Topical Application of Melatonin and Growth Hormone Accelerates Bone Healing around Dental Implants in Dogs

Fernando Muñoz, DVM, PhD;* Mónica López-Peña, DVM, PhD;† Natalia Miño, DVM, PhD;‡ Gerardo Gómez-Moreno, DDS, PhD;§ Javier Guardia, DDS, PhD;¶ Antonio Cutando, MD, PhD, DDS§

ABSTRACT

Background: Growth hormone (GH) and melatonin belong to the group of growth factors. These substances have been proposed to improve and accelerate osseous healing using topical applications.

Purpose: The aim of this study was to evaluate the effect of the topical administration of GH and melatonin on osseointegration of dental implants in Beagle dogs 2, 5, and 8 weeks after their insertion.

Materials and Methods: Twelve adult Beagle dogs and 48 implants were used in the study. The maxillary and mandibular premolars and molars were extracted. Each mandible received cylindrical screw implants of 3.25 mm in diameter and 10 mm in length. Prior to implanting, 4 IU of recombinant human GH and 1.2 mg of lyophilized powdered melatonin was applied to one osteotomy at each side of the mandible. None was applied at the control sites. The implants were retrieved at 2, 5, and 8 weeks for light microscopic examination, energy-dispersive x-ray microanalysis, and histomorphometric measurements in ground sections.

Results: At week 2, BIC was significantly higher in the melatonin-growth hormone group than in the implant control one (34.20 vs 25.05%; $p = .010$). The M-GH group also increased significantly the peri-implant bone area (64.72 vs 53.20%; $p = .038$) and interthread bone area (35.62 vs 25.08%; $p = .02$). At weeks 5 and 8, BIC and bone density around implants were similar to both groups. Significant differences were detected in bone neoformation at 8 weeks in ML-GH group (9.04 vs 7.53%; $p = .05$). Regarding the mineral composition, in ML-GH group increments in concentrations of phosphorus (10.70 vs 10.34; $p = .013$) were observed at 2 weeks and of magnesium (0.29 vs 0.25; $p = .019$) 5 weeks after implantation.

Conclusion: The present study confirms that GH and melatonin synergistically enhance new bone formation around titanium implants in early stages of healing.

KEY WORDS: dental implants, dog, growth factor, growth hormone, melatonin

INTRODUCTION

The long-term success of many dental implants depends on their ability to become well integrated in bone. Titanium (Ti) is the implant material of choice for use in dental applications. The stable oxide, formed easily on Ti surfaces, is attributed to its excellent biocompatibility. However, their surface properties are not well suited for bonding to bone. Modifications of both surface topography and surface chemistry have led to significant improvements in the integration of such materials in bone. Therefore, the attention has been focused on the surface preparation of the Ti implant.

Several measures have been proposed to improve and accelerate osseous healing using topical treatments.

*Research fellow, Department of Veterinary Clinical Sciences, University of Santiago de Compostela, Lugo, Spain; †professor, Department of Veterinary Clinical Sciences, University of Santiago de Compostela, Lugo, Spain; ‡research fellow, Rof Codina Foundation, Lugo, Spain; §professor, Department of Special Care in Dentistry, School of Dentistry, University of Granada, Granada, Spain; ¶collaborator, Department of Special Care in Dentistry, School of Dentistry, University of Granada, Granada, Spain

Reprint requests: Dr. Fernando Muñoz, Dpto. Ciencias Clínicas Veterinarias, Facultad de Veterinaria, Universidad de Santiago de Compostela, Campus Universitario s/n, E-27002, Lugo, Spain; e-mail: fernandom.munoz@usc.es

DOI 10.1111/j.1708-8208.2009.00242.x

They include the application of platelet-rich plasma (PRP), bone morphogenetic proteins (BMPs), and growth factors.[1] Growth hormone and melatonin belong to this group of substances and play a role in bone remodelation and neoformation.

Growth hormone (GH) is one of the most important regulatory substances in bone growth and bone remodeling.[2] Synthesized in the adenohypophysis and in almost all cells, including the osteoblasts,[3,4] it works directly on the liver and chondral tissue stimulating the synthesis and releasing insulin-like growth factor I (IGF-I), which stimulates the osteoblast differentiation, and IGF-II, which improves the collagen synthesis and is found in great concentrations in the bone matrix.[5]

Systemic administration of GH had been previously documented to stimulate bone fracture repair[6–8] and to increase bone mass. However, GH has rarely been administrated locally to enhance peri-implant bone growth. Tresguerres and colleagues[9] demonstrated that the local administration of recombinant human GH during the surgical placement of titanium sheets on the tibia of an osteoporotic rabbit model enhanced periosteal and transcortical reaction and mineralization of osteoid. In 2005, the same authors suggested that local administration of GH accelerated the remodeling process.[10] Gómez-Moreno and colleagues demonstrated that the topical application of the growth hormone around dental implants produced an increment in the bone implant contact ratio and bone area, 2 weeks after implantation.[11]

Melatonin is synthesized and secreted by the pineal gland and other organs. Systemically, melatonin seems to have a fundamental role as a regulating system in haematopoiesis and immune-enhancement. It appears to be closely involved in several fundamental aspects of host defense and it is potentially be useful as an adjuvant tumor immunotherapeutic agent.[12] In addition, melatonin may also be an effective hormone in the treatment of bone changes in estrogen deficiency states[13,14] and around dental implants placed in tibia of rat; its efficiency has been shown when used in combination with fibroblasts growth factor-2 (FGF-2).[15] Anyway, in the mouth, melatonin possesses antioxidant, free-radical scavenging, and immunoenhancing properties.[16] Recently, we have stated that salivary melatonin levels vary according to the degree of periodontal disease[17,18] and we have also demonstrated that topical application of melatonin activated osteogenesis around titanium implants in a canine mandibular model successfully.[19]

