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

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Abstract

Objective: The aim of this study was to evaluate the effects of topical applications of melatonin over implant surfaces placed immediately after extraction by means of histological and histomorphometric analysis of peri-implant tissues.

Material and methods: Six American foxhound dogs were used in the study; mandibular premolar distal roots were extracted. Thirty-six immediate conical implants were randomly assigned to the distal site on each site of the mandible in three groups: (Group CI) 12 titanium implants alone; (Group MI) 12 titanium implants supplemented with melatonin; and (Group DI) 12 titanium implants supplemented with vitamin D (DI). Prior to implanting test, implants (MI) were submerged in melatonin 5% solution, and implants from (DI) group were submerged in vitamin D 10% solution. No treatment was applied at control implants. After 12 weeks, animals were sacrificed. Block sections were obtained 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 analyzed.

Results: At 12 weeks, all implants were clinically stable and histologically osseointegrated. Total BIC values were $48.36 \pm 7.45^*$ for the MI group and 44.82 ± 10.98 for the CI group ($P = 0.035$) with statistically significant difference between groups. BIC% were 41.36 ± 3.93 for MI and 41.34 ± 9.26 for CI ($P > 0.05$). Inter-thread bone formation values were $15.99 \pm 2.43^*$ and 14.79 ± 3.62 ($P = 0.03$), MI showing significantly better results. No statistically significant differences in peri-implant new bone formation could be found between the two groups: MI 25.37 ± 2.32 , CI 26.55 ± 7.75 ($P > 0.05$). Linear measurements showed that the MI group showed significantly less lingual crestal bone loss (CBL) (MI $0.52 \pm 0.74^*$, CI 0.92 ± 1.98) ($P = 0.045$) and less lingual peri-implant mucosa (PIM) (MI $3.13 \pm 1.41^*$, CI 3.71 ± 1.81) ($P = 0.042$). No significant differences were observed in the buccal aspect.

Conclusions: Within the limitations of this animal study, the topical application of melatonin improved bone formation around immediate implants and reduced lingual bone and lingual peri-implant mucosa, after 12 weeks of osseointegration.

Immediate implants have proved to be an effective therapy not only for reducing the number of surgical procedures (Lazzara 1989; Paolantonio et al. 2001; Nemcovsky & Artzi 2002), but also for simplifying clinical techniques and reducing total treatment time. Furthermore, the procedure achieves survival outcomes similar to delayed implant placement (Lazzara 1989; Werbit & Goldberg 1992; Botticelli et al. 2008; Calvo-Guirado et al. 2011b, 2014c). When the immediate implant is restored immediately, this also

helps to maintain interdental papillae (Kan et al. 2011; Cosyn et al. 2012).

Although immediate implants generally show good results, the overall bone loss and soft tissue recessions that occur mainly during the first year after placement (Kan et al. 2009, 2011; Lang et al. 2012) can compromise functional as well as esthetic outcomes (De-gidi et al. 2008; Evans & Chen 2008; Cosyn et al. 2012; Lang et al. 2012; Tan et al. 2012). Provably, main reason for buccal bone and soft tissue recession is because the higher

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percentage of bundle bone in the buccal wall, which disappears after extraction, causing resorption of the buccal wall and so a reduction in bone volume (Pietrokovski & Massler 1967; Cardaropoli et al. 2003; Schropp et al. 2003; Araújo & Lindhe 2005a). This anatomical characteristic must be taken into consideration if pronounced buccal bone resorption is to be avoided following immediate implant placement (Covani et al. 2004; Araújo et al. 2005b, 2006a,b; Barone et al. 2011). Other possible causes of soft and hard tissue remodeling around have been formulated, which include surgical trauma (Esposito et al. 1998), implant abutment microgaps (Broggini et al. 2006), biologic width establishment (Berglundh & Lindhe 1996), apicocoronal implant position (Negri et al. 2012a,b; Calvo-Guirado et al. 2013a, 2014b), buccolingual position (Buser et al. 2004; Chen et al. 2007; Kan et al. 2009), implant abutment design (Degidi et al. 2008; Buser et al. 2011; Calvo-Guirado et al. 2011a, 2014c; Rodríguez et al. 2011), soft tissue grafting (Grunder 2011; Tsuda et al. 2011), alveolus defect shape (Kan et al. 2007) and defects around the immediate implant (Botticelli et al. 2004a; Kan et al. 2009; Ferrus et al. 2010).

