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

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Abstract

Objective: The aim of this study was to evaluate the effects on osseointegration of topical applications of melatonin vs. vitamin D over surfaces of immediate implants.

Materials and methods: Mandibular premolar distal roots (P₂, P₃, P₄) were extracted bilaterally from six American Foxhound dogs. Three conical immediate implants were randomly 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.

Results: At 12 weeks, all implants were clinically stable and histologically osseointegrated. Total BIC values were 49.20 ± 3.26 for the MI group, 49.86 ± 1.89 for DI group and 45.78 ± 4.21 for the CI group ($P < 0.018$) with statistically significant difference between the three groups. BIC percentage were 42.44 ± 2.18 for MI, 44.56 ± 1.08 for DI, and 41.95 ± 3.34 for CI groups respectively ($P > 0.05$). Inter-thread bone formation values were MI 17.56 ± 2.01 , for DI 19.87 ± 0.92 , and CI 14.56 ± 1.24 ($P > 0.05$). Statistically significant differences in peri-implant new bone formation were found between the three groups: MI 28.76 ± 1.98 , DI 32.56 ± 1.11 and CI 25.43 ± 4.67 , respectively ($P < 0.045$). Linear measurements showed that the MI group showed significantly less lingual crestal bone loss (CBL) (MI 0.59 ± 0.71), compared to DI (0.91 ± 1.21) and CI (0.93 ± 1.21) ($P < 0.042$), and less lingual peri-implant mucosa (PIM) (MI 3.11 ± 1.34), DI 3.25 ± 0.18 compared with CI 3.54 ± 1.81 ($P = 0.429$). 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 (1.11 ± 1.38) ($P = 0.078$).

Conclusions: Within the limitations of this animal study, topical applications of 5% Melatonin or 10% vitamin D improved bone formation around implants placed immediately after extraction and helped to reduce CBL after 12 weeks osseointegration.

Although immediate implants generally show good results, bone loss and soft tissue recession occur during the first year after implant placement (Kan et al. 2011), which can compromise functional as well as long-term esthetic outcomes (Degidi et al. 2008; Evans & Chen 2008; Cosyn et al. 2012; Lang et al. 2012; Tan et al. 2012). The main cause of soft and hard tissue recession is the presence of bundle bone in the buccal wall, which will inevitably disappear after dental extraction (Araújo & Lindhe 2005; Araújo et al. 2006a, 2006b). For this reason, several authors stress the need for filling the gap created between the implant and the buccal

wall to minimize bone resorption and to maintain as much volume as possible, thereby improving treatment outcomes (Botticelli et al. 2004a, 2004b, 2004c; Ferrus et al. 2010; Tsuda et al. 2011; Bottini et al. 2012). Gap filling involves placing a grafting material to minimize bone resorption and improve implant osseointegration. But new bone formation will be influenced by the chemical and physical properties of the grafting material (Schwarz et al. 2008). Moreover, no bone substitute is able to respond to physiological loads or biochemical stimuli in the way that living tissue does (Hench 2002).

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Recently, dental research has focussed on improving bone substitutes and implant surfaces by morphologic or biochemical modification to achieve faster and better osseointegration (Calvo-Guirado et al. 2010a; Delgado-Ruiz et al. 2011). The application of biological mediators over the implant surface or embedded in the biomaterial can induce specific cell and tissue responses. Biochemical modifications can improve the quality and the amount of peri-implant tissue, reducing economic costs and treatment times, and consequently improving long-term outcomes. Examples of biological mediators used around implants to improve osseointegration are: Growth Hormone (Gómez-Moreno et al. 2009; Calvo-Guirado et al. 2011), melatonin (Calvo-Guirado et al. 2009, 2010a, 2010b; Salomó-Coll et al. 2015a), and vitamin D (Akhavan et al. 2012; Salomó-Coll et al. 2015b).

Melatonin –or N-acetyl 5-methoxytryptamine – is a hormone synthesized mainly in the pineal gland that is involved in various functions such as circadian rhythms, cell protection, antioxidant, and bone formation and remodeling. Melatonin stimulates the proliferation of type I collagen fibers (Nakade et al. 1999) and acts directly on osteoclasts to inhibit bone resorption; as a consequence, melatonin appears to improve bone formation (Roth et al. 1999). Several studies have demonstrated the effects of melatonin application around dental implants (Cutando et al. 2008; Calvo-Guirado et al. 2009, 2010a, 2010b; Guardia et al. 2011; Tresguerres et al. 2012) but its effect on immediate implants remains unclear.

