Osseoinductive Elements Around Immediate Implants

A pilot study in Fox Hound Dogs

Oscar Salomó Coll

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To my family and Naroa for always
encouraging me to surpass myself

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Research Environment

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Summary

This PhD thesis is a compendium of three publications which sets out to broaden our knowledge and understanding of the topical application of biological mediators to dental implants, in particular the effects on osseointegration of topical applications of Melatonin or Vitamin D on implants placed in an animal model immediately after dental extraction, determined by histological and histomorphometric analysis.

Recent dental research has focused on improving bone substitutes and implant surfaces by morphologic or biochemical modification to achieve faster and better osseointegration. The application of biological mediators over the implant surface or embedded in the biomaterial itself can induce specific cell and tissue responses. Biochemical modifications can improve the both quantity and the quality of peri-implant tissue, which will reduce economic cost and treatment time, and also improve long-term outcomes.

Mandibular premolar distal roots (P_2, P_3, P_4) were extracted bilaterally from six American foxhound dogs. Three conical immediate implants were placed bilaterally in each mandible. Three randomized groups were created: melatonin 5% test group (MI), vitamin D 10% test group (DI) and control group implants (CI). Block sections were obtained after 12 weeks and processed for mineralized ground sectioning. Bone-to-implant contact (total BIC), new bone formation (NBF), inter-thread bone (ITB) and histological linear measurements (HLM) were assessed.

The present trial demonstrated that bone formation around immediate dental implants treated with melatonin and vitamin D improved in comparison with untreated control implants. Implants treated with Vitamin D and Melatonin showed higher bone-to-implant contact values and more new bone formation compared with control implants.

Vitamin D treated implants exhibited lower buccal crestal bone loss values compared with control implants. On the other hand, melatonin implants showed less lingual crestal bone loss, and less peri-implant mucosa on the lingual aspect, and exhibited better values for osseointegration compared with control implants.

Within the limitations of this animal study, the topical application of 5% melatonin or 10% vitamin D improved bone formation around implants placed immediately after extraction and helped to reduce crestal bone loss after 12 weeks osseointegration.

Table of Contents

1. List of Papers

This thesis is based on three papers previously published, which are the followings:

Paper 1

Osseoinductive elements for promoting osseointegration around immediate implants: a pilot study in the foxhound dog.

Salomó-Coll O, Maté-Sánchez de Val JE, Ramírez-Fernández MP, Satorres-Nieto M, Gargallo-Albiol J, Calvo-Guirado JL.

Clin Oral Implants Res. 2015 Apr 2. doi: 10.1111/clr.12596.

IMPACT FACTOR: 3.889

ISI JCR Ranking (2014): 3/88 (Dentistry Oral Surgery & Medicine)

Paper 2

Topical applications of vitamin D on implant surface for bone-to-implant contact enhance: a pilot study in dogs part II.

Salomó-Coll O, Maté-Sánchez de Val JE, Ramírez-Fernandez MP, Hernández-Alfaro F, Gargallo-Albiol J, Calvo-Guirado JL.

Clin Oral Implants Res. 2015 Sep 30. doi: 10.1111/clr.12707.

IMPACT FACTOR (JCR): 3.889

ISI JCR Ranking (2014): 3/88 (Dentistry Oral Surgery & Medicine)

Paper 3

Osseoinductive elements around immediate implants for better osteointegration: a pilot study in foxhound dogs

Salomó-Coll O, Maté-Sánchez de Val JE, Ramírez-Fernandez MP, Hernández-Alfaro F, Gargallo-Albiol J, Calvo-Guirado JL.

Clin Oral Implants Res. Feb 29. doi: 10.1111/clr.12809

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2. Background

Bone is a dynamic tissue that undergoes continuous remodeling via two reciprocal processes: bone formation and bone reabsorption. Remodeling requires a balance between the deposition of new bone by osteoblasts and reabsorption of old bone by osteoclasts, to renew, maintain, or adjust bone strength and mineral homeostasis in response to changing environmental influences. Bone remodeling requires the interaction of multiple bone cells including osteocytes, osteoblasts and osteoclasts. This process takes place as four main phases: activation, reabsorption, reversal and formation (1).

Initially, osteoclast precursors are activated and attach to a particular area of bone surface, where they differentiate into osteoclasts, which are responsible for the proteolytic digestion of the tissue (1). Osteoclasts are able to reabsorb existing bone through the production of free radicals, which are molecules or portions of molecules that possess an unpaired electron in their valence orbital. This electron-deficient state makes these agents highly reactive and so they damage adjacent molecules by abstracting or donating an electron, which leads to cell destruction (2). The generation of oxygen-derived free radicals may play a crucial role in reabsorbing bone under inflammatory conditions (2,3). During the inflammation phase provoked (for example) by dental extraction, free radicals such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and enzymes such as Cyclooxygenase (COX) (that catalyses the biosynthesis of prostaglandins) all show increased levels (3,4). An increase in free radical production coexists with a decrease in the antioxidant defense system. The imbalance between the pro-oxidant and the antioxidant may lead to further oxidative tissue damage (3,5).

In the reversal phase, bone reabsorption transitions to bone formation, and pre-osteoblasts are recruited to proliferate and differentiate into osteoblasts invading the reabsorption area and initiating new bone formation by secreting an osteoid matrix. During bone genesis, mesenchymal cells are subjected to a

series of stimuli such as growth factors (bone morphogenetic proteins) and hormones (estradiol, parathyroid hormone, grow hormone and others) (6), resulting in the expression of transcription factors (Runx2 and others), which leads to osteogenic differentiation. Fully differentiated osteoblasts are responsible for the formation of bone extracellular matrix, composed mainly of collagen type 1 and non-collagen proteins such as osteocalcin, osteopontin, osteonectin and sialoprotein (7).

Type 1 collagen is the most abundant and important matrix molecule in bone; it forms a three-dimensional network onto which non-collagenous proteins and hydroxyapatite crystals are deposited. These proteins play an important role as mineralization promoters, inhibiting or signaling molecules: bone sialoprotein, for example, is involved in the formation and mineralization of hydroxyapatite (HA), while osteopontin inhibits the formation and growth of HA. Once mineralized osteoblasts are trapped in bone, they become osteocytes. Osteocytes, once considered silent cells trapped in mineralized bone, have now been identified as key regulators of bone remodeling, which secrete soluble factors acting on cells and organs (1,7).

Nowadays, the dental implant-supported prosthesis has become a widely adopted treatment for oral rehabilitation, and is considered highly predictable, often offering the best option for single or multiple tooth replacement (9). Subsequent to implant insertion in bone, the osseous wound undergoes the following sequence (as occurs following bone fracture): hemostasis, inflammation, and proliferation (4). During osseous healing, the host bone close to the implant will be reabsorbed and gradually changed by new woven bone. Remodeling processes around the implant will continue for an extended period of time depending on species, the time of implant loading, and the bony template (8). This process, termed osseointegration, was first coined by Bränemark et al. in 1969, and is defined as "Structural and functional contact between the titanium surface and bone."

During the osseointegration process, osteoblast progenitor stem cells play an essential role in recruitment, adhesion, proliferation, differentiation, and the mineralization of the deposited bone matrix (10). The initial interactions that occur at the bone-to-implant interface will determine the success or failure of the implant; this bone formation process requires osteoblast precursor cells, their differentiation into secretory osteoblasts, the production of an un-mineralized extracellular osteoid matrix (osteoid matrix) and its calcification (11).

After dental extraction, bone resorption reduces alveolar bone volume height and width (12-14); resorption is more pronounced during the first four months (15). When implants are placed immediately after dental extraction, the remodeling process takes on greater importance because bone loss may compromise the aesthetic outcomes. In 2001, Paolantonio *et al*. (16) proposed placing implants immediately following extraction as a possible means of avoiding the process of bone and soft tissue modification (without applying biomaterials or membranes). But a later series of dog studies by Araújo and Lindhe et al. showed that immediate implant placement does not affect the bone remodeling process (17-20).

Nevertheless, immediate implants have proved to be an effective therapy not only for reducing the number of surgical procedures (16,21,22), but also for simplifying clinical techniques and reducing total treatment time. Furthermore, the procedure achieves similar survival rates to delayed implant placement (21-26). When the immediate implant is restored immediately, this also helps to maintain interdental papillae (27,28).

The buccal wall is thinner than the lingual wall and has a higher percentage of bundle bone, which disappears after extraction causing resorption of the buccal wall and so a reduction in bone volume (13,14,29). This anatomical characteristic must be considered if pronounced buccal bone resorption wants to be avoided following immediate implant placement (17,18,20,30).

Although immediate implants generally show good results, the overall bone loss and soft tissue recessions that occur mainly during the first year after placement (27,31,32) can compromise functional as well as aesthetic outcomes (15,28,32-34). The causes of soft and hard tissue remodeling around implants remain unknown, although several premises have been formulated, which include surgical trauma (35), the presence of implant-abutment microgaps (36), biologic width establishment (37), apico-coronal implant position (38,39,40,41), bucco-lingual position (31,42,43), implant abutment design (26,34,44,45), soft tissue grafting (46, 47), alveolus defect shape (48), and defects around the immediate implant (31,49,50).

When implants are placed immediately in post-extraction sockets, the inner space between the buccal wall and the implant is known as the *jumping distance* or *buccal gap* (51). After immediate implant placement, the buccal wall suffers both vertical and horizontal resorption due the loss of bundle bone (52), and so several authors stress the need for gap filling at the time of immediate implant placement, depending on the dimensions of the gap (47,50-55). This involves the introduction of a grafting material that aims to minimize bone resorption and improve implant osseointegration. But new bone formation will be influenced by the chemical and physical properties of the graft material and procedure (56). Many protocols, techniques and biomaterials have been proposed for improving bone healing after dental extraction, but given the heterogeneity of biomaterials and techniques, no single protocol has proved better than the others and none have achieved the full maintenance of initial bone volume (30,57-59). No bone substitute is able to respond to physiological loads or biochemical stimuli in the way that living tissue does (60).

