor connective tissue (Lindgren et al., 2010; Ramírez-Fernández et al., 2012).

After 4 weeks of healing, the histomorphometric analysis demonstrated a statistically significant increase in new bone formation, in ST and LT groups, compared with SC and LC respectively; and less residual graft in ST and LT. In the test sockets, there were abundant lining cells around newly formed bone which included MP3® particles. The resorption of the biomaterial was evident, the xenograft had been substituted by osseous trabecular tissue and, in this way, the amount of residual graft had been reduced. The stimulation of the production and differentiation of cells in the cortical area, which accelerated osteoid matrix formation, led to new bone formation and increased the length of cortical bone closure; demonstrated by higher percentages of Ca —indicative of the degree of bone mineralization— obtained from de EDX analysis. Moreover SEM images revealed bone density similar to adjacent cortical bone.

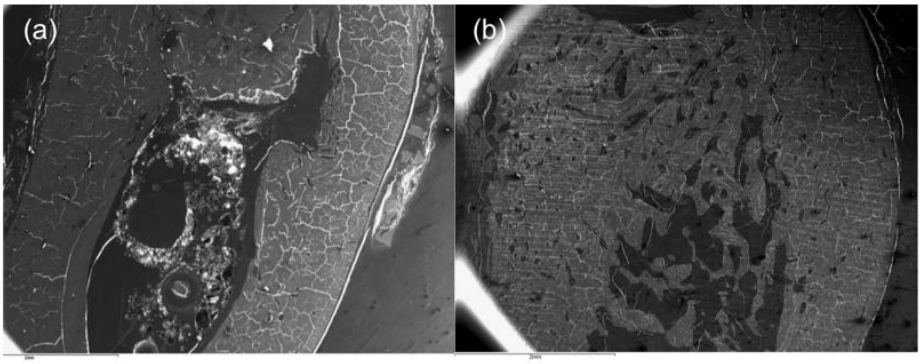


Fig. 8. SEM image of sockets filled with MP3® alone after 8 weeks of healing: (a) SC (small defects control), (b) LC (large defects control).

Table 1
EDX chemical elements analysis after 4 weeks healing. Data expressed as percentages ± standard deviation. Nonparametric Friedman test for multiple related samples.

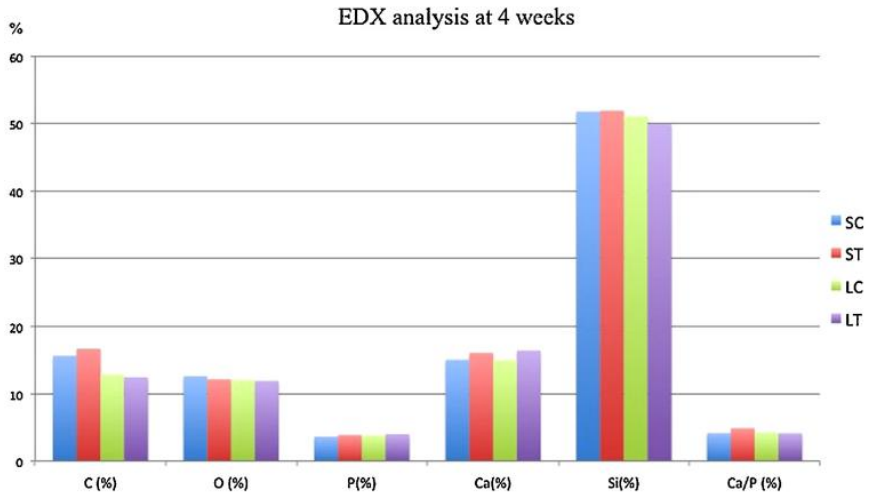
4 Weeks	C (%)	O (%)	P (%)	Ca (%)	Si (%)	Ca/P (%)
SC[a]	15.67 ± 0.33[c,d]	12.63 ± 0.20	3.64 ± 0.15	15.07 ± 0.25	51.81 ± 0.65	4.15 ± 0.23
ST[b]	16.72 ± 1.6[c,d]	12.17 ± 0.48	3.89 ± 0.15	16.1 ± 0.35[a,c]	51.95 ± 0.60	4.89 ± 0.22
LC[c]	12.91 ± 0.25	12.09 ± 0.38	3.83 ± 0.65	15.02 ± 0.36	51.11 ± 0.72	4.27 ± 0.67
LT[d]	12.48 ± 0.67	11.93 ± 0.46	4.01 ± 0.29	16.45 ± 0.39[a,c]	49.92 ± 0.91[a,b,c]	4.13 ± 0.39
p value	0.0002	0.0856	0.5880	0.0009	0.0164	0.6608