Vitamin D comprises a group of fat steroid hormones. These hormones can be found in several forms: ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). Vitamin D₃ is formed from cholesterol by the skin exposed to ultraviolet light, and its most active form is 1 α , 25-dihydroxyvitamin D₃ (1 α ,25-(OH)₂D₃); this active form is commonly known as calcitriol. This biomolecule has a fundamental role in bone and calcium homeostasis (Christakos et al. 2013), acting directly on calcium absorption in the intestines and kidney (Cooper 2000; Van Driel et al. 2006). It enhances bone resorption and reduces calcium and phosphate excretion. Vitamin D receptors are present in osteoblasts and have a direct effect on cells by regulating gene expression (Van Leeuwen et al. 2001; Van Driel et al. 2006; Cho et al. 2011; Christodoulou et al. 2013); it is also present in other proteins involved in bone formation such as osteocalcin (Ong et al. 1998).

The aim of this study was to determine whether the topical application of melatonin 5% or vitamin D₂ 10%, applied directly over the implant surface, improve osseointegration evaluated by histomorphometric analysis and histological linear measurements of bone-to-implant contact at 12 weeks after immediate implant placement.

Material and methods

Study design

Six American Foxhound dogs of approximately 1 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 test group (MI), vitamin D test group (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 35 μ g/kg (Medetor 1 mg, Virbac, CP-Pharma Handelsgesellschaft GmbH, Burgdorf 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 40 mg, 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 were sectioned in a bucco-lingual direction at the bifurcation using a tungsten-carbide bur (Fig. 1a). Distal roots were extracted individually using a periosteal elevator 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 test group (DI) and 12 implants alone (CI)), randomized into six dogs. Before implant placement, melatonin test group (MI) implants were submerged in melatonin at 5% in saline solution (TM-M5250; Sigma-Aldrich®, St. Louis, MO, USA), and vitamin test group (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

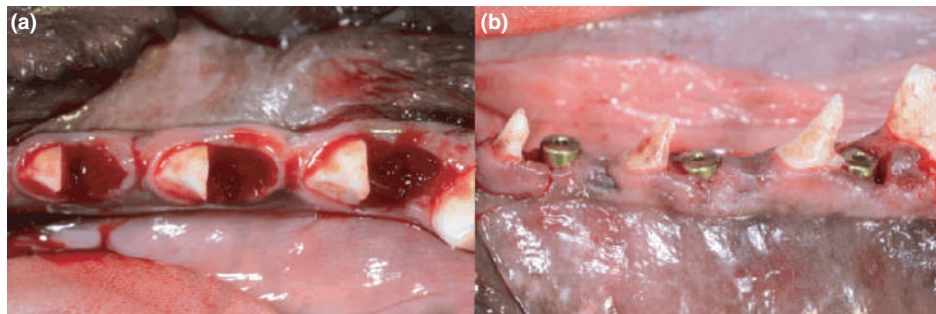


Fig. 1. (a): Lateral image of one hemiarch mandibular premolars odonto-sectioned (P₂, P₃, P₄) and (b) three implants were randomly placed immediately in the distal socket.

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 (Fig. 1). 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® Barlev, Israel) were placed to allow a non-submerged healing protocol (Fig. 1 b). 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 600 mg, Rimadyl, Pfizer, Madrid, Spain). The sutures were removed after 2 weeks. Animals were fed with a soft diet for 7 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) (Fig. 2). Afterwards, the animals were sacrificed (12 weeks after implant placement by means of pentothal sodium (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 Apparatebau, 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 100 µm thickness and ground to approximately 40 µm final thickness with an Exakt 400s CS grinding device (Exakt Apparatebau, Norderstedt, Hamburg, Germany).

Sections were stained Lévai Laczkó stain (Laczkó & Lévai 1975) 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 10× 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. 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 (Interthread 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 3a and 3b). Histomorphometric analysis was performed using a video camera (Sony 3CCD, Berlin, Germany) with 10× magnification. Interthread images were digitalized (Axiophot-System, Zeiss, Oberkochen, Germany), stored, and reference points were plotted.