In this context, implant osseointegration research has focused on quantifying bone-to-implant contact and developing different surface topographies and coatings to enhance architecture and the microenvironment around the implants (9).

Bone formation around implants, and consequently implant survival, will be conditioned by the implants' surface characteristics: roughness, texture, surface energy, and others (61). Multiple biomaterials such as titanium, zirconia, alumina, bioglass, polymeric compounds, calcium phosphates and composites have been extensively investigated (62). Biomaterials promote the morphologic preservation of the ridge by acting as scaffolds for bone formation, maintaining a space for bone ingrowth, and promoting wound healing. But these biomaterials have no osteogenic or osseoinductive properties, that sometimes prevents bone ingrowth; furthermore, graft particles can remain embedded in the newly formed bone for a long time, which can negatively influence peri-implant tissue heath and maintenance (61).

One goal of current implant research is to design a protocol capable of implementing controlled, guided and rapid bone healing. Several approaches to improving bone healing around implants have been investigated including physicochemical, morphologic or biochemical implant surface modifications. Implant surfaces can be textured using a number of methods such as grit blasting, plasma spraying, acid etching, anodic oxidation or a combination of these. Rough surfaces have shown greater initial cell attachment compared with smooth surfaces, increasing the osteoid matrix, levels of alkaline phosphatase (an indicator of mineralization), and levels of prostaglandins E2 (PGE2) and TGF beta (61).

Additional benefits may be derived from coating the surface with biologically active components. A bioactive material or implant surface is defined as one that has the potential to promote numerous molecular interactions, potentially forming a chemical bond between the material and bone (63). The goal of biochemical surface modifications is to apply biological mediators to induce specific cell responses (64). Hydroxyapatites, collagen, growth factors and enzymes have all been integrated into implant surfaces to enhance their biological efficacy and function (65). The surface properties of the bioactive implant can modulate cell responses such as cell attachment, spreading,

motility, proliferation and differentiation. Bioactive agents may endow the implant surface with enhanced osteogenic properties so that chemical, as well as physical bonding can take place, which could enhance prosthetic performance and longevity (63). It should be noted that the molecular mechanism underlying osseointegration is not yet fully understood; the specific genetic networks needed to target the evaluation of successful osseointegration have not been fully identified (7).

When developing biomaterials for implant surface modification, small molecules that promote osteoblast differentiation and maturation are particularly attractive. Increasing formation and maturation of osteoblasts or their mesenchymal progenitors could increase osseointegration and so improve prosthetic performance and longevity (66). These modifications can improve the quality and quantity of bone around dental implants, improving osseointegration, and so implant survival time (and also reduce economic costs and treatment times). Several biological mediators with osseoinductive properties have been applied to implant surfaces or into the biomaterial itself to induce specific cell and tissue responses, including bone morphogenetic proteins (BMP) (67), growth hormones (5, 45,68), melatonin (6,69, 70, 71), or Vitamin D (64,72).

Melatonin (N-acetyl-5-metoxy-tryptamine) is an amphiphilic hormone synthesized from serotonin. It is mainly synthesized in and secreted by the pineal gland by pinealocyte cells, but others areas such as eyes, skin or bone marrow can also produce melatonin. Melatonin can also be stored in the cell's nucleus or inside the mitochondria. As a hormone, melatonin's main function is to control and regulate circadian rhythms; it is also involved in other functions as a protector or anti-oxidant. Melatonin receptors have been identified in peripheral organs and in the cells of the central nervous system (73).

Melatonin plays an important role in protecting the oral cavity from tissue damage. This indomlamine has been found to be a significant modulator of calcium metabolism, preventing osteoporosis and hypocalcaemia, probably due

to its interaction with other bone regulatory factors, such as parathormone, calcitonin, and prostaglandins (73).

Melatonin can inhibit bone resorption in two different ways: it has the ability to detoxify free radicals, and can interfere in osteoclast functioning thereby inhibiting bone resorption. After dental extraction, osteoclasts generate reactive oxygen species (ROS) and reactive nitrogen species (RNS), in the bone's microenvironment. These contribute to the dissolution of bone matrix components, such as collagen, which is susceptible to oxidative damage by free radicals. Melatonin neutralizes the free radicals, limiting the potential damage to the surrounding tissues and consequently favoring healing (3-5). At the same time, melatonin is able to down-regulate RANKL expression, inhibiting osteoclast formation and activation, and so reducing healing time (69).

Melatonin has also demonstrated potential benefits in bone regeneration. Melatonin increases osteoblast differentiation and maturation, promoting the expression of type 1 collagen fibers and bone marker proteins (alkaline phosphatase, osteopontin, bone sialoprotein, and osteocalcin); it also induces a higher rate of maturation of pre-osteoblasts reducing differentiation time and perhaps inducing a higher rate of osteoid matrix production and calcification (69,74).

Vitamin D was discovered in 1922 during the quest to cure Rickets' disease. Originally classified as a vitamin, it is now considered a prohormone. Vitamin D compromises a group of fat-soluble sterols that includes Ergocalciferol (D_2) and Cholecalciferol (D_3). Vitamin D is obtained either by diet from sources such as oily fish, milk or food supplements, or can also be synthesized in the skin from 7-dehydrocholesterol upon exposure to UV radiation (62). Vitamin D is transported to the kidney where it is hydroxylated converting it into the active form 1,25-dihidroxivitamin D or calcitriol (75).

As a hormone, Vitamin D can develop multiple functions. The biologically active form of Vitamin D can bind to Vitamin D receptors and regulate a wide range of target genes (7). Epidemiological studies have found an association between vitamin D deficiency and various diseases such as cancer, autoimmune diseases, hypertension, and diabetes, which suggests that the vitamin plays many different roles in modulating the immune system and controlling various endocrine systems.

Vitamin D deficiency causes Rickets in children, and osteomalacia in adults, diseases characterized by inadequate mineralization of newly formed bone matrix. Vitamin D deficiency is believed to cause secondary hyperparathyroidism leading to increased bone turnover and consequently a generalized bone loss (76,77). There is evidence that lesser degrees of vitamin D insufficiency have deleterious effects on bone tissues (such as increased unmineralized matrix, which increases the risk of bone fractures).

Vitamin D also helps to reduce inflammation and helps improve the body's immune reactions, for example by increasing the expression of antimicrobial peptides such as defensins and cathelicidins, which enhance the microbicidal capability of monocytes (61,78).

Vitamin D has been shown top play an essential role in bone mineral homeostasis and it may act as a bioactive protein to promote new bone formation. In low doses, calcitriol has been shown to act as a potent stimulator of bone resorption, but at high doses it increases osteoblast activity and so maintains positive bone remodeling (10).

The main function of vitamin D is to regulate calcium and phosphorous absorption in the intestine, resulting in increasing plasma concentrations, but the exact role of vitamin D on osteoclastogenesis remains unclear. Vitamin D receptors have been found in osteoblast and osteoclast precursors, and have a direct effect on the cells by regulating the expression of several genes. This fact

explains vitamin D's possible effects on bone remodeling; it has also been shown to regulate collagen modification and maturation, which has been proved important in the early stages of bone formation (63).

Vitamin D has been shown to have osteoinductive properties: it promotes osteoblast differentiation and bone matrix formation by increasing the synthesis of collagen type 1, osteonectin (ONC), osteopontin (OPN) and by increasing alkaline phosphatase (ALP) (79,80). ALP is one of the marker proteins produced in the early stages of osteoblast differentiation (62). Vitamin D can enhance bone mineralization and expression of osteocyte markers such as dentin matrix protein 1 and fibroblast growth factor 23. Vitamin D is sometimes called a promoter of osteoblast-osteocyte transition (79). But when levels of vitamin D are low, the PTH hormone promotes high bone turnover leading to cortical bone loss and osteoporosis, reducing bone regeneration, and preventing bone fractures from healing. Vitamin D supplements have been shown to reduce PTH levels and reduce bone remodeling. Calcitriol or any form of vitamin D will decrease PTH levels and this may contribute to implant osseointegration. Several studies have demonstrated the potential benefits of Vitamin D supplementation around implants or its use in guided bone regeneration (GBR), whereby it accelerates bone formation, increases bone-to-implant contact (BIC), bone volume, resistance to implant removal, and helps to protect peri-implant bone tissue (81-89).

The aim of the present study was to determine whether the topical applications of melatonin 5% or vitamin D 10% applied directly over the implant surfaces improve osseointegration, evaluated by histomorphometric analysis and histological linear measurements of bone-to-implant contact twelve weeks after immediate implant placement.

3. Objectives

Main Objective

The aim of the present study was to evaluate the effects of Melatonin and Vitamin D, topically applied over implants surfaces placed immediately after extraction, on osseointegration by means of histological and histomorphometric analysis of peri-implant tissues.

Objectives of the Papers

Paper 1

The aim of this paper was to evaluate the effects of topical applications of melatonin over the surfaces of implants placed immediately after extraction, by means of histological and histomorphometric analysis of peri-implant tissues at twelve weeks.

Paper 2

The aim of this paper was to evaluate the effects of topical applications of vitamin D over the surfaces of implants placed immediately after extraction, by means of histological and histomorphometric analysis of peri-implant tissues at twelve weeks.