The level of significance was set at $p < 0.05$.

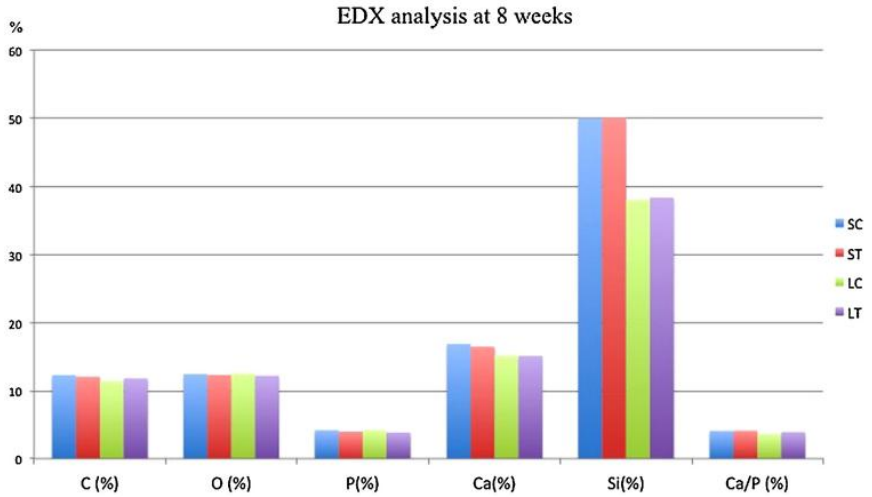
Table 2
EDX chemical elements analysis after 8 weeks healing. Data expressed as percentages ± standard deviation. Nonparametric Friedman test for multiple related samples.

8 Weeks	C (%)	O (%)	P (%)	Ca (%)	Si (%)	Ca/P (%)
SC[a]	12.30 ± 0.47	12.47 ± 0.22	4.2 ± 0.71	16.89 ± 0.35[c,d]	49.95 ± 0.9[c,d]	4.11 ± 0.71
ST[b]	12.07 ± 0.58	12.35 ± 0.38	4.01 ± 0.29	16.5 ± 0.17[c,d]	49.99 ± 0.39[c,d]	4.13 ± 0.33
LC[c]	11.41 ± 1.11	12.54 ± 0.49	4.2 ± 0.61	15.17 ± 0.42	38.01 ± 0.36	3.67 ±0.57
LT[d]	11.85 ± 0.41	12.22 ± 0.38	3.88 ±0.38	15.15 ± 0.32	38.37 ± 0.23	3.94 ± 0.44
p value	0.2875	0.7281	0.8358	0.0004	0.0000	0.6805

The level of significance was set at $p < 0.05$.



Graph 1. EDX chemical elements analysis based on samples located inside the sockets for all groups at 4 weeks of healing.



Graph 2. EDX chemical elements analysis based on samples located inside the sockets for all groups at 8 weeks of healing.

After eight weeks of healing, EDX analysis of Ca revealed higher amounts of immature bone in large defect groups LC and LT. For small defects (groups SC and ST), Ca/P ratios were slightly higher, in accordance with histomorphometric analysis, where a statistically significant greater amount of new bone formation was observed in ST sockets. In SEM description of control small and large defects (SC and LC), bone formation appeared to be similar to the test socket groups ST and LT at eight weeks. In both control and test groups, trabecular bone density was similar to the adjacent mineralized cortical bone, especially in the coronal third, and both groups achieved complete cortical closure. As in the SEM, no histological differences could be observed between control and test groups at 8 weeks. The woven tissue completely surrounded MP3® particles in the phase of resorption and substitution, and porcine bone was partially resorbed at this period in time.