The following buccal and lingual measurements were made at ×10 magnification (Fig. 4):

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).

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

Factors such as individual difference and position of the implant could be excluded as not significant. Despite having several

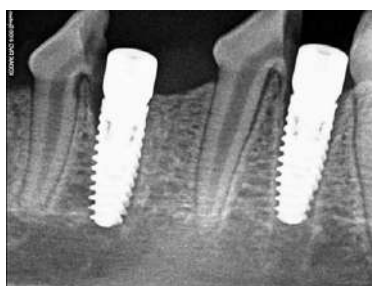


Fig. 2. Digital radiographs taken after 12 weeks of healing.

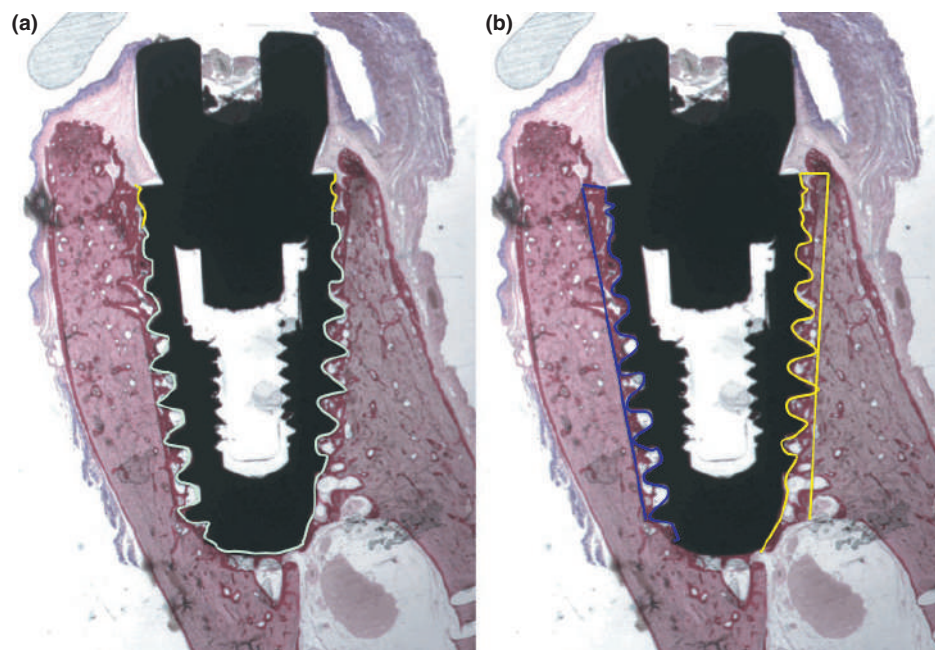


Fig. 3. BIC scheme: (a) Green lines show Total BIC, and red line shows crestal bone loss (b) yellow line delimitates interthread space, and blue line shows New Bone Formation.

samples on the same specimen, the dogs were considered independent variable. Dependent variables included the histomorphometric measurements previously described. Values were expressed as medians, means, and standard deviation. As the distribution of data was not normal, a nonparametric Wilcoxon signed rank test was applied. The medians of differences between groups were analyzed using a Brunner-Langer test (nonparametric repeated measures analysis of variance) for the three groups was applied. The level of statistical significance was established at $P < 0.05$. Because of the limited amount of samples, no correction test could be performed, and major amount of false-positive results could be expected. All histomorphometric parameters were analyzed using descriptive methods (SPSS 21.0 software, Chicago, IL, USA).

Results

Histological and histomorphometric analysis

The results of histomorphometric measurement are shown in Table 1. At 12 weeks, Total BIC values were 49.86 ± 1.89 for DI group, 49.20 ± 3.26 for the MI group and 45.78 ± 4.21 for the CI group ($P < 0.018$). BIC % were 44.56 ± 1.08 for DI, 42.44 ± 2.18 for MI, and 41.05 ± 3.34 for CI ($P > .05$). Interthread bone formation values were MI 15.99 ± 2.43 , for DI 19.56 ± 0.78 , and CI 15.51 ± 3.62 ($P > .05$). 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 ($P < 0.05$).