The mechanisms by which melatonin protects bone may occur through its actions on both osteoclasts and osteoblasts and through an interaction with estrogens.[20] At micromolar concentrations, melatonin stimulates the synthesis of collagen type I fibers in human osteoblasts in vitro.[21] In addition, it increases the genic expression of bone sialoprotein and other protein markers of bone, including alkaline phosphatase, osteopontine, and osteocalcine in preosteoblasts, reducing the osteoblast differentiation period from 21 to 12 days.[22–25]

Melatonin, by means of its indirect antioxidant and direct free-radical scavenging action and its actions on down-regulation of RANKL, may interfere with the activity of osteoclasts and, thereby, inhibit bone resorption[22–24,26–28] through specific melatonin receptors.[29,30]

Considering the next process of the application of melatonin and growth hormone in dentistry, we thought that it was beneficial to introduce the effects of melatonin and growth hormone in implant treatment, and we hypothesized that when applied topically, melatonin-growth hormone (M-GH) would promote peri-implant bone formation. The purpose of this study was to examine whether or not the topical application of M-GH induced or not histological and histometric differences in the newly formed peri-implant bone in mandibles of dogs.

OBJECTIVES

Therefore, the aim of our study was to determine whether the topical administration of GH and melatonin at the time of the surgery would induce histological and histometric differences in the newly formed peri-implant bone in dog mandibles at several intervals of time.

MATERIAL AND METHODS

Animals

Twelve Beagle dogs, about 18 months old, were used. The protocol was approved by the Ethical Committee of the University of Granada, Spain. All surgical procedures were performed using general anesthesia induced with ketamine and chlorbutol, previously premedicated with acepromazine (0.5–1 mL/kg) and atropine (0.05 mg/kg) under veterinary supervision.

Implants

The implants used (Microdent® system mini micro, Implant Microdent® System, Santa Eulalia de Ronsana,

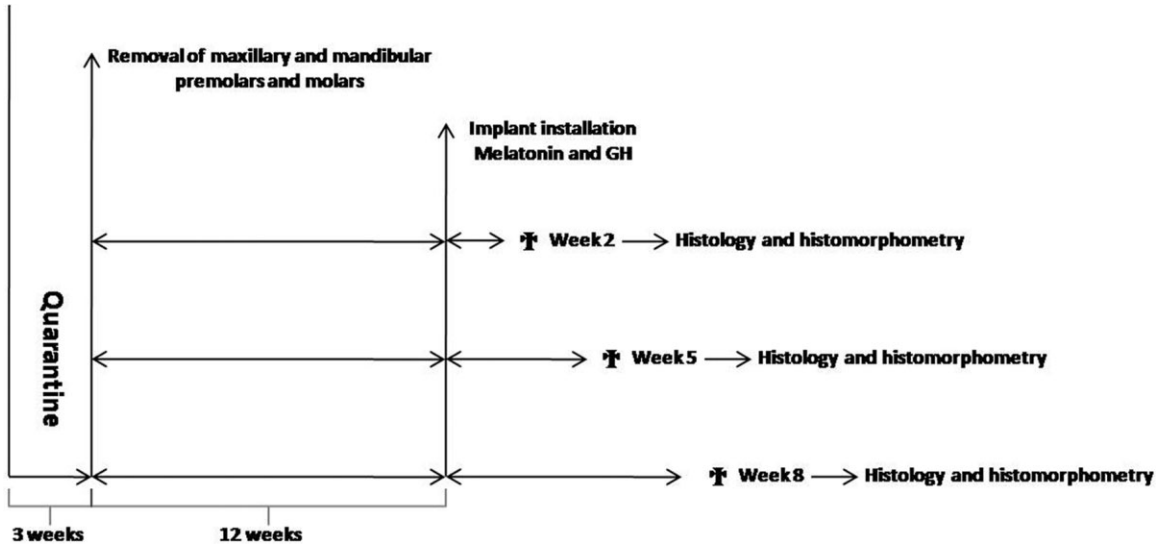

Figure 1 Design of the study.

Barcelona, Spain) had a diameter of 3.5 mm and included a 10 mm long intraosseous portion and a 1 mm high transmucosal collar. The endosseal portion had an acid-etched surface.

Surgical Procedures

The outline of the experiment is presented in Figure 1. The maxillary and mandibular premolars and molars were extracted. Three months later, a bilateral crestal incision was made in the edentulous mandibular region. Buccal and lingual mucoperiosteal flaps were raised and osteotomy preparations to a depth of 10 mm were made in eight sites. Prior to implanting, one osteotomy test site at each side of the mandible was treated with 4 IU of recombinant human GH (Saizen®; Serono Laboratories, Madrid, Spain) and 1.2 mg of lyophilized powdered melatonin (Helsinn Chemicals SA, Biasca, Switzerland). No treatment was applied at the two control sites. The other four implants placed were treated according to a protocol already published.[11,19] In order to avoid that growth factors affect other sockets, a space of at least 5 mm was left between implants. Implant treatment was randomly assigned to mesial and distal sites on each side of the mandible. The number of implants treated in each position and time was the same for all animals. Cover screws were placed and flaps were adjusted and sutured. Perioperatively, an antibiotic (amoxicillin) and an analgesic (buprenorphine) were administered for 7 and 3 days, respectively.