Few studies have used EDX analysis as a tool for understanding a biomaterial's degradation process, and most of them have focused on bone response after maxillary sinus floor elevation (Slater et al., 2008; Ramírez-Fernández et al., 2012), or were used to assess bone remodelling around titanium implants (Haga et al., 2009). A study conducted by Calvo-Guirado et al. (2012), to investigate critical size defects in rabbits filled with Ossceram®. Ca/P mapping revealed increased Ca and p values at a 60-day final study period, finding more mineralized bone than in control samples. They also observed that Ca and P ions promoted new bone growth with trabecular formation within the medullar area.

In the present study, in the early phase (four weeks), significantly higher amounts of Ca were observed in the test group, indicating a more rapid calcification process, and the Ca/P ratio was slightly higher for the ST group (4.89 ± 0.22). The present study demonstrated the effectiveness of the EDX analysis to examine changes in the Ca/P ratio of the biomaterial during the healing process. On the other hand, the present study demonstrated that pamidronate promotes the calcification and formation of new bone with faster resorption and substitution of the biomaterial particles, especially during the earlier phases of socket healing. However these results have to be interpreted with caution due to the limitations of the study. The animal model studied, the limited sample size and study duration might have influenced the results; more well-designed studies are needed to confirm our results.

5. Conclusions

Within the limitations of this experimental study, SEM description and EDX elemental microanalysis have been demonstrated to be useful techniques in assessing bone remodelling of small and large defects. Both techniques have shown increased bone formation in test groups (pamidronate combined with collagenized porcine bone) after four and eight weeks of healing.

Ethical approval

Approved by the Ethics Committee for Animal Research of the University of Murcia (Spain), following the European Union Council Directive of February 1st 2013 (R.D.53/2013).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aanat.2016.09.009

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Paper III

Do topical applications of bisphosphonates improve bone formation in oral implantology? A systematic review.

Medicina Oral Patología Oral Cirugía Oral, 2017

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Dr. Oscar Salomo-Coll
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Dear Dr,

Thank you for submitting your article for our consideration.

Your above referenced article with the following authors: Oscar Salomo-Coll,Naroa Lozano-Carrascal,Sergio Alexandre Gehrke,Jordi Gargallo-Albiol,Federico Hernández-Alfaro,Jose Luis Calvo-Guirado , has been evaluated by the reviewers. We are happy to inform you that they have recommended accepting the manuscript for publication in Medicina Oral Patologia Oral Cirugia Bucal.

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Editor Med Oral Patol Oral Cir Bucal
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12. Annexes

1. Resumen

Esta tesis doctoral es un compendio de tres artículos, sobre nuestro trabajo en el campo de la aplicación tópica de bifosfonatos, solos o mezclados con un injerto óseo, en defectos óseos alveolares, con el objetivo de evaluar la capacidad de los mismos para preservar/potenciar la neo-formación ósea.

En los últimos años, la investigación sobre substitutos óseos se ha centrado en conseguir regeneraciones óseas mejorados y más rápidos, mediante la modificación morfológica y bioquímica de los mismos. Los bifosfonatos son un grupo de fármacos que reducen la reabsorción ósea inhibiendo la formación, reclutamiento y actividad de los osteoclastos maduros; además de promoviendo su apoptosis. Adicionalmente algunos tipos de bifosfonatos son capaces de potenciar la diferenciación y actividad de los osteoblastos. Por lo cual, se ha demostrado que una aplicación única y tópica de determinados bifosfonatos, puede minimizar la reabsorción ósea alveolar después de cirugías con elevación de colgajos a espesor total o en procesos de peri-implantitis; mejorar las propiedades osteoconductivas y regenerativas de biomateriales; reducir la reabsorción de los injertos óseos autólogos en bloque; o reducir los cambios dimensionales de alveolos post-extracción.