In immediate implants, the space between the buccal wall and the implant is called *jumping distance* or *buccal gap* (Botticelli et al. 2003); several authors stress the need for gap filling at the time of immediate implant placement, depending on the dimensions of the gap to minimize bone loss (Botticelli et al. 2004a,b,c; Ferrus et al. 2010; Tsuda et al. 2011; Bottini et al. 2012). 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 (Schwarz et al. 2008). 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 (Barone et al. 2011; Calvo-Guirado et al. 2011c, 2013b; Vignoletti et al. 2012). No bone substitute is able to respond to physiological loads or biochemical stimuli in the way that living tissue does (Hench 2002).

In recent years, research has focussed on improving bone substitutes and implant surfaces to achieve faster and better osseointegration by morphologic or biochemical modification (Calvo-Guirado et al. 2010a;

Delgado-Ruiz et al. 2011). These modifications can improve bone quality and quantity around dental implants, reducing economic costs and treatment times, improving osseointegration and consequently survival time. Biochemical modifications consists of the application of biologic mediators over the implant surface or into the biomaterial to induce specific cell and tissue responses, such as growth hormone (Gómez-Moreno et al. 2009; Calvo-Guirado et al. 2011a; Muñoz et al. 2012), melatonin (Calvo-Guirado et al. 2009, 2010a,b) or vitamin D (Akhavan et al. 2012).

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 remodeling. Several studies have shown that melatonin is an important mediator in angiogenesis (Ramírez-Fernández et al. 2013) and bone formation (Roth et al. 1999). 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 would appear to improve bone formation (Roth et al. 1999). Several studies have demonstrated the effect of melatonin application around dental implants (Cutando et al. 2008; Calvo-Guirado et al. 2009; Calvo-Guirado et al. 2010a,b; Guardia et al. 2011; Tresguerres et al. 2012), but its effect on immediate implants remains unclear. Nevertheless, melatonin may play an important role during the regeneration of alveolar bone after immediate implant placement through the stimulation and modulation of inflammatory, osteoblastic and osteoclastic activity.

The aim of this study was to determine whether the topical application of melatonin over the implant surface is effective in improving osseointegration using histomorphometric analysis and histological linear measurements to evaluate bone-to-implant contact (BIC) at 12 weeks after immediate implant placement.

Material and methods

Study design

Six American foxhound dogs of approximately 1 year of age, each weighing approximately 13–15 kg, were used in the study. Three conical implants were inserted in each hemiarch and divided randomly into three different groups. This study includes results obtained from two of the groups ($n = 24$;

results for the other group will be published in Part 2, because of the amount of data collected and difficulties on statistical analysis.

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 antirabies vaccine, antihelminthics, and vitamins. 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. Clinical examination established that the dogs were in good general health.

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 µg/kg (Medetor 1 mg, Virbac, CP-Pharma Handelsgesellschaft GmbH, Germany). The mixture was injected intramuscularly in the femoral quadriceps. Animals were then taken to the operating theater 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 buccolingual direction at the bifurcation using a tungsten-carbide bur (Fig. 1a and b). Distal roots were extracted individually using a periosteal elevator and forceps without damaging the bony walls (Fig. 1c). 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>.