Paper 3

The aim of this paper was to evaluate the effects on osseointegration of topical applications of melatonin or vitamin D over surfaces of immediate implants by means of histological and histomorphometric analysis of peri-implant tissues after twelve weeks osseointegration.

4. Matherial and Mehoths

Study design.

Six American Foxhound dogs of approximately one year of age, each weighing 13-15 kg were used in the study. Three conical implants were inserted bilaterally in each hemiarch, and divided randomly into three different groups: melatonin (MI), vitamin D (DI) and control implants (CI). The Ethics Committee for Animal Research at the University of Murcia (Spain) approved the study protocol, which followed guidelines established by the European Union Council Directive February 1st 2013/53/CEE. Animals were quarantined for application of anti-rabies vaccine and anthelmintics. Pre- and postoperatively, the animals were kept in kennel cages; they received appropriate veterinary care with free access to water and standard laboratory nutritional support throughout the trial period. All animals presented intact maxillas, without any oral viral or fungal lesions.

Surgical Procedures

First stage

The animals were pre-anesthetized with 10% zolazepam at 0.10 ml/kg, 0.12% acepromazine maleate (Calmo-Neosan, Pfizer, Madrid) at 0.25 mg/kg, and medetomidine 35µg/kg (Medetor 1mg, Virbac, CP-Pharma Handelsgesellschaft GmbH, Burgodorf Germany). The mixture was injected intramuscularly in the femoral quadriceps. Animals were then taken to the operating theatre where, at the earliest opportunity, an intravenous catheter was inserted (diameter 22 or 20 G) into the cephalic vein and propofol was infused at the rate of 0.4 mg/kg/min at a slow constant infusion rate. Anesthetic maintenance was obtained using volatile anesthetics; the animals were submitted to tracheal intubation with a Magill probe for adaptation of the anesthetic device and for administration of oxygen-diluted volatile isoflurane (2%). In addition, local

anesthesia (Articaine 40mg, 1% epinephrine [Normon, Madrid, Spain]) was administered at the surgical sites. These procedures were carried out under the supervision of a veterinary surgeon.

Bilateral mandibular premolar extractions (P2, P3, P4,) were performed in each dog. Teeth where sectioned in a bucco-lingual direction at the bifurcation using a tungsten-carbide bur. Distal roots were extracted individually using a periotome and forceps without damaging the bony walls. Mesial roots were filled with mineral trioxide aggregate (MTA) and composite to maintain minimum function during implant healing and to prevent endodontic pathology.

A randomization scheme was generated using the web site http://www.randomization.com. A scheme was created for the 36 implants (12 implants with melatonin (MI), 12 implants with vitamin D [DI] and 12 implants alone [CI]), randomized into six dogs. Before implant placement, test (MI) implants were submerged in melatonin at 5% in saline solution (TM–M5250; Sigma-Aldrich®, St. Louis, MO USA), and test (DI) implants were submerged in vitamin D at 10% in acetone solution (ref740578, Sigma-Aldrich®, St. Louis, MO, USA). No treatment was applied to control implants (CI). Minimal full-thickness mucoperiosteal flaps were elevated, and the implants were placed. Each dog received six conical C1 implants (MIS, Barlev, Israel) with sandblasted and acid etched surfaces, three per hemiarch randomly assigned bilaterally in the mandible. Implant positions were determined in relation to the shape and volume of each alveolar process and the buccal wall position. The apical portion of each socket was prepared using a conventional drilling protocol as recommended by the manufacturer. All implants were placed at the same buccal bone level, with the implant shoulder level with the buccal bone crest. Each mandible received six conical screw implants (3.75×10 mm). After implant placement, standard 4 mm height healing abutments (CS-HS475, MIS® Implant Technologies, Carmiel, Israel) were placed to allow a non-submerged healing protocol. After abutment placement, occlusion was checked to avoid interferences during biting. No graft materials were used in the gaps remaining

between bony plates and implants. The flaps were closed with simple interrupted non-resorbable sutures (Silk® 4-0, Lorca Marin, Lorca, Spain).

After the surgical procedures, the animals received antibiotics twice daily (Amoxicillin 500 mg. Clamoxyl L.A., Pfizer, Madrid, Spain), and analgesics three times a day (Ibuprofen 600mg, Rimadyl, Pfizer, Madrid, Spain). The sutures were removed after two weeks. Animals were fed with a soft diet for seven days after surgery. The animals had free access to water and were fed with moistened balanced dog chow. The wounds were inspected daily for any clinical signs of complications and the healing screws were cleaned.

Second stage

Digital radiographs were taken at 12 weeks (Kodak 6100, Eastman Kodak, Rochester, NY, USA) . Afterwards, the animals were sacrificed (12 weeks after implant placement by means of pentothal natrium (Abbot Laboratories, Madrid, Spain) perfused through the carotid arteries with a fixative containing a mixture of 5% glutaraldehyde and 4% formaldehyde. The mandibles were dissected and block sections including the implant sites and surrounding soft and hard tissues were removed using a saw.

Histological and histomorphometric analysis:

Biopsies were processed for ground sectioning according to the methods described by Donath and Breuner. Samples were dehydrated in increasing grades of ethanol up to 100%, infiltrated with methacrylate, polymerized, and sectioned at the buccal-lingual plane using a diamond saw (Exakt Apparatebeau, Norderstedt, Hamburg, Germany). Two sections were cut from each biopsy specimen. The first was cut from the center of the implant and the second from the surrounding bone. Each block was sectioned with a high-precision diamond disk to about 100µm thickness and ground to approximately

40µm final thickness with an Exakt 400s CS grinding device (Exakt Apparatebau, Norderstedt, Hamburg, Germany).

Sections were stained with toluidine blue, and a semi-quantitative evaluation of BIC was performed. To obtain a single digitally processable overview image of each implant, four images of the same implant were taken with a 10X lens and assembled into a single image (Image-Pro Plus 4.5, Media Cybernetics Inc., Immagini & Computer Snc., Milan, Italy). A 1 mm wide zone around the implant surface, reaching up to the original implantation level, was defined as the region of interest (ROI). Within the ROI, hard tissue was defined digitally as old bone or newly formed bone. In order to improve the differentiation between native and newly formed bone, light and dark blue chromaticity were digitally enhanced. Finally, the contact length between bone and implant surface (BIC) was determined.

Bone-to-implant contact (BIC) in each histological section was calculated by measuring the length of the implant surface in contact with bone tissue, in comparison with the total length of the implant surface, expressed as a percentage (Total BIC). The percentage of mineralized bone in direct contact with the titanium surface was determined by counting inside the threaded zone (Inter-thread Bone). New bone formation was calculated as the inter-thread bone and the bone in direct contact with the implant perimeter (New Bone Formation). BIC percentages were calculated around the entire implant body, from the first point of bone-to-implant contact, at the most coronal point, evaluating mineralized bone in contact with the implant surface linearly (BIC%) (Figs. 1 & 2).

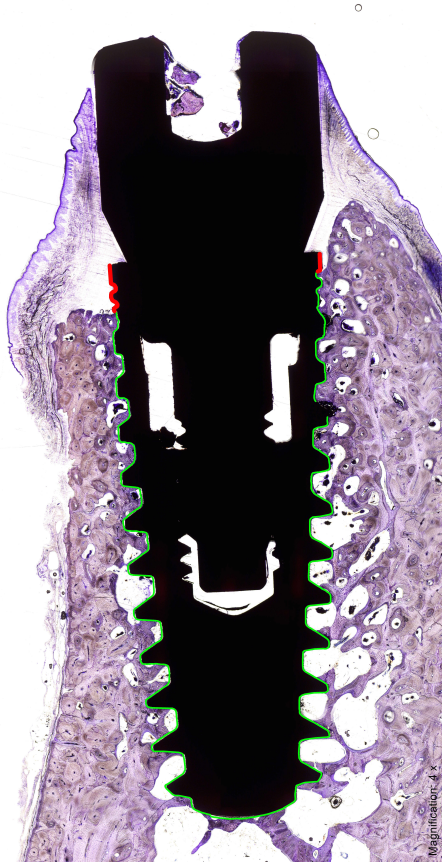

Fig. 1. BIC scheme.Green lines shows Total BIC and red line show crestal bone loss.

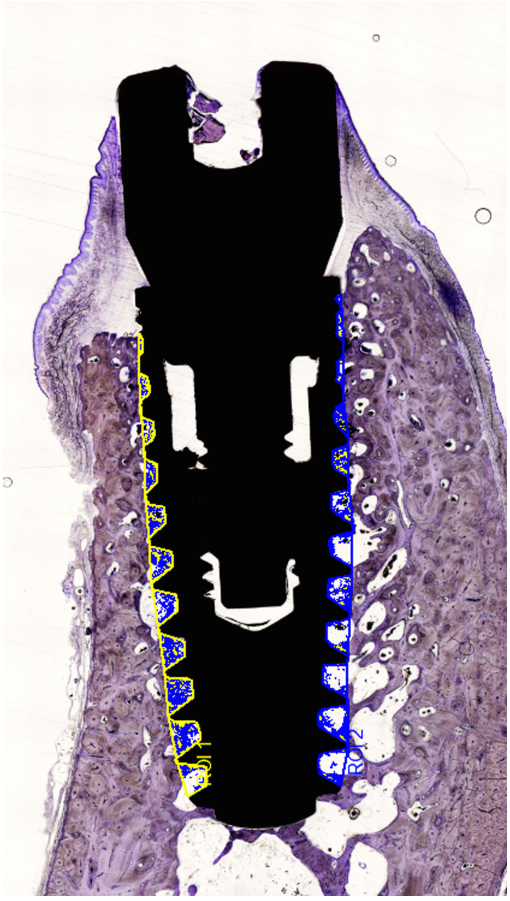

Fig. 2. BIC scheme. Yellow line delimitates interthread space and blue line shows New bone formation

Histomorphometric analysis was performed using a video camera (Sony 3CCD, Berlin, Germany) with 10X magnification. Inter-thread images were digitalized (Axiophot-System, Zeiss, Oberkochen, Germany), stored, and reference points were plotted.