Para realizar el estudio experimental se utilizaron seis perros machos de raza Foxhound, a los que se les extrajo los segundos premolar (P2) y primeros molares (M1) mandibulares. Los alveolos P2 fueron asignados como defectos pequeños (SD) y los M1 como defectos grandes (LD). Cuatro grupos randomizados fueron creados: SC (defectos pequeños control rellenados con MP3® xenoinjerto porcino), ST (defectos pequeños test rellenados con MP3® + pamidronato), LC (defectos grandes control rellenados con MP3®), and LT (defectos grandes test rellenados con MP3® + pamidronato). Tras cuatro y ocho semanas de cicatrización los perros fueron sacrificados y se obtuvieron

mediante análisis histológico e histomorfométrico los porcentajes de neo-formación ósea (NB), partículas residuales (RG) y de tejido conectivo (CT). Con el fin de realizar un análisis más exhaustivo de las muestras y entender mejor el proceso de cicatrización y degradación de los biomateriales, las muestras fueron analizadas mediante microscopio electrónico de barrido (SEM), y los elementos químicos presentes en el biomaterial y tejido circundante extraídos mediante espectroscopia de rayos X de energía dispersa (EDX).

Para una mayor comprensión la aplicación tópica de los bifosfonatos en implantología, se realizó una revisión sistemática de la literatura científica publicada al respecto entre Enero del 2000 y Diciembre del 2016. Se buscaron artículos que evaluasen *en vivo* los efectos de la aplicación tópica de bifosfonatos en la regeneración/preservación ósea de defectos óseos alveolares. Se identificaron un total de 154 resúmenes, 18 artículos potencialmente interesantes fueron seleccionados, para finalmente incluir nueve en la revisión.

El análisis histológico e histomorfométrico del estudio experimental demostró tras 4 y 8 semanas de cicatrización, una mayor neo-formación ósea para los grupos test (ST y LT) tratados de forma tópica con pamidronato, en comparación con los grupos SC y LC, respectivamente; el porcentaje de partículas residuales fue significativamente mayor en ambos grupos control (SC y LC) en comparación con los test (ST y LT); y la cantidad de tejido conectivo fue mayo en los defectos de mayor tamaño (LC y LT). El SEM reveló mayor cantidad de tejido óseo mineralizado en los grupos test (ST y LT) en comparación con los controles, lo cual quedó demostrado mediante el mayor porcentaje de Ca obtenido en el análisis de espectroscopia EDX.

Pese a las limitaciones del estudio experimental, podemos sugerir que los xenoinjertos porcinos (MP3®) modificados con pamidronato tópico favorecen la neo-formación ósea y la sustitución de las partículas de xenoinjerto, tras 4 y 8 semanas de curación. Estas conclusiones están en conformidad con las obtenidas de la revisión sistemática, a pesar de la difícil comparación de los

resultados, debido a la heterogeneidad en los procedimientos. Podemos concluir que la aplicación de soluciones tópicas de bifosfonatos parece favorecer la neo-formación ósea en defectos alveolares, potenciar la capacidad regenerativa de diferentes biomateriales, y por consiguiente aumentar la densidad ósea alveolar.

2. Acceptance Ph.D. Protocol

Universitat Internacional de Catalunya | Escola de Doctorat

Barcelona, 9 de enero de 2014

Sra. Naroa Lozano Carrascal
Sant Antoni Mª Claret 20
08304, Mataró

Estimada Sra.

Por la presente, le comunico que la Comisión Académica del Doctorado en Ciencias de la Salud, en la su sesión del 10 de diciembre de 2013, y una vez estudiada su solicitud ha acordado:

Se acuerda admitir a la Sra. Naroa Lozano Carrascal al Periodo de Investigación del Doctorado de Odontología.

Se acuerda aprobar el Proyecto de Tesis titulado "Alteraciones Dimensionales De Los Alveolos Postextracción Con Diferentes Técnicas De Preservación: Estudio Experimental En Perros", y nombrar al Dr. Jordi Gargallo Albiol como Director de la Tesis y al Dr. José Luis Calvo-Guirado como Codirector.