The sutures were removed 2 weeks after implant placement. A plaque control program, including daily cleaning of teeth and implants with toothbrush and a chlorhexidine gel, was initiated.

The dogs were divided into three groups (four animals per group), and at 2, 5, and 8 weeks were sacrificed by overdose of Sodium Pentobarbital (Dolethal®, Vétoquinol, France).

Ground Sections

The lower jaw was removed and immersed in buffered formalin for 1 week. Tissue blocks containing the implant and the surrounding soft and hard tissues were dissected and processed for ground sectioning according to the method described by Donath and Breuner (1982).[31] From each tissue block, one buco-lingual section was prepared and reduced to a thickness of approximately 20 µm (Exakt® Apparatebau, Norderstedt, Germany). The sections were stained using the Levai Laczkó technique.[32]

Histometric Measurements

One calibrated masked examiner performed the histometric analysis using light microscopy and a PC-based image analysis system (Microimage 4.0, Media Cybernetic, Silver Springs, MD, USA). One section per implant was used for analysis.

The bone-to-implant contact ratio was defined as the length of the bone surface border in direct contact with the implant/complete implant periphery (×100 [%]) starting at the shoulder of the implant. The interthread bone density was defined as the area of bone inside the threads/complete area inside the threads

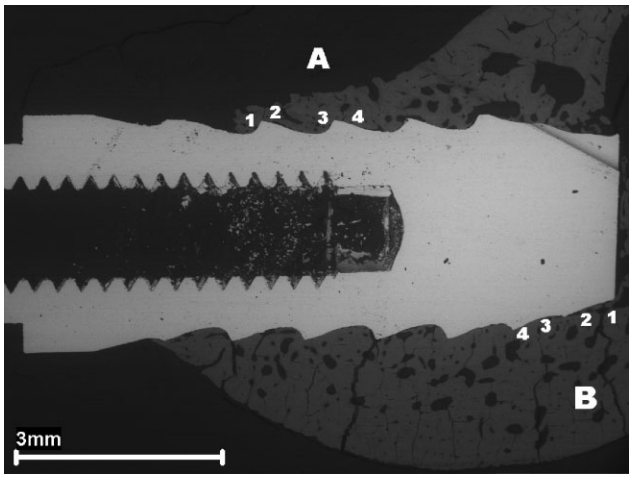

Figure 2 Scanning electron image showing the points where the quantification for minerals was carried out by EDX.

(×100 [%]) beginning with the first thread down to the most apical thread. Surrounding the implant, up to a lateral distance of 1 mm, the peri-implant bone density was determined as bone area/tissue area (×100 [%]), starting again with the first thread down to the most apical thread. The percentage of bone neoformation was defined as the area of new bone that was formed after implant insertion. The newly formed bone was situated in the peri-implant area and between the implant threads.

Scanning Electron Microscope (SEM) and Energy-Dispersive X-Ray Microanalysis

The PMMA embedded specimens obtained from the histological preparation were mounted on a SEM (LEO–435 VP) with beam voltages of 0.3–30 kV. Quantification for calcium (Ca) (mol %), phosphorus (P) (mol %), and magnesium (Mg) (mol %) was determined by wavelength dispersive x-ray spectroscopic analysis and carried out at 8 points in each implant sample: four in the bone situated around the apical threads and four around the most coronal threads of the implant (Figure 2). The average values were calculated. The ratio of calcium to phosphorus (Ca/P) was calculated by dividing the values obtained from calcium (mol %) and phosphorous (mol %) at each point. This ratio is unit-less. Unfortunately, it was not possible to analyze the samples obtained at 8 weeks after implantation.

Statistical Analysis

All data were expressed as the mean ± standard deviation (SD). The *t*-test was used to analyze differences among variables. Statistical analyses were carried out using the SPSS 11.0 computer program (SPSS Inc., Chicago, IL, USA). The level of statistical significance was established at $p < .05$.

RESULTS

Histological Findings

For both implant types, a thin layer of apparently newly formed bone in direct contact with the implant was evident at 2 weeks of healing. At this early time point, a scaffold of woven bone formation was observed (Figure 3). In the pitch regions of the screw and alveolar crest, modeling areas with bone resorption and apposition took place. The adjacent non-mineralized granulation tissue contained many vascular structures and a few inflammatory cells (Figure 4). Osteoclasts were present in the bone marrow spaces resorbing necrotic and dislocated bone produced during site preparation and fitting on the implant. We observed in the M-GH group areas of woven bone remodeled by osteoclastic cells (Figure 5).

At 5 weeks, bone remodeling was extensive around all implants, within a zone lateral to the implant that contained remnants of the old bone that had been partly replaced by newly formed lamellar and woven bone (Figure 6). Bone density increased, mainly in the interthread region, as indicated by the reinforcement of woven bone trabeculae by the deposition of

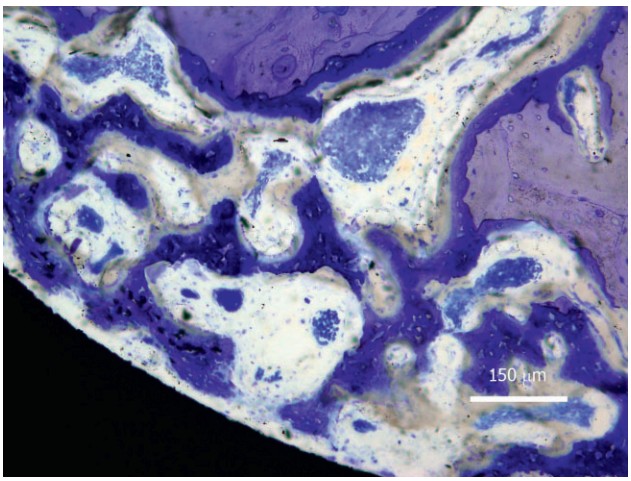

Figure 3 Histological appearance of bone apposition at 2 weeks in melatonin-GH-treated implant. Woven bone is characterized by the intense staining of the mineralized matrix and the numerous osteocytes located in large lacunae (undecalcified ground sections, surface stained with Levai Laczkó). The bars show the magnification.