No gaps at the implant-bone interface were founded in both test implant groups (melatonin and Vitamin D). No inflammatory cell infiltrate, foreign body reaction cells, or multinucleated giant cells were found in both implant groups. The histomorphometry showed no significant differences ($P < 0.05$) in the measurements when the mean distance between the original level of the coronal bone level to the top of the implant were compared for the test group implants versus control groups implants.

All histological images showed good bone healing without any signs of inflammation, but control implant group (CI), showed more buccal crest bone resorption compared with vitamin D implant (DI) and Melatonin implant group (MI), respectively (Fig. 5).

The cortical bone seemed to have undergone a small buccal bone resorption and apposition process, where some of the bone that was in close contact to the implant in Vitamin D group, compared with melatonin group (MI) (Fig. 6). Newly formed bone was similar for both groups: in direct contact with implant surface, 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. 7). Fig. 8 shows a

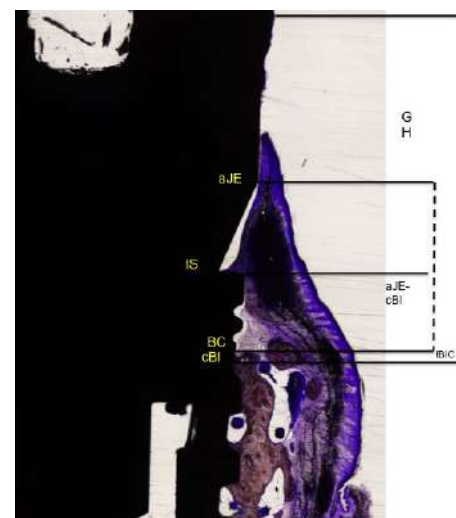


Fig. 4. Linear measurements scheme: P, top of the peri-implant mucosa; aJE, apical portion of the junctional epithelium; fBIC, first point of bone-to-implant contact; IS, implant shoulder; BC, buccal crest.

histological lingual section of a bone to implant contact treated with topical application of vitamin D 10% (DI); direct bone-to-implant contact with increased interthread bone can be observed, with an increment in bone growth and vascular tissue compared with melatonin and test group (Figs 9 and 10).

Linear measurements

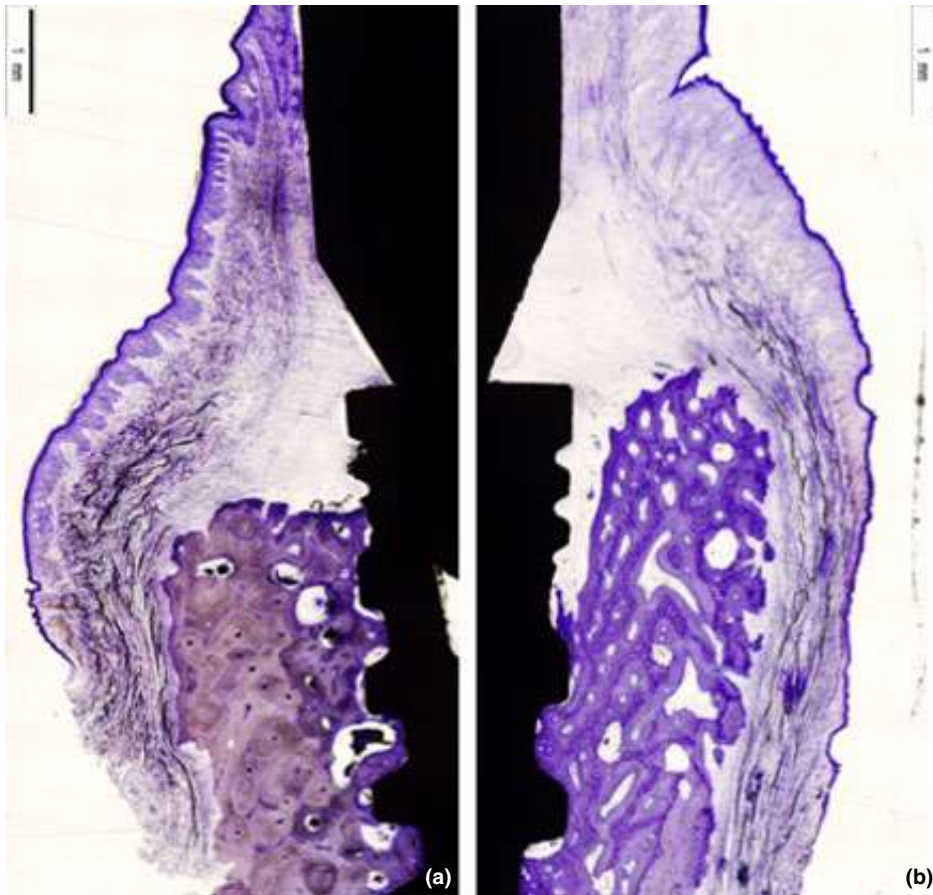
The results of the various linear measurements are presented in Table 2. Statistically significant differences were found in the buccal and lingual areas. Linear measurements showed that the MI group showed significantly less lingual crestal bone loss (CBL) (MI $0.52 \pm 0.71^*$, compared to DI (0.96 ± 1.21) and CI (0.99 ± 1.21) ($P < 0.05$), and less lingual peri-implant mucosa (PIM) (MI 3.11 ± 1.34 , compared with CI 3.54 ± 1.81). 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) ($P = 0.038$). Average and total ranges are expressed in Table 3.