Adicionalmente, se le informa que la normativa de la UIC establece que debe obtener una evaluación favorable del Comité de Ética en la Investigación, antes de la puesta en marcha de la investigación. Deberá aportar este informe cuando lo obtenga.

Aprovecho la oportunidad para saludarla cordialmente,

Jaime Oliver Serrano
Secretario Comisión Académica
Doctorado en Ciencias de la Salud

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3. Ethical Committee of Investigation (U. Murcia)

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INFORME DE LA COMISIÓN DE BIOÉTICA DE LA UNIVERSIDAD DE MURCIA

Jaime Peris Riera, Catedrático de Universidad y Secretario de la Comisión de Bioética de la Universidad de Murcia

CERTIFICA:

Que D. José Luis Calvo Guirado ha presentado el proyecto de investigación titulado *"Dimensional alterations of extraction sites after various socket preservation techniques"* a la Comisión de Bioética de la Universidad de Murcia.

Que dicha Comisión analizó toda la documentación presentada, y de conformidad con lo acordado el día 10 de febrero de 2012[1], acuerda por unanimidad emitir informe favorable desde el punto de vista bioético, **CONDICIONADO** a que en el proyecto se haga expresa mención al lugar donde se encuentran los animales de experimentación (Granada o Murcia) dado que si es en Granada el informe debe ser emitido por aquella Universidad. Con todo en el proyecto se deberá incluir la referencia específica y se tenga en cuenta el contenido de lo dispuesto en la legislación española, en concreto el RD 1201/2005, de 10 de octubre, sobre protección de animales utilizados para experimentación y otros fines científicos, en la Ley 32/2007 de 7 de noviembre para el cuidado de los animales en su explotación, transporte, experimentación y sacrificio, así como lo establecido en la Directiva 2010/63/UE del Parlamento Europeo y del Consejo de 22 de septiembre de 2010 relativa a la protección de los animales utilizados para fines científicos (Diario Oficial de la Unión Europea)

Y para que conste y tenga los efectos que correspondan, firmo esta certificación, con el visto bueno del Presidente de la Comisión, en Murcia 10 de febrero de 2012.

Vº Bº
EL PRESIDENTE DE LA COMISIÓN
DE BIOÉTICA DE LA UNIVERSIDAD DE MURCIA

Fdo.: Gaspar Ros Berruezo

[1] A los efectos de lo establecido en el art. 27.5 de la Ley 30/1992 de 26 de noviembre de Régimen Jurídico de las Administraciones Públicas y del P.A.C. (B.O.E. 27-11), se advierte que el acta de la sesión citada está pendiente de aprobación

4. Ethical Committee for Research (UIC)

CER Comitè d'Ètica de Recerca | Universitat Internacional de Catalunya

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Codi de l'estudi: CIR-ELB-2014-01
Versió del protocol: 1.0
Data de la versió: 01.04.2014
Títol: Alteraciones dimensionales de los alveolos postextracción con diferentes técnicas de preservación: estudio experimental en perros

Sant Cugat del Vallès, 10 de juny de 2012

Investigador: Naroa Lozano

Títol de l'estudi: Alteraciones dimensionales de los alveolos postextracción con diferentes técnicas de preservación: estudio experimental en perros

Benvolgut(da),

Valorat el projecte presentat, el CER de la Universitat Internacional de Catalunya, considera que, des del punt de vista ètic, reuneix els criteris exigits per aquesta institució i, per tant, ha

RESOLT FAVORABLEMENT

emetre aquest CERTIFICAT D'APROVACIÓ per part del Comitè d'Ètica de la Recerca, per que pugui ser presentat a les instàncies que així ho requereixin.

Em permeto recordar-li que si en el procés d'execució es produís algun canvi significatiu en els seus plantejaments, hauria de ser sotmès novament a la revisió i aprovació del CER.

Atentament,

Dr. Josep Argemí
President CER-UIC

1