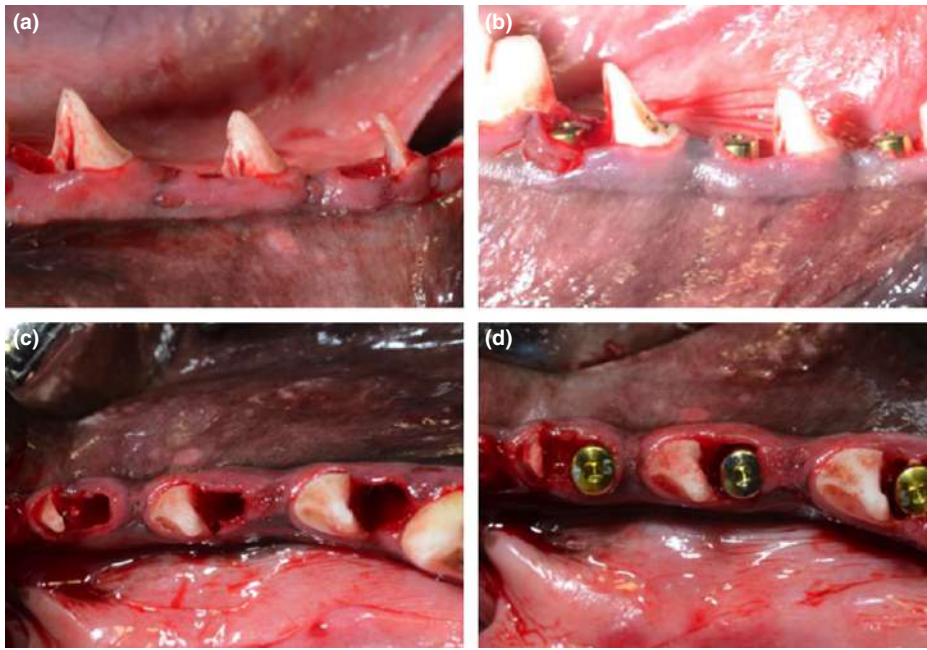


Fig. 1. (a) Lateral image of one hemiarch mandibular premolars (P_2 , P_3 , P_4); (b) A crestal incision performed bilaterally in the premolar region of the mandible with teeth odontosection; (c) Clinical photograph in occlusal view showing extraction of the distal root in a single hemiarch. (d) Three implants were randomly placed immediately in the distal socket (c) Titanium dental implant with Melatonin 5% dipping surface.

com. A scheme was created for the 36 implants (12 implants with melatonin 5% [MI], 12 implants with vitamin D 10% and 12 implants alone [CI], randomized into six dogs. Before implant placement, test implants were submerged in melatonin at 5% in solution (TM-M5250; Sigma-Aldrich®, USA) (Fig. 1d). No treatment was applied to control implants (CI). Minimal full-thickness mucoperiosteal flaps were elevated, and implants were placed. Each dog received six conical C1 implants (MIS, Medical Implants System, Israel) randomly assigned in the mandible. Implant positions were determined in relation to the shape and volume of each alveolar process and the buccal wall position (Fig. 1e). 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 (Fig. 1e). 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 grafting materials were used in the gaps remaining between bony plates and implants. The flaps were closed with simple inter-

rupted 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). The sutures were removed after 2 weeks. Animals were fed 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 cleaned.

Second stage

Digital radiographs were taken at 12 weeks (Kodak 6100, Eastman Kodak, Rochester, NY, USA) (Fig. 2). Afterward, 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%,

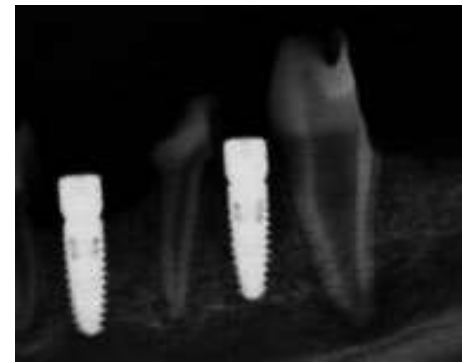


Fig. 2. Digital radiographs taken after 12 weeks of healing.

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 mm thickness and ground to approximately 40 mm final thickness with an Exakt 400s CS grinding device (Exakt Apparatebau, Norderstedt).

Sections were stained with toluidine blue, and a semiquantitative 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 $10\times$ lens and assembled into a single image (Image-Pro Plus 4.5, Media Cybernetics Inc., Immagini & Computer Snc.). 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 (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