The following buccal and lingual measurements were made at X10 magnification (Fig.3):

D1: Distance from the top of the peri-implant mucosa and the apical portion of the junctional epithelium (P-aJE).

D2: Distance from the apical portion of the junctional epithelium to the first point of bone-to-implant contact (aJE-fBIC).

D3: Distance from the implant shoulder to the first point of bone-to-implant contact (IS-fBIC) (Crestal Bone Loss: CBL).

D4: Distance from the top of the peri-implant mucosa to the first point of bone-to-implant contact (P-fBIC) (Peri-implant Mucosa: PIM).

D5: Distance from the implant shoulder to the bone crest (IS-BC).

D6: Distance from the first point of bone-to-implant contact to the bone crest (fBIC-BC).

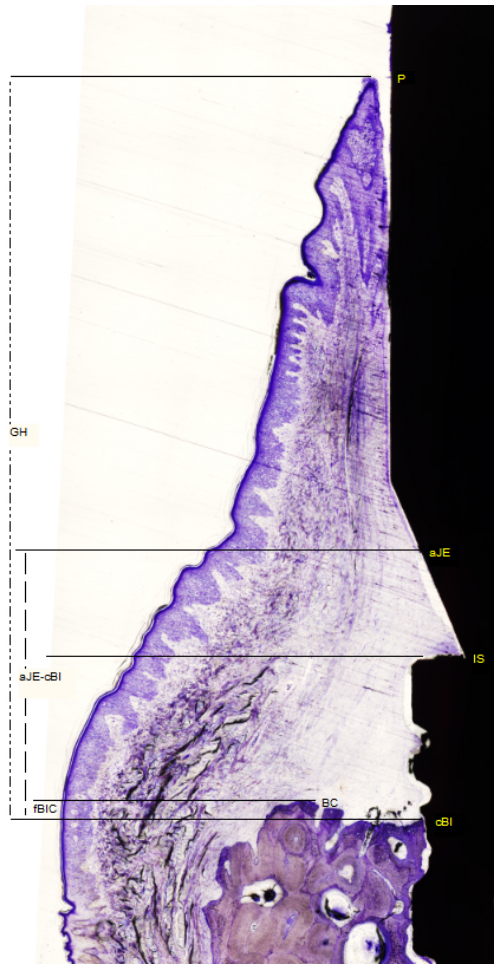

Fig. 3. Linear Mesurements scheme: P:top of the peri-implant mucosa. aJe: apical portion of the junctional epithelium. fBIC: first point of bone-to-implant contact. IS: Implant shoulder. BC, buccal crest.

Measurements were performed with a light microscope (Laborlux S, Leitz, Wetzlar, Germany) connected to a high-resolution video camera (3CCD, JVC KY-F55B, JVC®, Yokohama, Japan) and interfaced to a monitor and PC (Intel Pentium III 1200 MMX, Intel®, Santa Clara, CA, USA). This optical system was connected to a digitizing pad (Matrix Vision GmbH, Oppenweiler, Germany) and a histometry software package with image capture capabilities (Image-Pro Plus 4.5, Media Cybernetics Inc., Immagini & Computer Snc., Milan, Italy).

Radiographic evaluation

Digital radiographs were taken at the time of implant placement and 12 weeks later to verify implant osseointegration and to assess changes to post-surgical crestal bone levels (Kodak 6100, Eastman Kodak, Rochester, NY, USA). Exposure parameters were standardized. No radiolucent images or signs of osseointegration disorders were observed. X-ray analysis revealed that all 36 implants showed uneventful osseointegration.

Statistical analysis

Firstly, factors such as individual difference and position of the implant could be excluded as not significant. Despite having several samples of the same specimen, implants were considered as samples rather than dogs; for this reason samples were considered independent. To compare independent samples with continuous variables, differents tests were applied depending on the sample to study. These tests work with the median rather than the mean to perform comparative analysis of quantitative and qualitative variables. The level of statistical significance was establish at $p<0.05$. All histomorphometric parameters were analyzed using descriptive methods (SPSS 21.0 software, Chicago, IL, USA). In cases of statistically significant differences, Bonferroni correction method for multiple comparisons was applied.

5. Results

Paper 1

Histological and histomorphometric analysis

The results of histomorphometric measurement are presented in Table 1. BIC% values at 12 weeks varied slightly between the melatonin group MI (41.36±3.93) and the CI group (41.34±9.26) although without statistically significant difference (P > 0.05). Analyzing total BIC, statistically significant differences were found between MI (48.36±7.45)* and CI (44.82±10.98) (P = 0.036). For inter-thread bone, the MI group showed a statistically significant increase (P = 0.035) in bone density around implants (15.99±2.43)* compared with control implants CI (14.79±3.62).

For new peri-implant bone formation, the melatonin group did not show statistically significant differences (P > 0.05) compared with the control group: MI (25.37±2.32), CI (26.55±7.75).

Table 1. Medians, means and standard deviation for total BIC, BIC%, new bone formation and interthread bone after 12 weeks of healing.

Variable	Alone (n = 12) Mean ± SD	Melatonin (n = 12) Mean ± SD	P value
New bone formation (NBF)	26.55 ± 7.749	25.37 ± 2.323	0.622
Inter-thread bone (ITH)*	14.79 ± 3.622	15.99 ± 2.435	0.035*
Total BIC*	44.82 ± 10.975	48.36 ± 7.451	0.036*
BIC%	41.34 ± 9.257	41.36 ± 3.933	0.995

*Significant differences in inter thread group and total BIC (P < 0.05).

Figures 4 and 5 show histological images of control and test implants. Figure 4 shows an untreated implant [CI], with a small amount of inter-thread bone and small quantity of bone surrounding the implant. Figure 5 shows an implant treated with a topical application of melatonin 5% [MI]; direct bone-to-implant contact with increased inter-thread bone can be observed with an increase in bone growth and vascular tissue.

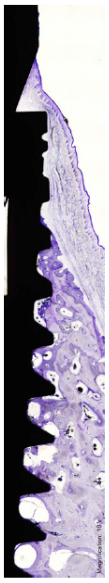

Fig. 4. Buccal ground section of an untreated implant [CI] after 12 weeks healing. Hematoxilin eosin stain, original magnification X10.

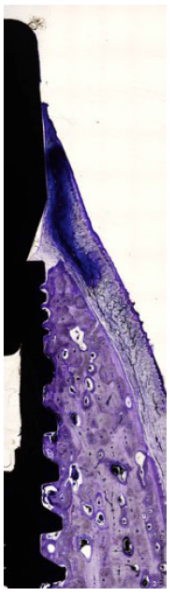

Fig. 5. Buccal ground section of a test implant [MI] after 12 weeks healing. Hematoxilin eosin stain, original magnification X10.

Linear measurements

Tables 2 and 3 show linear measurements. Statistically significant differences were only found on the lingual aspect. The melatonin group showed statistically less peri-implant mucosa (PIM) MI (3.13±1.41)*, CI (3.71±1.81) (P = 0.042) and less lingual crestal bone loss (CBL) MI (0.52±0.74)* compared with control implants CI (0.92±1.98) (P = 0.045).

Table 2. Medians, means and standard deviation for linear measurements at 12 weeks of healing at the buccal bone crest.

Buccal bone crest	Alone (n = 12) Mean ± SD	Melatonin (n = 12) Mean ± SD	P value
P-aJE buc	2.67 ± 0.83	2.44 ± 0.43	0.242
aJE-cBI buc	0.87 ± 1.08	0.69 ± 0.59	0.291
IS-cBI buc (CBL)	1.24 ± 1.19	1.09 ± 1.38	0.551
P-cBI buc (PIM)	4.78 ± 0.63	4.22 ± 0.47	0.160
IS-BC buc	1.71 ± 1.06	1.16 ± 1.12	0.114
fBIC-buc	0.47 ± 0.79	−0.07* ± 0.61	0.114

*Differences between values achieving statistical significance ($P < 0.05$).

Table 3. Medians, means and standard deviation for linear measurements at 12 weeks of healing at the lingual bone crest.

Lingual bone crest	Alone (n = 12) Mean ± SD	Melatonin (n = 12) Mean ± SD	P value
P-aJE lin	2.16 ± 0.91	1.56 ± 0.57*	0.001*
aJE-cBI lin	0.63 ± 1.08	0.65 ± 1.57	0.347
IS-cBI lin (CBL)	0.92 ± 1.98	0.52 ± 0.74*	0.045*
P-cBI lin (PIM)	3.71 ± 1.81	3.13 ± 1.41*	0.042*
IS-BC-lin	1.16 ± 0.82	0.88 ± 1.12*	0.039*
fBIC-lin	0.21 ± 1.24	0.06 ± 1.59	0.977

*Differences between values achieving statistical significance ($P < 0.05$).

Radiographic analysis

X-rays did not show radiolucency or evidence of great bone loss around implants. Adequate contact between the small diameter implants and the host bone was observed in all cases. All 24 implants showed uneventful osseointegration at 12 weeks. No statistical values could be obtained due to the difficulty of x-ray standardization.