Evaluating the fBIC, suggesting a much smaller amount of bone loss or remodeling in the Melatonin and Vitamin D implant group, both on the buccal and lingual sides, compared with more severe remodeling and bone loss for the Control implant group.

The Vitamin D and Melatonin implant groups showed at the histologic examination appeared to be well integrated into mature mineralized bone (Fig. 9). The overall percentage of bone-to-implant contact was lower at the control implant group compared with the test sites. (Fig. 10).

Table 1. Mean values (% $\pm$ SD) Brunner–Langer (nonparametric mixed model). Summarizing the mean values and standard deviation of BIC. The bold numbers expressed the level of significance $P < 0.05$

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

**Fig. 5.** Ground sections illustrating the 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 Leivai Lacsco stain, original magnification $\times 10$.

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 was observed. No statistical values could be obtained because of the difficulties of x-ray standardization.

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. (Calvo-Guirado et al. 2010a; Tresguerres et al. 2012; Cutando et al. 2008). The literature reports links between melatonin (Nakade et al. 1999; Roth et al. 1999; Ramírez-Fernández et al. 2013), or vitamin D and bone metabolism (Kato et al. 2015). 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 and vitamin D improved in comparison with non-treated control implants. Implants treated with

melatonin showed higher bone-to-implant contact values ($P = 0.026$), and more new bone formation ($P = 0.045$). The melatonin implants showed less lingual CBL ($P = 0.044$), and less PIM on the lingual aspect ($P = 0.020$). 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 (Nakade et al. 1999; Roth et al. 1999). The BIC and new bone formation values registered in the present study agree with an experimental study by Tresguerres et al. (2012) of New-Zealand rabbits, which found that the topical application of melatonin (3 mg) 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. (2010b), implants treated with melatonin inserted in rabbit tibiae exhibited better results for the melatonin-treated group. Similar results have been reported by Calvo-Guirado et al. (2015) and Cutando et al. (2008). The authors claim that the effect of melatonin is more powerful at the early stages of bone formation, which 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 et al. (2008) and Guardia et al. (2011) (who used a Beagle model with similar methodology) at five- and 8-week evaluations, found statistically significant differences for inter-thread bone and new bone formation. At 5 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 8 weeks, the same parameters showed statis-



Fig. 6. Ground section illustrating the healing after 12 weeks of a Vitamin D implant group (DI). (a) A proper soft and hard tissue adaptation around the neck is observed. (b) Bone remodeling is clearly visible around the crestal bone region of the implant, more relevant in buccal bone crest related to lingual crest. Levai Lacsco stain, original magnification $\times 10$.

tically 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. 2012 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 2-, 5-, and 8-week observation times. They concluded that topical administration of GH-ML significantly increased all osseointegration parameters – BIC, total peri-implant bone area, and percentage of new bone formation – at 2 weeks compared with control implants; similar results were obtained at 5 weeks, but at 8 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. 2009, 2010a, 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 4-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 4 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 in a period of 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 CBL on the lingual aspect ($P = 0.036$), which can be explained by the fact that melatonin acts as an antioxidant and has a capability to neutralize free radicals, inhibiting osteoclast activity and so crestal bone resorption (Cutando et al. 2007a, 2007b; Calvo-Guirado et al. 2010a). 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 PIM ($P = 0.042$).