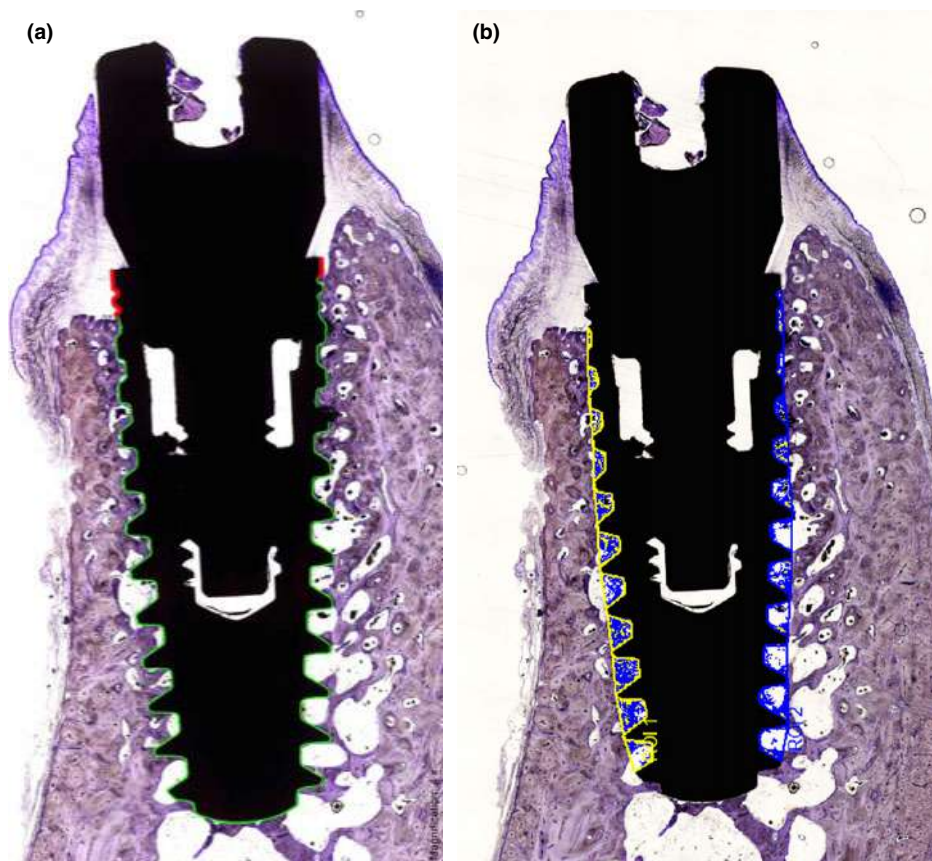


Fig. 3. (a) BIC scheme: Green lines shows Total BIC and red line show crestal bone loss. (b) BIC scheme: Yellow line delimitates interthread space and blue line shows New Bone Formation.

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

The following buccal and lingual measurements were made at ×10 magnification (as illustrated in 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 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 implant placement and 12 weeks later to verify implant osseointegration and to assess changes to postsurgical crestal bone levels (Kodak 6100, Eastman Kodak). Exposure parameters were standardized. No radiolucent images or signs of osseointegration disorders

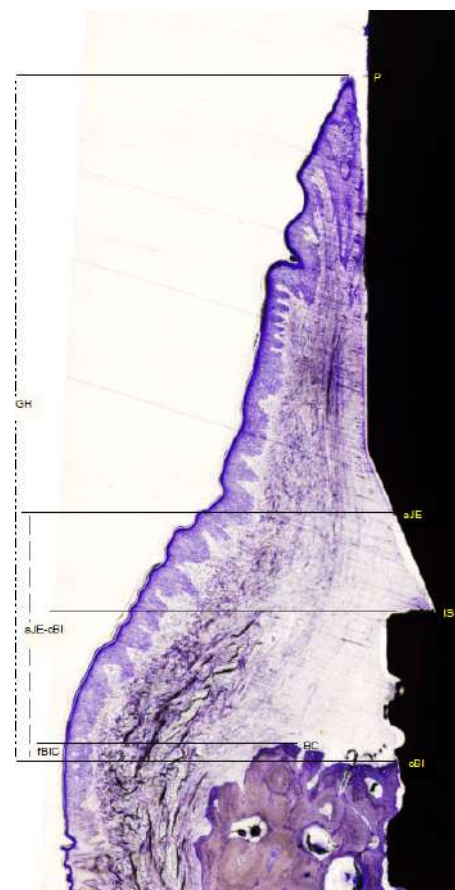


Fig. 4. Linear Measurements scheme: P, top of the peri-implant mucosa; aJE, apical portion of the junctional epithelium; fBIC: first point of bone-to-implant contact; IS, Implant shoulder; BC, buccal crest.

were observed. X-ray analysis revealed that all 24 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 on the same specimen, implants were considered as samples rather than dogs; for this reason, samples were considered independent. Values were expressed as means ± standard deviation. To compare two samples with continuous variables and independent samples, normality was checked using the Shapiro-Wilk test. As the distribution of data was not normal, the Mann-Whitney *U*-test was applied; this test works with the median rather than the mean to perform comparative analysis of quantitative and qualitative variables. 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

Table 1. Means and standard deviation for total BIC, BIC%, new bone formation and inter-thread bone after 12 weeks of healing

Variable	Alone (<i>n</i> = 12) Mean ± SD	Melatonin (<i>n</i> = 12) Mean ± SD	<i>P</i> 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 ($P < 0.05$).

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 presented in Table 1. BIC% values at 12 weeks varied slightly for the melatonin group MI (41.36 ± 3.93) compared with CI (41.34 ± 9.26) but 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).