Paper 2

Histomorphometric and histological results

Histomorphometric measurements are presented in Table 4. BIC% values at 12 weeks were slightly higher in the vitamin D group (DI) (43.59±0.98), although without statistically significant differences (P < 0.05) compared with control implants (CI) (42.67±1.45). Analyzing total BIC, statistically significant differences were found between DI (48.96±2.14) and CI (44.56±1.75) (P > 0.05). DI inter-thread bone values were 19.56±0.78, while CI values were 16.23±1.24 (P < 0.05). Implants treated with vitamin D [DI] showed significantly more new bone formation (31.87±1.23) than control implants [CI] (27.18±2.38) (P= 0.021).

Table 4. Medians, means and standard deviation for total BIC, BIC%, new bone formation and interthread bone after 12 weeks of healing. Nonparametric Wilcoxon signed rank test for related samples

	Control group C1		Test group D1		
Variable	Mean ± SD %	Median	Mean ± SD %	Median	*P* value
New Bone Formation	27.18 ± 2.38	26.3	31.87 ± 1.23	30.6	0.021*
Interthread Bone (ITH)	16.23 ± 1.24	15.9	19.56 ± 0.78	18.6	0.345
Total BIC	44.56 ± 1.75	43.6	48.96 ± 2.14	47.9	0.035*
BIC%	42.67 ± 1.45	41.8	43.59 ± 0.98	42.7	0.167

*Differences between values achieving statistical significance ($P < 0.05$).

Figures 6 and 7 show histological images (buccal and lingual, respectively) of a control implant [CI]. Figures 8 and 9 show a test implant [DI] (buccal and lingual, respectively). All histological images revealed good bone healing without any signs of inflammation. Newly formed bone was similar in both groups, in direct contact with implant surface and showing active remodeling activity with mixed patterns of densely organized mature and immature bone. No fibrous connective tissue was observed at the bone-to-implant interface.

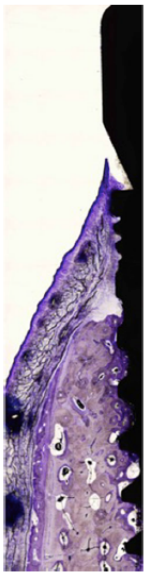

Fig. 6. Buccal ground section of an untreated implant [CI] after 12 weeks healing. Hematoxilin eosin stain, original magnification X10.

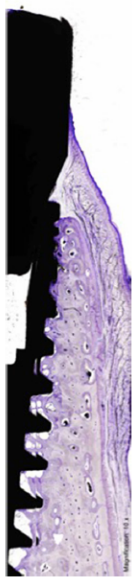

Fig. 7. Lingual ground section of an untreated implant [CI] after 12 weeks healing. Hematoxilin eosin stain, original magnification X10.

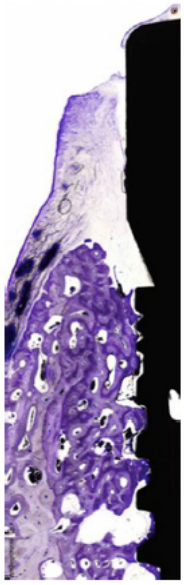

Fig. 8. Buccal ground section of a test implant [DI] after 12 weeks healing. Hematoxilin eosin stain, original magnification X10.

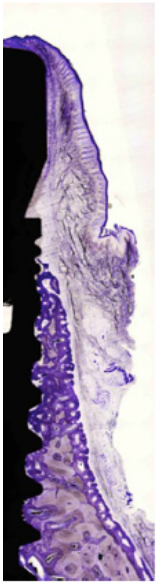

Fig. 9. Lingual ground section of a test implant [DI] after 12 weeks healing. Hematoxilin eosin stain, original magnification X10.

Linear measurements

The results of the different linear measurements are presented in Table 5. Few statistically significant differences were found among the variables. The vitamin D group DI showed statistically less buccal crestal bone loss (0.37±0.12) than the control group CI (1.26±0.81) (P=0.032), and less lingual junctional epithelium in DI (1.58±0.43)* than CI (2.18±0.48) (P=0.028).

Table 5. Medians, means and standard for linear measurements at weeks of healing.

	Control group C1 (n = 12)		Test group D1 (n = 12)		
Buccal	Mean ± SD	Median	Mean ± SD	Median	P value
P-aJE buc	2.59 ± 0.71	2.11	2.78 ± 0.24	2.67	0.034*
aJE-cBI buc	0.91 ± 1.04	0.87	1.97 ± 0.98	1.71	0.154
IS-cBI buc (CBL)	1.26 ± 0.81	0.91	0.37 ± 0.12*	0.29	0.032*
P-cBI buc (PIM)	3.98 ± 0.32	3.11	4.68 ± 0.97	3.91	0.023*
IS-BC buc	1.73 ± 1.05	1.12	1.89 ± 0.95	1.79	0.214
fBIC buc	0.51 ± 0.71	0.47	0.45 ± 0.82	0.39	0.281
	ALONE (n = 12)		VITAMIN D (n = 12)		
Lingual	Mean ± SD	Median	Mean ± SD	Median	P value
P-aJE lin	2.18 ± 0.48	1.97	1.58 ± 0.43	1.1	0.028*
aJE-cBI lin	0.66 ± 0.93	0.60	0.79 ± 0.65	0.143	0.590
IS-cBI lin (CBL)	0.96 ± 0.62	0.89	0.98 ± 1.23	0.478	0.187
P-cBI lin (PIM)	3.67 ± 1.74	3.12	3.58 ± 0.53	0.443	0.378
IS-BC-lin	1.18 ± 0.43	0.92	1.22 ± 0.37	0.052	0.143
fBIC-lin	0.22 ± 1.11	0.18	0.44 ± 0.99	0.410	0.016*

*Differences between values achieving statistical significance ($P < 0.05$). Nonparametric Wilcoxon signed rank test for related samples.

Radiographical analysis

No evidence of major bone loss or radiolucency was observed around the implants. X-rays showed good contact between the implants and the host bone. All 24 implants showed uneventful osseointegration at 12 weeks. No statistical values could be obtained because of difficulties of X-ray standardization.

Paper 3

Histological and histomorphometric analysis

The results of histomorphometric measurement are shown in Table 6. At 12 weeks, Total BIC values were 49.86±1.89 for the DI group, 49.20±3.26 for the MI group and 45.78±4.21 for the CI group ($P < 0.018$). BIC % was 44.56±1.08 for DI, 42.44±2.18 for MI, and 41.05±3.34 for CI ($P > 0.05$). Inter-thread bone formation values were 15.99±2.43 for MI, 19.56±0.78 for DI, and 15.51±3.62 for CI ($P > 0.05$). Statistically significant differences in peri-implant new bone formation were found between the three groups, DI 32.56±1.11, MI 28.76±1.98 and CI 25.43±4.67 ($P < 0.05$).

Table 6. Mean values. Brunner-Langer (nonparametrix mixed model). Summarizing the mean values and

	DI Group (n = 12) B		MI Group (n = 12) C		CI Group (n = 12) A		
Variable	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	*P* value
New Bone Formation (NBF)	32.56 ± 1.11	30.01 ± 36.62	28.76 ± 1.98	26.21 ± 31.54	25.43 ± 4.67	22.41 ± 28.14	**0.045 AB/BC**
Inter-thread Bone (ITB)	19.87 ± 0.92	17.98 ± 22.53	17.56 ± 2.01	15.67 ± 20.87	14.65 ± 1.24	12.34 ± 17.23	0.064
Total BIC	49.86 ± 1.89	46.89 ± 52.76	49.20 ± 3.26	47.52 ± 52.78	45.78 ± 4.21	42.31 ± 48.71	**0.018 AB/AC**
BIC%	44.56 ± 1.08	41.27 ± 47.86	42.44 ± 2.18	40.34 ± 45.67	41.05 ± 3.34	40.56 ± 46.82	0.84

No gaps were observed at the implant-bone interface in either of the test implant groups (melatonin and vitamin D). No inflammatory cell infiltrate, foreign body reaction cells, or multinucleated giant cells were found in any of the implant groups.

All histological images showed good bone healing without any signs of inflammation but control implants (CI) showed greater buccal crestal bone resorption compared with vitamin D implants (DI) and Melatonin implants (MI). (Fig. 10)

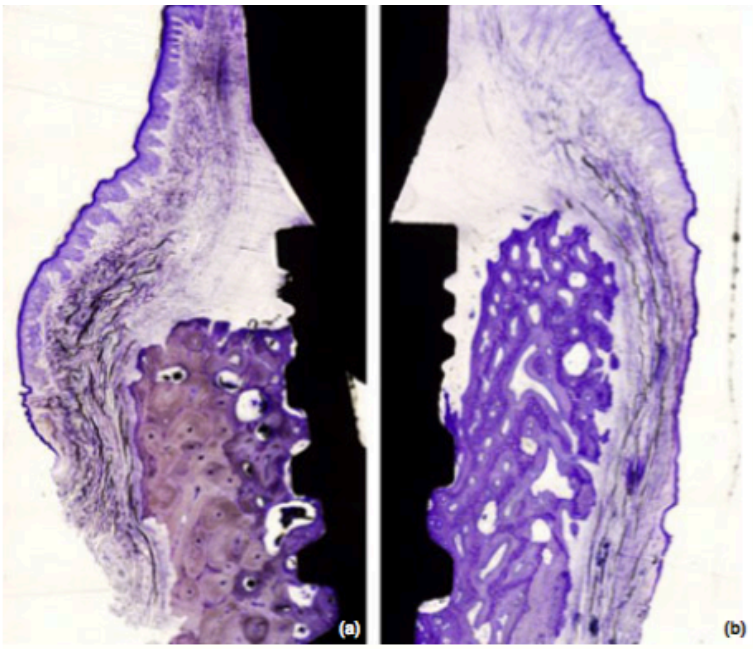

Fig. 10. Ground sections illustrating healing after 12 weeks of an untreated control implant group (CI). (a) Implants were integrated in mature mineralized bone. (b) The buccal bony wall and the peri-implant mucosa were located more apically compared with the lingual bone crest. Levai Lacsko stain, original magnification x10.