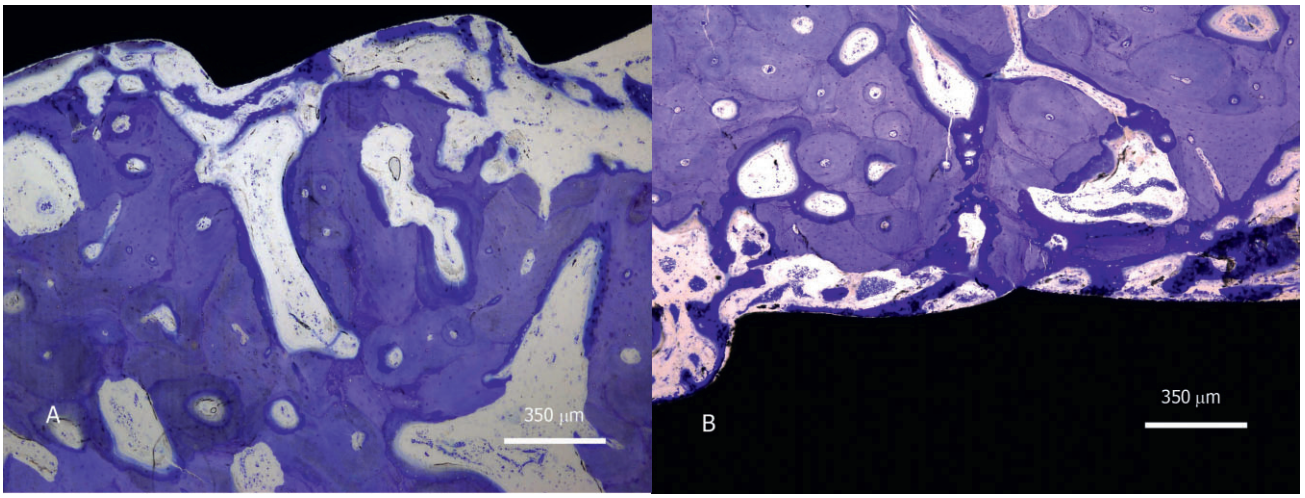

Figure 4 Control- (A) and melatonin-GH-treated (B) implants at 2 weeks. The bone-implant interface is mainly formed by woven bone and many vascular structures (undecalcified ground sections, surface stained with Levai Laczkó). The bars show the magnification.

parallel-fibered bone (Figure 7). Bone remodeling around the alveolar crest persisted, showing no differences in both groups (Figure 8).

After 8 weeks, typical secondary osteons with concentric lamellae and a central haversian canal were observed in the lamellar bone around the implants (Figure 9). Bone density increased again, with slight differences between the remodeling in both groups, showing an increment in new bone and bone maturation in M-GH group (Figure 10). Bone remodeling around crestal bone, mainly in bucal, persisted during that period (Figure 11).

Histomorphometrical Findings

The results from the histometric measurements are presented in Table 1. At week 2, BIC was significantly higher in the melatonin-growth hormone group than in implant control group ($p = .010$). M-GH group also increased significantly the peri-implant bone area ($p = .038$) and interthread bone area ($p = .02$) compared with the control group.

At weeks 5 and 8, BIC and bone density around implants were similar to both groups. Interthread bone area was increased in the ML-GH group compared with