Figures 5 and 6 showed histological images of control and test implants. Figure 5 shows untreated implant [CI], small amount of inter-thread bone and small quantity of bone surrounding the implant. Figure 6 shows implant treated with topical application of melatonin 5% [MI], direct bone-to-implant contact with increased inter-thread bone can be observed with an increment in bone growth and vascular tissue.

Linear measurements

The results of the different linear measurements are presented in Tables 2 and 3. 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 the control implants CI (0.92 ± 1.98) ($P = 0.045$). Medians and ranges are expressed in Table 4.

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.

Discussion

The use of biocompatible materials to improve bone formation around dental implants has been widely documented in the

literature. A wide variety of substances have been studied in the aim of improving bone regeneration and peri-implant bone responses that include growth hormone, morphogenetic proteins, calcium coatings, fluorine coatings, magnesium, etc. (Cutando et al. 2008; Calvo-Guirado et al. 2010a; Tresguerres et al. 2012). The literature reports a number of links between melatonin and bone metabolism, as well as its stimulatory effect on osteoblast proliferation and differentiation (Nakade et al. 1999; Roth et al. 1999), and on bone repair and angiogenesis (Ramírez-Fernández et al. 2013). However, the effect of the topical application of osteoinductive elements such as melatonin on immediate implants remains a subject for research.

The present trial demonstrated that bone formation around immediate dental implants

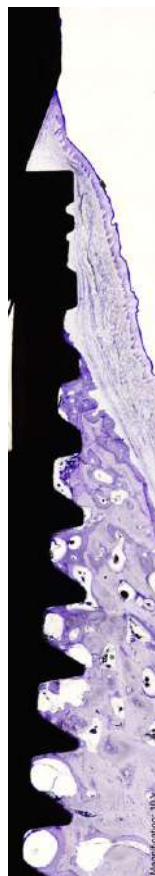


Fig. 5. Buccal ground section of an untreated implant [CI] after 12 weeks of healing. Hematoxylin eosin stain, original magnification $\times 10$.

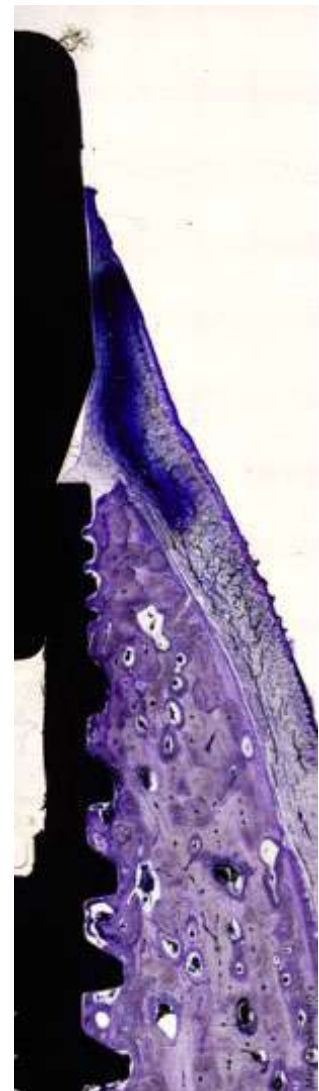


Fig. 6. Buccal ground section of a test implant [MI] after 12 weeks of healing. Hematoxylin eosin stain, original magnification $\times 10$.

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

Buccal bone crest	Alone (n = 12)		Melatonin (n = 12)	P value
	Mean ± SD	Mean ± SD		
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.** Means and standard deviation for linear measurements at 12 weeks of healing at the lingual bone crest

Lingual bone crest	Alone (n = 12)		Melatonin (n = 12)	P value
	Mean ± SD	Mean ± SD		
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 ($P < 0.05$).

treated with melatonin was improved in comparison with non-treated control implants. Implants treated with melatonin showed higher bone-to-implant contact values ($P = 0.036$) and more inter-thread bone formation ($P = 0.035$). The melatonin implants showed less crestal bone loss ($P = 0.045$) and less peri-implant mucosa on the lingual aspect ($P = 0.042$). These results may be explained by the stimulatory effect of melatonin on human osteoblast proliferation and differentiation, also in the synthesis of collagen type I, and other bone matrix proteins, which have been described in several *in vitro* studies (Nakade et al. 1999; Roth et al. 1999). However, no differences could be found on the buccal aspect, probably due to the resorption of bundle bone. Some authors claim that after dental extraction, bundle

bone resorption is unavoidable (Araújo et al. 2005b, 2006a,b). Differences in BIC values could be due to implant macrodesign; conical implants have less surface area than cylindrical implants, a factor that could produce varying BIC values. 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) and 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 and evaluated at 15, 30, 45 and 60 days after

surgery found that melatonin increased the length of cortical bone ($95.13 \pm 0.42\%$) compared with control implants ($62.91 \pm 1.45\%$). The authors claimed 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 matrix and so hastens osseointegration.