Cortical bone appeared to have undergone slight buccal bone resorption and in the vitamin D (DI) group; some of the bone that was in close contact to the implant showed greater appositional growth than the melatonin group (M1) (Figs. 11 & 12). Newly formed bone was similar in both groups [DI and MI]: in direct contact with the implant surfaces, showing active remodeling with a mixed pattern of densely organized mature and immature bone. No fibrous connective tissue was observed at the bone-to-implant interface (Fig. 12).

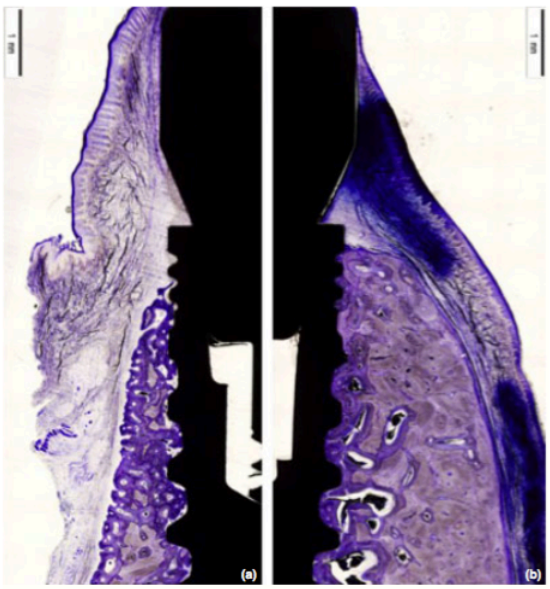

Fig. 11. Ground sections illustrating healing after 12 weeks of a Vitamin D implant group (DI). (a) Proper soft and hard tissue adaptation around the neck is observed. (b) Bone remodeling is clearly visible around the implant's crestal bone region, more relevant in the buccal bone crest than lingual crest. Levai Lacsko stain, original magnification x10.

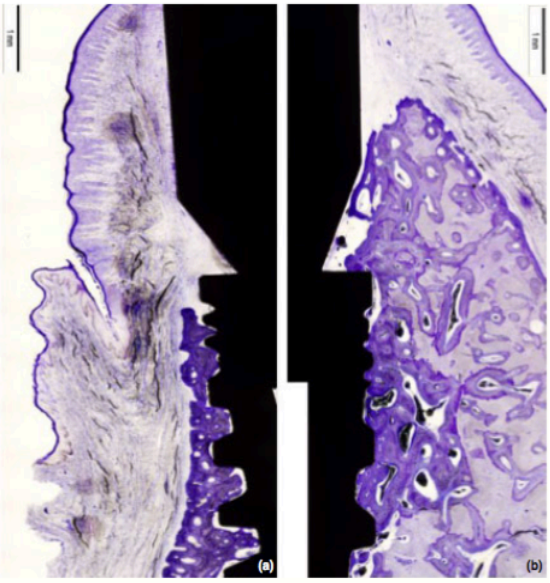

Fig. 12. Ground sections illustrating healing after 12 weeks of a Melatonin implant group (MI). (a-b) The soft and hard tissues are well adaptated around the implant neck, and new bone formation can be seen at the top of the buccal and lingual crests. Levai Lacsko stain, original magnification x10.

Fig. 13 shows a lingual histological section of a DI implant treated with a topical application of vitamin D 10%; direct bone-to-implant contact with increased inter-thread bone can be observed, and an increment in bone growth and vascular tissue compared with Melatonin implants and Control impants group (Figs. 14 & 15).

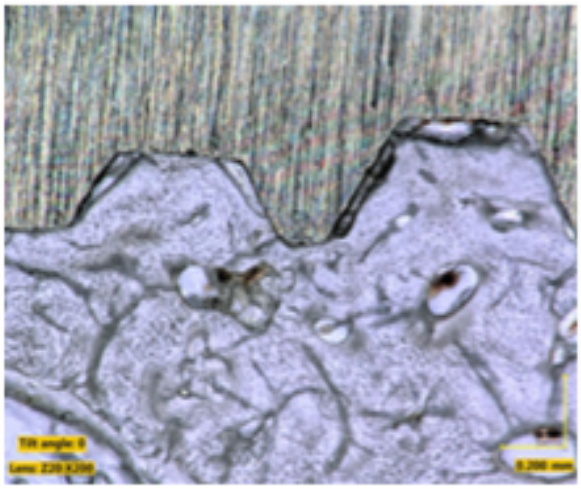

Fig. 13. Intra-thread bone in close contact with the implant in vitamin D implant group; Histological images obtained after 12 weeks healing. Magnification x200.

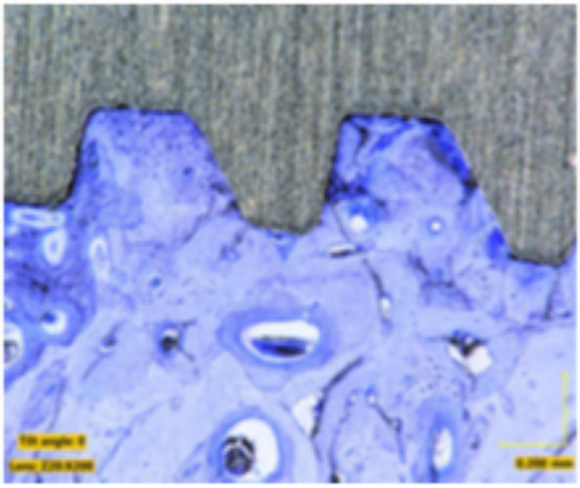

Fig. 14. Intra-thread bone in close contact with the implant in Melatonin implant group; similar percentages of newly formed bone were found in Vitamin D group. Magnification x200.

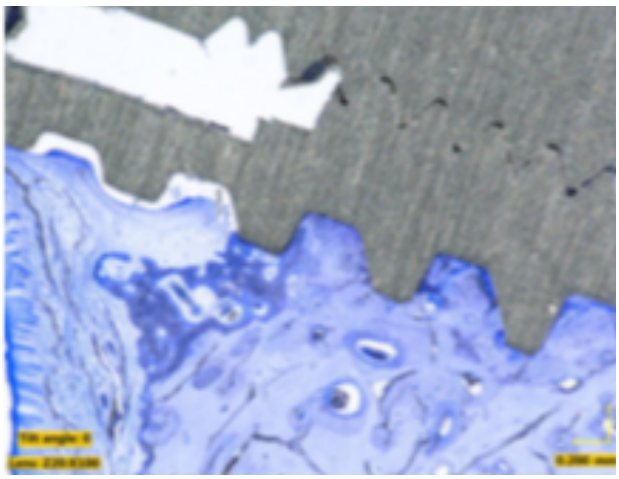

Fig. 15. Small amount of newly bone formation was found in Control implant group compared with Vitamin D and Melatonin Groups. Magnification x100.

Linear measurements

The results of the various linear measurements are presented in Table 7. Statistically significant differences were found in the buccal and lingual areas. Linear measurements showed that the MI group showed significantly less lingual crestal bone loss (CBL) (MI 0.52±0.71*), compared with DI (0.96±1.21) and CI (0.99±1.21) ($P < 0.05$). Linear measurements of buccal CBL showed significantly less buccal bone loss in test DI (0.36±0.12) than CI (1.34±1.23) and MI (1.05±1.38) ($P = 0.038$).

	DI Group (n = 12) B		MI Group (n = 12) C		CI Group (n = 12) A		
Variable	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	P value
P-aJE Buc	2.81 ± 0.24	2.41 ± 3.56	2.47 ± 0.43	2.42 ± 2.53	2.72 ± 0.78	2.67 ± 2.79	0.436
aJE-cBI Buc	1.97 ± 0.98	1.56 ± 2.34	0.64 ± 0.59	0.59 ± 0.69	0.91 ± 1.11	0.87 ± 1.10	0.342
IS-cBI Buc (CBL)	0.36 ± 0.12	0.36 ± 0.42	1.05 ± 1.38	1.30 ± 1.41	1.34 ± 1.23	1.30 ± 1.39	**0.038 AB/BC**
P-cBI Buc (PIM)	3.78 ± 0.97	3.73 ± 3.83	3.56 ± 0.47	3.51 ± 3.60	4.39 ± 0.56	4.31 ± 4.42	0.232
IS-BC Buc	1.88 ± 0.95	1.83 ± 1.94	1.141 ± 12	1.09 ± 1.21	1.78 ± 1.23	1.71 ± 1.83	0.387
fBIC-Buc	0.46 ± 0.82	0.41 ± 0.49	0.09 ± 0.61	0.05 ± 0.13	0.53 ± 0.87	0.49 ± 0.59	0.068
P-aJE Lin	1.59 ± 0.41	1.51 ± 1.63	2.43 ± 0.52	2.39 ± 2.49	2.14 ± 0.76	1.99 ± 2.21	0.354
aJE-cBI Lin	0.78 ± 0.43	0.72 ± 0.83	0.66 ± 1.51	0.59 ± 0.69	0.62 ± 1.11	0.61 ± 0.72	0.257
IS-cBI Lin (CBL)	0.96 ± 1.21	0.92 ± 1.05	0.52 ± 0.71	1.11 ± 1.15	0.99 ± 1.21	0.87 ± 1.12	**0.042 AB/BC**
P-cBI Lin (PIM)	3.25 ± 0.58	3.49 ± 3.59	3.11 ± 1.34	3.95 ± 4.17	3.54 ± 0.67	3.48 ± 3.61	0.429
IS-BC Lin	1.24 ± 0.34	1.18 ± 1.31	1.11 ± 1.14	1.07 ± 1.18	1.11 ± 1.14	1.07 ± 1.18	0.278
fBIC-Lin	0.45 ± 0.93	0.41 ± 0.49	0.06 ± 1.54	0.03 ± 0.09	0.22 ± 0.66	0.19 ± 0.27	0.066

Evaluations of the first point of bone-to-implant contact (fBIC) pointed to a much smaller amount of bone loss or remodeling in the melatonin and vitamin D implant groups on both buccal and lingual sides, compared with more severe remodeling and bone loss in the control implant group.