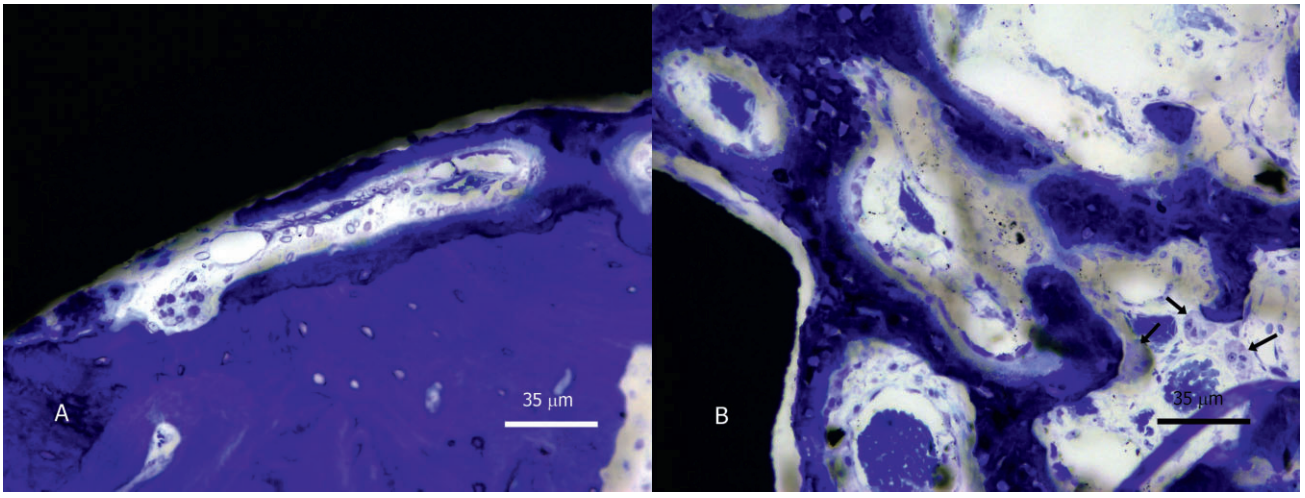

Figure 5 Control- (A) and melatonin-GH-treated (B) implants at 2 weeks. The osteoclast resorbs bone adjacent to the implant. In *B*, the woven bone is being remodeled by osteoclasts (black arrows). We have not detected this fact in control group (undecalcified ground sections, surface stained with Levai Laczkó). The bars show the magnification.

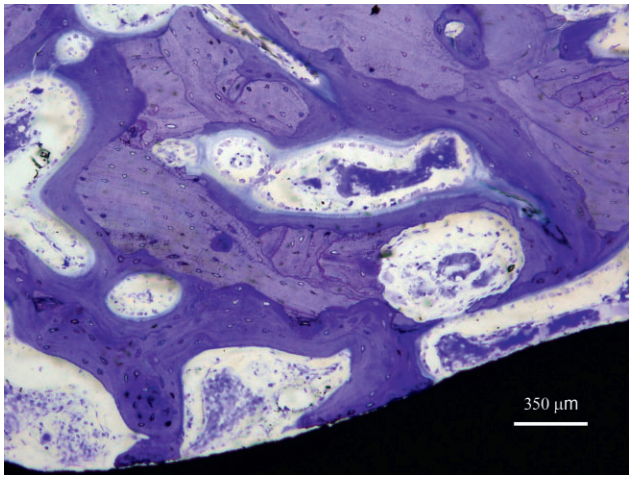

Figure 6 Histological appearance of bone apposition at 5 weeks. Lateral to implants, the old bone have been partially replaced by lamellar and woven bone. We observed osteoblasts located around blood vessels (undecalcified ground sections, surface stained with Levai Laczkó). The bars show the magnification.

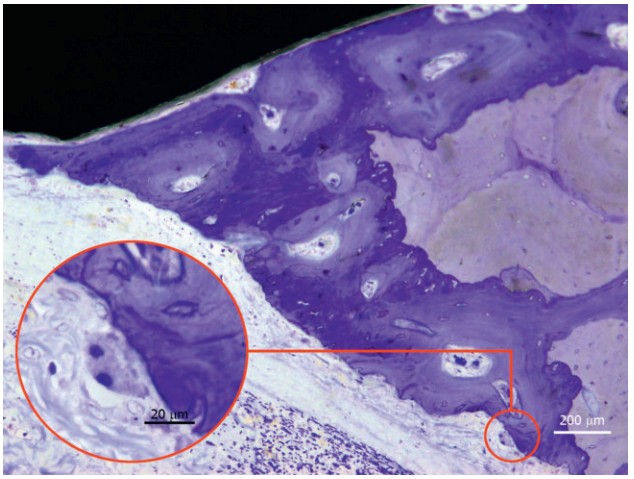

Figure 8 Histological appearance of alveolar crest at 5 weeks. Detail of an osteoclast during remodeling (undecalcified ground sections, surface stained with Levai Laczkó). The bars show the magnification.

the control group at 5 weeks, but the difference was not significant. Relevant differences were detected in bone neoformation at 8 weeks in the ML-GH group ($p = .05$).

SEM-EDX Findings

About the mineral composition of bone around implants obtained by EDX (Table 2), increments in concentrations of calcium, phosphorus, and magnesium in the ML-GH group were observed, but they were not significant, except in phosphorus at 2 weeks and in magnesium at 5 weeks after implantation. The Ca/P ratios were similar in both groups, decreasing along the implantation period.

DISCUSSION

Links between melatonin, growth hormone. and bone metabolism have been documented in many studies.[21–24] GH has been administered to increase bone mass and to favor the repair of tibia fractures in rats.[6,7] Also, several investigations have been carried out to check the stimulating effects of GH applied locally on bone formation in

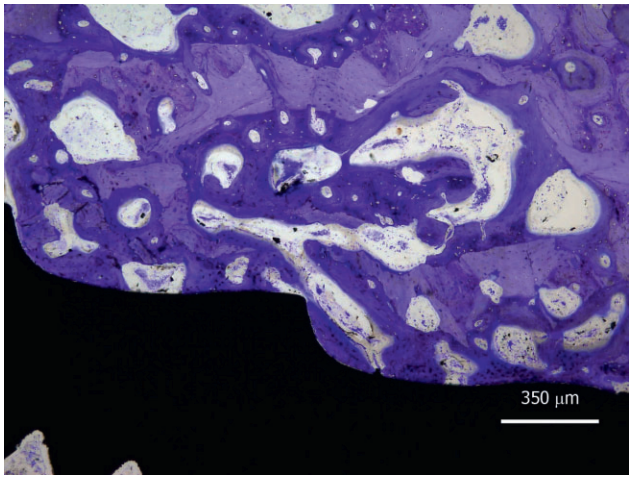

Figure 7 Histological appearance of bone apposition at 5 weeks. The bone density is incremented in the interthread region (undecalcified ground sections, surface stained with Levai Laczkó). The bars show the magnification.