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 CBL 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. 2009; in a study performed in rats deprived of vitamin D intake and light exposure, found that at 2 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. 2012; found that at six and 8 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. 2012 in a study performed in healthy Beagle dogs created surgical defects and supplemented the dogs with high-doses of vitamin D and calcium for 4 weeks. At 4 weeks the VitD/Ca-supplemented group presented increased new bone formation, increased bone density, and reduced CBL when compared with non-supplemented groups. Naito et al. 2014, 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

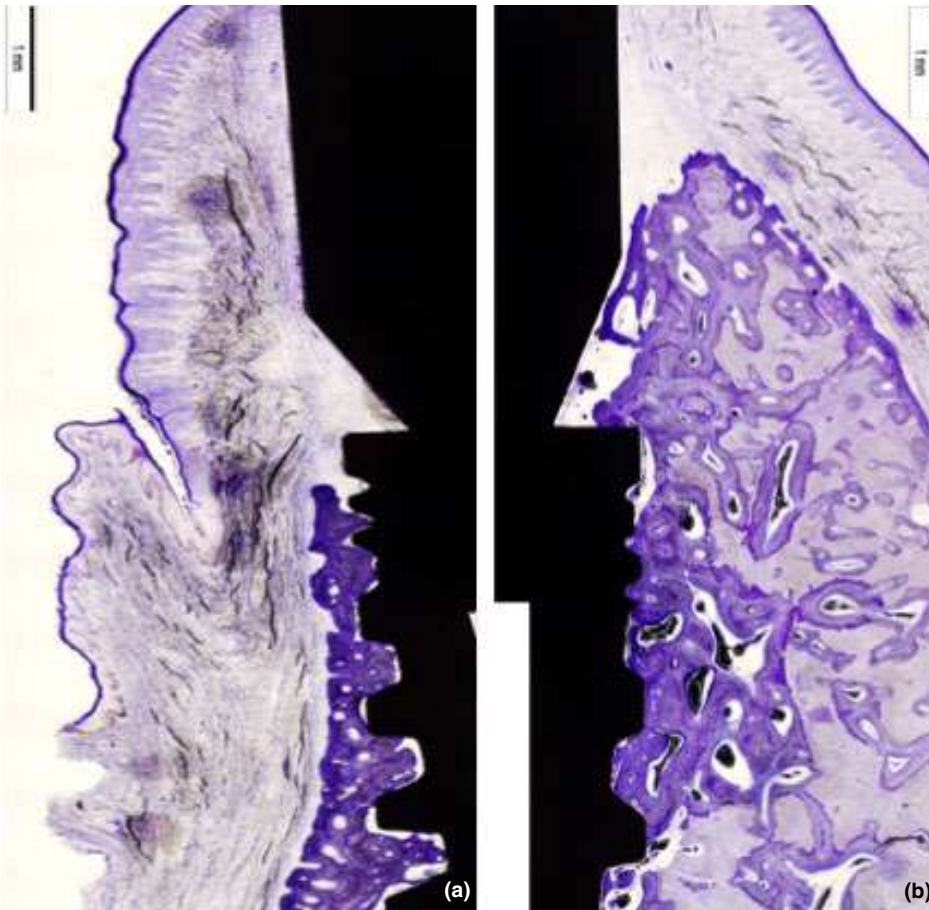


Fig. 7. Ground section illustrating the healing after 12 weeks of a melatonin implant (MI). (a–b) The soft and the hard tissues are well adapted around the implant neck, and new bone formation was founded at the top of buccal and lingual crest. Levai Lacsco stain, original magnification $\times 10$.