Histological analysis found that implants treated with vitamin D and melatonin appeared to be well integrated in mature mineralized bone. The overall percentage of bone-to-implant contact was lower in the control implant group compared with the test groups.

Radiographic analysis

No evidence of major bone loss around implants (or presence of radiolucency) was observed. X-ray analysis found that all 36 implants showed uneventful osseointegration at 12 weeks and good contact between the implants and the host bone. No statistical values could be obtained because of the difficulties of x-ray standardization.

6. General Discussion

In recent years, research has assayed a wide variety of substances in the aim of improving bone regeneration and peri-implant bone responses including growth hormone, bone morphogenetic proteins, calcium coatings, fluorine coatings, magnesium, etc. (6,84,90).

The literature reports links between melatonin (4,74,91), and vitamin D, and bone metabolism (79). But their effect on immediate implants remains a subject for investigation.

The present trial demonstrated that bone formation around immediate dental implants treated with melatonin or vitamin D improved in comparison with untreated control implants. Implants treated with melatonin showed higher bone-to-implant contact values, and more new bone formation. The melatonin implants showed less lingual crestal bone loss, and less peri-implant mucosa on the lingual aspect. These results may be explained by the stimulatory effect of melatonin on human osteoblast proliferation and differentiation, as well as its effect on the synthesis of collagen type I and other bone matrix proteins (74,91).

The BIC and new bone formation values registered in the present study agree with an experimental study by Tresguerres *et al*. (6) of New Zealand rabbits, which found that the topical application of melatonin (3mg) to implant sites improved trabecular bone-to-implant contact when compared with control implants (13.62±1.44 vs. 24.61±2.87); it also improved new bone formation (8.68±1.61 vs. 4.02±0.36). In a similar study carried out by Calvo-Guirado *et al*. (71), implants treated with melatonin inserted in rabbit tibiae exhibited better results for the melatonin-treated group. Calvo-Guirado *et al*. (73) and Cutando *et al*. (84) have reported similar results, claiming that the effect of melatonin is more powerful at the early stages of bone formation. This may be explained by the fact that melatonin stimulates cell production and accelerates

cell differentiation of the osteoid matrix, resulting in earlier synthesis and mineralization of the bone matrix.

In the present study, slightly greater BIC and new bone formation was observed in the test groups. In agreement with these results, Cutando *el al*. (84) and Guardia *et al*. (70) (who used a Beagle model with similar methodology) found statistically significant differences for inter-thread bone and new bone formation at five- and eight-week evaluations. At five weeks melatonin applied to delayed implants increased inter-thread bone (72.56±3.62 vs. 64.08±8.68) and new bone formation (80.48±1.99 vs. 72.53±4.54). At eight weeks, the same parameters showed statistically significant differences for inter-thread bone (82.49±3.87 vs. 80.57±2.28), and new bone formation (90.71±2.25 vs. 88.09±1.38).

Muñoz *et al*. (68) conducted a study to evaluate the effect of topical applications of growth hormone (GH) and melatonin (ML) on dental implant osseointegration in a Beagle dog model at two-, five- and eight-week observation times. They concluded that the topical administration of GH-ML significantly increased all osseointegration parameters (BIC, total peri-implant bone area, and percentage of new bone formation) at two weeks compared with control implants. Similar results were obtained at five weeks, but at eight weeks statistically significant differences were only found in the percentage of new bone formation (9.04% vs. 7.53%). The authors suggest that both molecules promote osseointegration by stimulating osteoblasts and inhibiting osteoclasts, and so improve implant osseointegration.

Calvo-Guirado *et al*. (69,90) performed a study in Beagle dogs testing three different implant surface treatments: non-melatonin surface, melatonin-treated surface and melatonin mixed with porcine bone-treated surface. At the four-week evaluation, the group with implant surfaces treated with melatonin alone showed significant increases in bone in direct contact with the implants, bone density, and new bone formation, in comparison with control implants. At four

weeks, the researchers observed a higher number of osteoblasts in the peri-implant zone, and greater quantities of mineralized bone matrix around the melatonin-treated implants; as a consequence, BIC values and total peri-implant bone were significantly higher. It was seen that pre-osteoblasts matured into osteoblasts within 12 days in melatonin-treated implants, while in the control group pre-osteoblasts took 21 days to mature.

The present study found differences in linear measurements on the lingual aspect. The melatonin group showed significantly less crestal bone loss on the lingual aspect, which can be explained by the fact that melatonin acts as an antioxidant and is able to neutralize free radicals, inhibiting osteoclast activity and so crestal bone resorption but provably ineffective on avoiding bundle bone resorption(2,3,90). The fact that melatonin has an anti-inflammatory effect through its neutralization of the free radicals generated by bacteria could explain the lower levels of inflammation observed and so the smaller amounts of peri-implant mucosa.

Vitamin D implants (DI) showed higher BIC values compared with CI, and greater new bone formation compared with CI and MI. Vitamin D treated implants exhibited less buccal crestal bone loss values compared to control and melatonin implants. The absence of larger, statistically significant differences might be explained by the fact that specimens were in good general health, and vitamin D has been demonstrated to have the greatest effect on populations with low levels of vitamin D.

Kelly *et al*. (92), in a study performed in rats deprived of vitamin D intake and light exposure, found that at two weeks implants in test groups showed statistically less BIC compared with control implants. In this study, the authors affirm that vitamin D deficiency significantly impaired the osseointegration of titanium implants. In agreement with these results, Dvorak *et al*. (85), found that at six and eight weeks, vitamin D deficiency led only to significantly decreased cortical BIC; the authors explain this by the fact that their specimens

only suffered moderate vitamin D deficiencies in comparison with Kelly's specimens that suffered severe deficiencies. The authors concluded that the overall process of peri-implant bone formation is not substantially altered by vitamin D deficiency.

In agreement with the present results, Hong *et al*. (80) in a study performed in healthy Beagle dogs created surgical defects and supplemented the dogs with high-doses of vitamin D and calcium for four weeks. At four weeks the Vitamin D/Calcium supplemented group presented increased new bone formation, increased bone density, and reduced crestal bone loss when compared with non-supplemented groups.

Naito *et al*. (63), in an experimental study in rabbit tibiae, evaluated BIC and new bone formation around 28 implants at 6 weeks. In this study, the authors used machined implants with three degrees of vitamin D coating and compared them with a control group (untreated machined implants). After six weeks, no statistically significant differences could be found among the four groups. The authors explain this by the fact that either the machined surfaces cannot sustain sufficient concentrations of vitamin D or that the protein underwent damage caused by the coating process. The authors use machined implants to potentiate the effect of vitamin D; they suggest that as rough implants improve osseointegration, this could cause an alteration of the potential effect of vitamin D.

Cho *et al*. (64), inserted implants with a vitamin D coating in rabbit tibiae. Osseointegration was determined by calculating BIC values at four and 12 weeks. At four weeks, test implants showed higher total BIC values (37.08±10.18) compared with control implants (28.01±8.70) ($p<.028$). At 12 weeks, statistically significant differences were also found between the two groups (29.53±9.49) and (39.10±7.68) respectively ($P = 0.013$).

Contrary to all these results, Akhavan *et al*. (72) and Fügl *et al*. (89) found that vitamin D oral supplements administered to vitamin D-deficient specimens had no effect on BIC formation and were not time-dependent. These authors

suggest that the absence of statistically significant results could be due to inadequate doses of vitamin D intake. Moreover, they suggest that age; body weight, sex, environmental factors, or genetic variations of vitamin D receptors might also influence the effects of vitamin D.

In general, most studies have shown better outcomes in melatonin-treated groups than the present study, and the effect of this hormone would appear to be greater during the early stages of osseointegration. But most of these studies have used healed areas. Differences in findings could be due to the time of implant placement (delayed or immediate) and varying observation periods. The process initiated by dental extraction can modify the healing of immediately placed implants. Experimental model, dose, vitamin D deficiency, Calcium supplementation, administration route, receptor polymorphisms, sun exposure, and observation period all seem to influence research outcomes. So the potential effect of oral or local vitamin D supplementation around titanium implants remains unclear.

Nevertheless, the present study demonstrated that after twelve weeks osseointegration, melatonin and vitamin D had a positive effect on bone formation around implants when these were placed immediately after dental extraction. However, the results of this animal pilot study should be interpreted with caution, as the heterogeneity of methodologies across different studies appears to cause different outcomes.

7. Conclusions

Paper 1

Within the limits of this foxhound dog study, topical applications of melatonin on implants placed immediately after extraction improved dental implant osseointegration and reduced lingual bone resorption and peri-implant mucosa thickness after twelve weeks osseointegration.

Paper 2

Within the limits of this foxhound dog study, topical applications of vitamin D to implants placed immediately after extraction would appear to reduce crestal bone loss and increase bone-to-implant contact after 12 weeks osseointegration.