A recent study by Calvo-Guirado et al. (2014a) assessed BIC% of micro-grooved zirconia implants and titanium implants supplemented with melatonin, in comparison with micro-grooved zirconia implants and titanium implants without melatonin, after 1- and 4-week periods. At 1 week, melatonin-supplemented titanium ($29.7 \pm 2.14\%$) and micro-grooved zirconia implants ($28.9 \pm 1.3\%$) showed higher percentages of BIC in comparison with the control groups. At 4 weeks, melatonin-supplemented micro-grooved zirconia implants presented the highest BIC values ($47.5 \pm 2.2\%$). These results agreed with the ones obtained in the present study and demonstrate that topical applications of melatonin increase BIC during the early stages of osseointegration regardless of implant surface materials/characteristics.

In the present study, slightly greater BIC and inter-thread bone formation were observed in the test group, but comparisons of these findings with other animal studies should be treated with caution because of the differences in methodology and metabolism between dogs and rabbits. Moreover, different studies have used different observation time frames. According to Cutando et al. (2008) and Guardia et al. (2011) (who used a Beagle model with the same methodology) at 5- and 8-week evaluations, statistically significant differences were found for inter-thread bone

Table 4. Medians and ranges for linear measurements after 12 weeks of healing

Variable	Alone (n = 12)		Melatonin (n = 12)		U de Mann-Whitney	P value
	Range average	Total range	Range average	Total range		
IS-BC buc	10.17	122.00	14.83	178.00	44.00	0.114
IS-BC lin*	9.54	114.50	15.46	185.50	36.50	0.039*
IS-cBI buc (CBL)	11.58	139.00	13.42	161.00	61.00	0.551
IS-cBI lin (CBL)*	9.63	115.50	15.38	184.50	37.50	0.045*
P-aJE_buc	14.25	171.00	10.75	129.00	51.00	0.242
P-aJE_lin*	17.25	207.00	7.75	93.00	15.00	0.001*
aJE-cBI buc	14.08	169.00	10.92	131.00	53.00	0.291
aJE-cBI lin	11.08	133.00	13.92	167.00	55.00	0.347
fBIC-buc	14.83	178.00	10.17	122.00	44.00	0.114
fBIC-lin	12.54	150.50	12.46	149.50	71.50	0.977
P-cBI lin (PIM)	14.54	174.50	10.46	125.50	47.50	0.160
P-cBI lin (PIM)*	15.42	185.00	9.58	115.00	37.00	0.042*

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

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 statistically 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), confirming the premise that melatonin action peaks during the early stages of bone formation.

The present and other studies demonstrated higher values of BIC and inter-thread bone in implants treated with melatonin. From our point of view, this phenomenon is based on the direct action of melatonin on the osteoblasts; which induces a higher rate of maturation of pre-osteoblasts to osteoblasts and perhaps a larger number of osteoblasts with a higher rate of production of the osseous matrix and its corresponding calcification.

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. These findings are similar to the present study in that not all parameters showed statistical differences at 8 weeks; this supports the concept that melatonin is more effective during the early stages of healing, given that BIC and inter-thread values did not show significant differences at 8 weeks. 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 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. At 12 weeks, melatonin combined with porcine bone produced higher BIC percentages ($84.5 \pm 1.5\%$), compared with melatonin-treated ($75.1 \pm 1.4\%$) and non-melatonin-treated ($64 \pm 1.4\%$) implants. The present study found differences in linear measurements on the lingual aspect. The melatonin group showed statistically less crestal bone 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 neu-

tralize free radicals, inhibiting osteoclast activity and so crestal bone resorption (Cutando et al. 2007a,b; 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 lesser amounts of peri-implant mucosa ($P = 0.042$). However, no differences were observed at the buccal aspect probably because of the inevitability of bundle bone resorption. Nevertheless, the present study demonstrated that after twelve weeks of treatment, melatonin still had an effect on bone formation around implants when these were placed immediately after dental extraction.

Conclusions

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

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