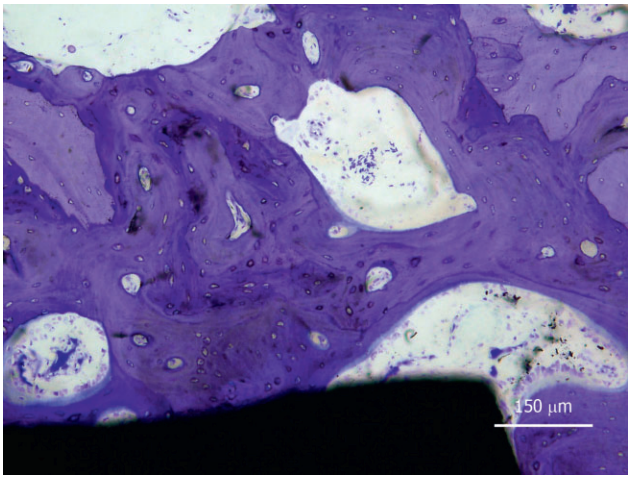

Figure 9 Lamellar bone around a ML-GH implant at 8 weeks of healing. Typical osteons around a central haversian canal are observed. Some osteoblastic activity persists in areas around blood vessels (undecalcified ground sections, surface stained with Levai Laczkó). The bars show the magnification.

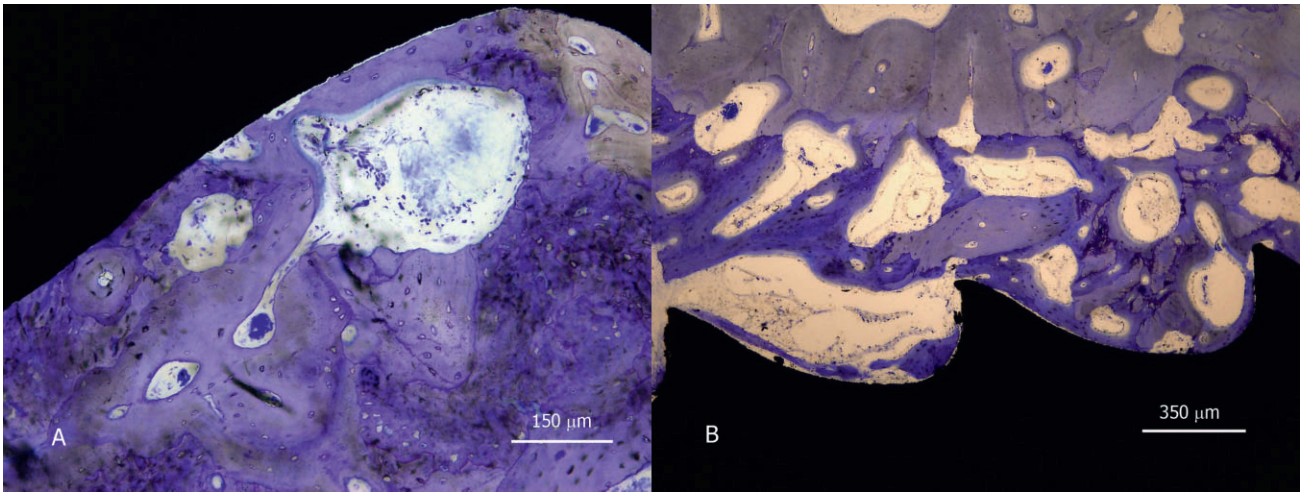

Figure 10 Melatonin-treated (A) and control (B) implants at 8 weeks showing slight differences in remodeling. In *B*, an increment in osteoblastic activity is shown and the implant interface was not completely remodeled (undecalcified ground sections, surface stained with Levai Laczkó). The bars show the magnification.

rat mandibles,[33] using a phosphate/calcium matrix[34] and around titanium sheets in osteoporotic rabbit tibiae.[9]

It has been shown that melatonin, administered orally, modifies bone remodeling after ovariectomy in rats,[27] increasing bone mineral density. This effect is produced through releasing growth hormone and cortisol stimulated by melatonin or through suppression of bone resorption, by down-regulating RANK-mediated osteoclast formation and activation.[25] This effect becomes more marked when adequate concentrations of estradiol are present.[27]

On the other hand, several studies have demonstrated that melatonin and GH separately can stimulate the differentiation of primary osteoblasts in culture, as well as the synthesis of collagen type I and other proteins of the bone matrix, such as sialoprotein, alkaline