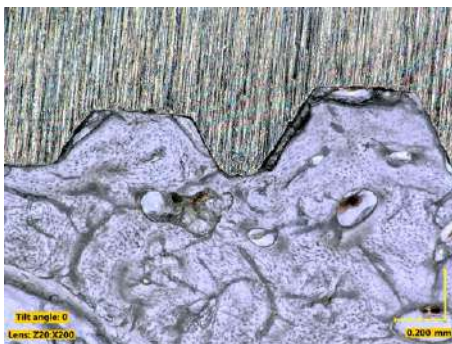


Fig. 8. Intrathread bone that was in close contact to the implant in Vitamin D implant group after 12 weeks of follow-up. magnification $\times 200$.

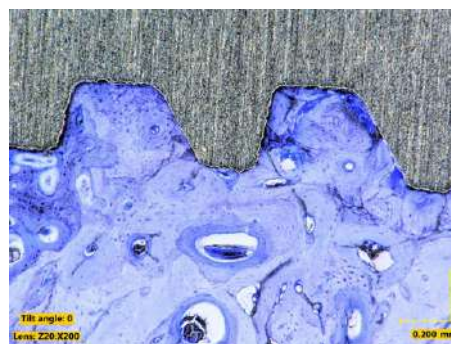


Fig. 9. Intrathread bone that was in close contact to the implant in Melatonin implant group after 12 weeks of follow-up similar percentage of newly formed bone in Vitamin D group was found. magnification $\times 200$.

6 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 can cause an alteration of the potential effect of vitamin D.

Cho et al. 2011 inserted implants in rabbit tibiae with a vitamin D coating. At four and 12 weeks, osseointegration was determined through BIC values. At 4 weeks, test implants showed higher Total BIC values (37.08 ± 10.18) compared with control

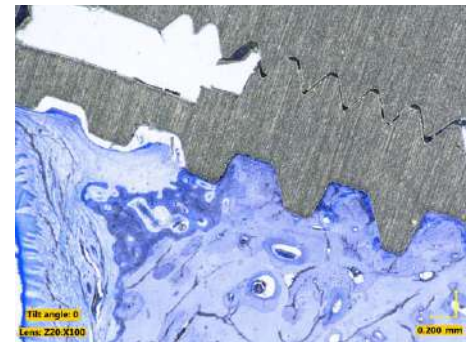


Fig. 10. Small amount of newly bone formation was founded in Control implant group magnification $\times 200$.

implants (28.01 ± 8.70) ($P < .028$). At 12 weeks, statistically significant differences were found between the two groups (29.53 ± 9.49) and (39.10 ± 7.68) ($P < .013$). Contrary with all these results, Fögl et al. 2015 and Akhavan et al. 2012 found that vitamin D oral supplements administered to vitamin D-deficient specimens had no effect on BIC formation and was 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 between findings could be due to the time of implant placement (delayed or immediate) and 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 12 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.

Table 2. Mean values (% ± SD) Brunner–Langer (nonparametric mixed model) of mean values and standard deviation of Linear Measurements Buccal and Lingual respectively. The bold numbers expressed the level of significance $P < 0.05$

Variable	DI Group (n = 12) B		MI Group (n = 12) C		CI Group (n = 12) A		P value
	Mean ± SD	Median	Mean ± SD	Median	Mean ± SD	Median	
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

Table 3. Average and total ranges for Linear Measurements after 12 weeks of healing

Variable	CI Group (n = 12) A	DI Group (n = 12) B	MI Group (n = 12) C	P value
	Average Range	Average Range	Average Range	
IS-BC Buc	10.25	14.67	14.83	0.366
IS-BC Lin	9.63	15.29	15.46	0.071
IS-cBI Buc (CBL)	10.58	8.42	13.42	0.037
IS-cBI Lin (CBL)	10.53	13.58	15.38	0.044
P-aJE_Buc	14.71	9.83	10.75	0.483
P-aJE_Lin	16.59	9.08	7.75	0.012
aJE-cBI Buc	12.88	13.33	10.92	0.274
aJE-cBI Lin	10.71	14.67	13.92	0.074
fBIC-Buc	12.67	14.50	10.17	0.063
fBIC-Lin	13.13	11.29	12.46	0.861
P-cBI Buc (PIM)	14.19	11.17	10.46	0.242
P-cBI Lin (PIM)	14.53	11.38	9.58	0.020

The bold numbers expressed the level of significance $P < 0.05$.

Conclusions

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.

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