Paper 3

Within the limitations of this foxhound dog study, the topical application of 5% Melatonin or 10% Vitamin D improved bone formation around implants placed immediately after extraction and helped to reduce crestal bone loss after 12 weeks of osseointegration.

General

Within the limitations of this foxhound dog study, the topical application of bioactive elements Melatonin 5% and Vitamin D 10% applied directly over the surface of implants placed immediately after extraction, enhance osseointegration determined by histological and histomorphometric parameters at twelve weeks.

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12. Annexes

1. Resumen

Esta tesis doctoral es un compendio de tres publicaciones sobre la aplicación tópica de mediadores biológicos aplicados sobre la superficie de implantes dentales oseointegrados, más concretamente sobre los efectos de la Melatonina y la Vitamina D aplicados de forma tópica sobre la superficie de implantes dentales colocados inmediatamente después de la extracción dental. Este estudio se desarrollo en un modelo animal con el fin de mejorar la oseointegración bajo análisis histológico e histomorfométrico.

Actualmente la investigación en el campo de la implantología, se ha centrado en desarrollar sustitutos óseos y superficies de implantes modificados bioquímicamente para conseguir una mayor y mas rápida oseointegración. La aplicación de mediadores biológicos sobre la superficie de los implantes dentales o integrados en biomateriales son capaces de inducir una respuesta específica sobre células y tejidos, mejorando la cantidad y calidad de los tejidos peri-implantários, reduciendo los costes y los tiempos de tratamiento, además de mejorar los resultados a largo plazo.

Para el desarrollo de este estudio se realizaron las extracciones las raíces distales de los premolares mandibulares (P_2, P_3, P_4) de seis perros American Foxhound. En los alveolos post-extracción se colocaron seis implantes inmediatos divididos tres grupos aleatorios.: Grupo test 1 Implantes tratados con melatonina 5% (MI), Grupo test 2 implantes tratados con vitamina D 10% (DI) Grupo Control, implantes sin tratamiento (CI). Se obtuvieron cortes histológicos a las 12 semanas tras la intervención. Se analizó el Bone-to-implant Contact (total BIC),

la neo-formación ósea (NBF), el hueso inter-rosca (ITB), además se realizaron mediciones histológicas a las muestras procesadas (HLM).

El presente estudio demostró que los implantes inmediatos tratados con melatonina y vitamina D mostraron mejores valores de oseointegración y de mediciones histológicas comparado con los implantes control. Los implantes tratados con Vitamina D mostraron valores mas altos para BIC y mayor neo-formación ósea. Además, estos implantes mostraron menor pedida ósea crestal bucal comparado con los implantes control. Por otro lado, los implantes tratados con Melatonina mostraron mayores valores de BIC comparados con los implantes control y mayor neo-formación ósea comparado con los sin tratamiento, además de presentar menor perdida ósea crestal en la zona lingual.

Dentro de las limitaciones de este estudio animal, la aplicación tópica de Melatonina al 5% y de Vitamina D al 10% sobre la superficie de los implantes colocados de manera inmediata tras la extracción, mostraron mejores resultados de oseointegración, además de reducir la perdida ósea crestal tras 12 semanas de oseointegración.

2. Acceptance Ph.D. Protocol

Universitat Internacional de Catalunya | Escola de Doctorat

Barcelona, 9 de enero de 2014

Sr. Oscar Cosme Salomó Coll
Sant Antoni Mª Claret 20
08304 Mataró

Estimado Sr.

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 al Sr. Oscar Cosme Salomó Coll al Periodo de Investigación del Doctorado de Odontología.

Se acuerda aprobar el Proyecto de Tesis titulado "Evaluación Histológica E Histomorfométrica De La Neofromación Ósea Con Implantes Inmediatos C1 Impregnados Con Melatonina Y Vitamina D: 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 saludarlo cordialmente,

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

Universitat Internacional de Catalunya
VICERECTORAT DE RECERCA

Universitat Internacional de Catalunya
REGISTRE GENERAL
Sortida 00011
Data 10 01 14

3. Ethical Committee of Investigation (U. Murcia)

Región de Murcia
Consejería de Agricultura y Agua
Dirección General de Ganadería y Pesca

Servicio de Sanidad Animal
Plaza Juan XXIII s/n
30008 Murcia

T. 012
T.968.368913
F. 968 362863
F.968.394378
www.carm.es/cagric

Región de Murcia
Consejería de Agricultura y Agua
REGISTRO DE SALIDA
Fecha 21 ABR 2014
Nº 91.601

JOSÉ LUÍS CALVO GUIRADO
DEPARTAMENTO DE ODONTOLOGÍA
INTEGRADA DE ADULTOS.
HOSPITAL MORALES MESEGUER, 2ª
PLANTA
C/ MARQUÉS DE LOS VÉLEZ
30007 MURCIA

RESOLUCION

Vista la solicitud presentada con fecha 10/04/2014 y nº de registro de entrada 3000Nº201400174941, para la autorización de la realización de un proyecto de investigación con animales presentada por D. José Luís Calvo Guirado, como responsable del mismo.

Vista la propuesta del proyecto denominado:"Clinical evauation of bone to implant contact around inmediate implants with melatonin and vitamin D coating: An experimental study in dogs"

Visto el informe favorable del comité ético del establecimiento usuario con código REGA ES300305440012.

Visto el resumen no técnico del proyecto.

Visto el resultado favorable de la evaluación del proyecto por el órgano habilitado "Comité Ético de Experimentación Animal (CEEA) de la Universidad de Murcia".

Visto que en dicha evaluación se indica que no es necesario llevar a cabo la evolución retrospectiva del proyecto.

Visto que en la propuesta del proyecto se indica que hay doce colaboradores que van a participar en el mismo y no poseen la capacitación previa adecuada, cuyos nombres son: Julián Andrés Balanta Melo, Liliana Otero, Daniel Henao, Antonio Aguilar Salvatierra Raya, Carlos Pérez Albacete Martinez, Cristina Pérez Sánchez, Laura López Marí, Jordi Gargallo, Marta Satorres Nieto, Giovanna Lezzi, Marcus Abboud y Eugenio Velasco Ortega.

Visto que dichos colaboradores, según se indica en la propuesta del proyecto serán supervisadas por el investigador responsable del proyecto, que sí dispone de capacitación.

Visto el Real Decreto 53/2013, de 1 de febrero, por el que se establecen las normas básicas aplicables para la protección de los animales utilizados en experimentación y otros fines científicos, incluyendo la docencia.

Visto el Informe emitido por el Jefe del Servicio de Sanidad Animal el 15 de abril de 2014.

Proyecto de investigación A1320140401

Región de Murcia
Consejería de Agricultura y Agua
Dirección General de Ganadería y Pesca

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Considerando la competencia que tiene atribuida esta Dirección General sobre la base de lo dispuesto en el Decreto 26/2011, de 25 de febrero, por el que se establece la estructura orgánica de la Consejería de Agricultura, y Agua y con independencia de otras actuaciones que esta u otra Administración puedan emprender, esta Dirección General

RESUELVE

Conceder autorización a D. José Luís Calvo Guirado, para la realización del proyecto solicitado, como responsable y usuario del mismo, asignándole el código de identificación **Nº A1320140401**, teniendo esta autorización una validez que se corresponderá con la duración prevista en su memoria, con un máximo de cinco años, siempre y cuando no se produzca una modificación relevante en dicho procedimiento, en cuyo caso sería necesario efectuar una nueva solicitud de autorización a la autoridad competente.

El proyecto se llevará a cabo en el establecimiento usuario con código REGA ES300305440012.

Se autoriza a los doce colaboradores cuyos nombres son: Julián Andrés Balanta Melo, Liliana Otero, Daniel Henao, Antonio Aguilar Salvatierra Raya, Carlos Pérez Albacete Martinez, Cristina Pérez Sánchez, Laura López Marí, Jordi Gargallo, Marta Satorres Nieto, Giovanna Lezzi, Marcus Abboud y Eugenio Velasco Ortega, a participar en los procedimientos dentro del marco del proyecto citado siempre que se trabaje bajo la supervisión del investigador responsable del proyecto.

Lo que en cumplimiento del Art. 58 de la Ley de Régimen Jurídico de las Administraciones Públicas y del Procedimiento Común (Ley 30/1992, de 26 de noviembre) se le NOTIFICA, significándole que contra dicha RESOLUCIÓN cabe Recurso de Alzada ante el Excmo. Sr. Consejero de Agricultura y Agua en el plazo de UN MES desde la recepción de la presente notificación, sin perjuicio de poder ejercitar, en su caso, cualquier otro que se estime pertinente.

Murcia, a 15 de abril de 2014
La Directora General de Ganadería y Pesca

Consejería de Agricultura y Agua
Región de Murcia
Dirección General de Ganadería y Pesca

Carmen T. Morales Cuenca

Proyecto de investigación A1320140401

4. Ethical Committee for Research (UIC)

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

CARTA APROVACIÓ PROJECTE PEL CER

Codi de l'estudi: CIR-ELB-2014-02
Versió del protocol: 1.0
Data de la versió: 01.04.2014
Títol: Evaluación histológica e histomorfométrica de la neoformación ósea con implantes inmediatos C1 impregnados con Melatonina y Vitamina D: estudio experimental con perros

Sant Cugat del Vallès, 10 de juny de 2012

Investigador: Oscar Salomó Coll

Títol de l'estudi: Evaluación histológica e histomorfométrica de la neoformación ósea con implantes inmediatos C1 impregnados con Melatonina y Vitamina D: estudio experimental con 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