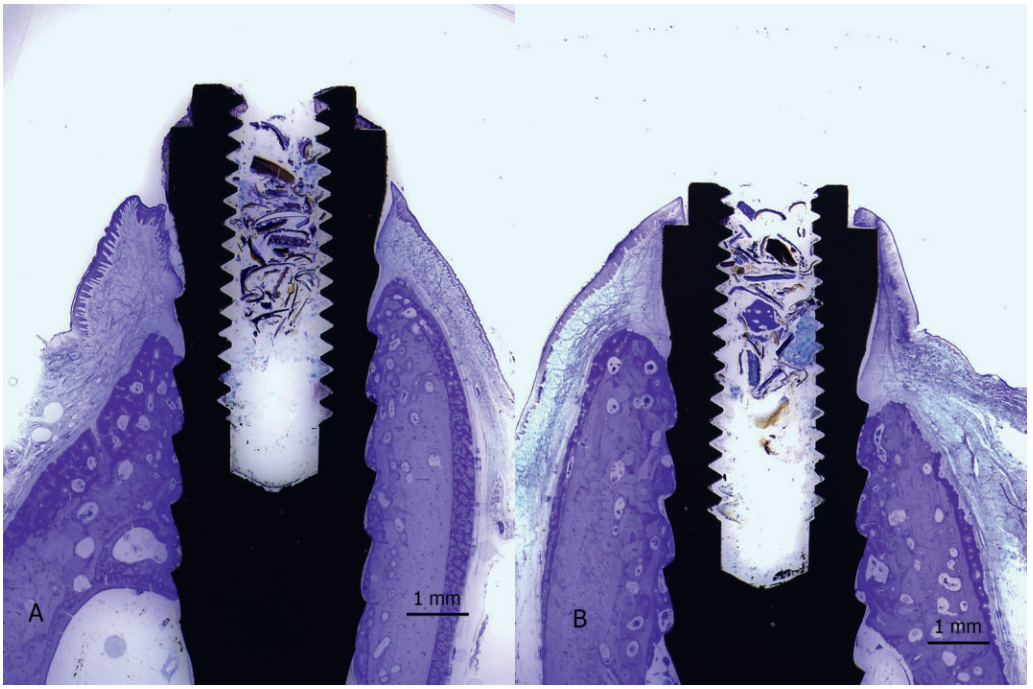

Figure 11 Melatonin-treated (A) and control (B) implants at 8 weeks. In both groups, bone remodeling around crestal bone persisted during this period (undecalcified ground sections, surface stained with Levai Laczkó). The bars show the magnification.

TABLE 1 Histomorphometric Parameters for Osteointegration in Control and Melatonin-GH-Treated Implants, 2, 5, and 8 Weeks after Placement

	2 Weeks		5 Weeks		8 Weeks	
	ML + GH	Control	ML + GH	Control	ML + GH	Control
BIC	**34.20 ± 11.02**	**25.05 ± 11.90**	36.43 ± 6.02	34.33 ± 8.13	31.47 ± 10.69	33.15 ± 11.35
	***p* = .010**					
Total peri-implant bone area (%)	**64.72 ± 16.45**	**53.40 ± 22.41**	63.83 ± 11.08	68.93 ± 13.76	71.51 ± 6.47	71.07 ± 5.57
	***p* = .038**					
Interthread bone (%)	**35.62 ± 2.25**	**25.08 ± 3.47**	80.76 ± 11.80	72.56 ± 12.54	83.50 ± 14.51	82.43 ± 13.41
	***p* = .02**					
Bone neoformation (%)	4.21 ± 1.27	3.89 ± 1.56	8.01 ± 1.42	8.45 ± 1.41	**9.04 ± 1.71**	**7.53 ± 1.69**
					***p* = .05**	

Bold numbers indicate statistically significant differences.

phosphate, osteopontin, and osteocalcin,[4,21,24] and in the production of IGF-I, which stimulates osteblasts and bone remodeling.[5]

It is a fact that osteoclasts generate high levels of superoxide anions during bone resorption that contribute to the degradation process. Administration of both GH and melatonin has been shown to stimulate several antioxidative enzymes that reduce the oxidative stress and improve antioxidant defenses.[23,35] Therefore, the effect of both substances in preventing osteoclast activity in bone may depend in part on the free radical scavenging properties.

In our study, the effects of local administration of melatonin and growth hormone on osseointegration of a dental implant were analyzed. We speculated that both substances worked synergically to increment the bone apposition rate around dental implants and to accelerate the bone healing.

The data obtained in our study related with the local administration of ML-GH showed extremely significant histomorphometric differences between treated implants and controls 2 weeks after implant placement. Topical ML-GH significantly increased osteointegration parameters: BIC, total peri-implant bone area, and percentage of bone neoformation. These results are consistent with the data reported in the studies carried out by Treguerres and colleagues[9,10] after local administration of growth hormone around titanium implants or sheets inserted in the tibia of osteoporotic and non-osteoporotic rabbits, but only with regard to BIC values. They observed an increased response from periosteal and endosteal bone, but that was only a tendency. In our results, the bone density differences were significant. Cutando and colleagues[19] also detected differences related to BIC and bone density around dental implants topically treated with melatonin at 2 weeks.

TABLE 2 Bone Composition in Control Implants and Melatonin-GH-Treated Implants, 2 and 5 weeks after Placement

	2 Weeks		5 Weeks	
	ML + GH	Control	ML + GH	Control
Ca	20.22 ± 1.66	19.82 ± 1.96	18.95 ± 0.62	17.69 ± 1.78
P	**10.70 ± 0.66**	**10.34 ± 0.78**	10.12 ± 0.56	9.35 ± 0.81
	***p* = .013**			
Mg	0.28 ± 0.04	0.27 ± 0.05	**0.29 ± 0.04**	**0.25 ± 0.04**
			***p* = .019**	
Ca/P Ratio	1.90 ± 0.05	1.91 ± 0.06	1.88 ± 0.07	1.89 ± 0.04

Bold numbers indicate statistically significant differences.

About the histometrical differences at 5 and 8 weeks after implantation, our results agree with Tresguerres and colleagues,[10] who showed differences between GH treatment and control at 6 weeks in BIC ratio. However, Tresguerres used young rabbits, perhaps not the best experimental model (as the author discussed). The differences may be caused by the differences in the metabolism of dogs and rabbits. Anyway, the circulating half-life of melatonin[36] and GH[37] are 23 and 20 minutes, respectively, and at 5 weeks, the bone remodeling process around a dental implant in the dog mandible is almost healed. We believe that it is interesting to use a carrier, in order to release it gradually and to increase the half-life in the bone-implant interface.

In the microanalysis by EDX, slightly more calcium was found in the treated group, showing significant differences in phosphorus concentrations at 2 weeks and in magnesium ratios at 5 weeks. Ca/P ratio values had been previously described in dogs and did not differ from the values found.[38] Magnesium is essential to the synthesis of alkaline phosphatase[39] and osteocalcin, affecting the osteoblastic activity[40] and increasing the bone strength.[41] Significant increment of both cations in the treated group may indicate an increased mineral apposition rate and magnesium, individually, may increment the osteoblastic activity and accelerate the bone maturation process.

These findings strongly suggest that these two molecules have the potential to promote osseointegration. GH could act synergistically with melatonin in the induction of bone formation. Although the exact roles of these molecules during osteogenesis are not well understood, it is likely that GH and melatonin work through some related mechanisms but, separately, each of them plays an important and different role. The stimulatory effects of these two molecules are not identical: GH is typically thought to control osteoprogenitor cell proliferation whereas melatonin is more important in osteoblast differentiation and osteoclast inhibition. In any case, we speculate that ML-GH therapy on bone in the oral cavity will not only focus on dental implantology, but will also extend to other areas such as periodontal disease, which exhibits progressive bone loss in its advanced stages.

In conclusion, the present study shows that GH and melatonin, each of which has nearly equal stimulatory effects on osteogenesis, synergistically enhanced new bone formation around titanium implants in early stages of healing.

ACKNOWLEDGMENTS

The authors thank the Spanish Ministries of Health and Education for funding this work through FIS project PI041610, CICYT project PETRI 95-0885-OP, MAT2005-07244-C03-02 and Microdent Implant System (Barcelona, Spain) that provided the implants.

REFERENCES

1. Zechner W, Tangl S, Tepper G, et al. Influence of platelet-rich plasma on osseous healing of dental implants: a Histologic and Histomorphometric Study in minipigs. Int J Oral Maxillofac Implants 2003; 18:15–22.
2. Ohlsson C, Bengtsson BA, Isaksson OG, Andreassen TT, Slootweg MC. Growth hormone and bone. Endocr Rev 1998; 19:55–79.
3. Harvey S, Kull KL. Growth hormone, a paracrine growth factor? Endocrine 1998; 7:267–279.
4. Ohlsson C, Vidal O. Effects of growth hormone and insulin-like growth factors on human osteoblasts. Eur J Clin Invest 1998; 28:184–186.
5. Kassem M, Blum W, Ristelli J, Mosekilde L, Eriksen EF. Growth hormone stimulates proliferation and differentiation of normal human osteoblast-like cells in vitro. Calcif Tissue Int 1993; 52:222–226.
6. Bak B, Jørgensen PH, Andreassen TT. Increased mechanical strength of healing rat tibial fractures treated with biosynthetic human growth hormone. Bone 1990; 11:233–239.
7. Bak B, Andreassen TT. The effect of growth hormone on fracture healing in old rats. Bone 1991; 12:151–154.
8. Nielsen HM, Bak B, Jørgensen PH, Andreassen TT. Growth hormone promotes healing of tibial fractures in the rat. Acta Orthop Scand 1991; 62:244–247.
9. Tresguerres IF, Clemente C, Donado M, et al. Local administration of growth hormone enhances periimplant bone reaction in an osteoporotic rabbit model. An histologic, histomorphometric and densitometric study. Clin. Oral Impl. Res 2002; 13:631–636.
10. Tresguerres IF, Alobera MA, Baca R, Tresguerres JAF. Histologic, morphometric, and densitometric study of peri-implant bone in rabbits with local administration of growth hormone. Int J Oral Maxillofac Implants 2005; 20:193–202.
11. Gómez-Moreno G, Cutando A, Arana C, et al. The effects of growth hormone on the initial bone formation around implants. Int J Oral Maxillofac Implants 2009. (In press)
12. Miller SC, Pandi PSR, Esquifino AI, Cardinali DP, Maestroni GJM. The role of melatonin in immuno-enhancement: potential application in cancer. Int J Exp Pathol 2006; 87:81–87.
13. Turgut M, Uslu S, Uysal A, Yurtseven ME, Ústün H. Changes in vascularity of cartilage endplate of degenerated intervertebral discs in response to melatonin administration in rats. Neurosurg Rev 2003; 26:133–138.

14. Oktem G, Uslu S, Vatansever SH, Aktug H, Yurtseven ME, Uysal A. Evaluation of the relationship between inducible nitric oxide synthase (iNOS) activity and effects of melatonin in experimental osteoporosis in the rat. Surg Radiol Anat 2006; 28:157–162.
15. Takechi M, Tatehara S, Satomura K, Fujisawa K, Nagayama M. Effect of FGF-2 and melatonin on implant bone healing: a histomorphometric study. J Mater Sci Mater Med 2008; 19:2949–2952.
16. Cutando A, Gómez-Moreno G, Arana C, Acuña-Castroviejo D, Reiter RJ. Melatonin: potential functions in the oral cavity. J Periodontol 2007; 78:1094–1102.
17. Cutando A, Galindo P, Gómez-Moreno G, et al. Relationship between salivary melatonin and severity of periodontal disease. J Periodontol 2006; 77:1533–1538.
18. Gómez-Moreno G, Cutando A, Arana C, et al. Melatonin expresión in periodontal disease. J Periodontal Res 2007; 42:536–540.
19. Cutando A, Gómez-Moreno G, Arana C, et al. Melatonin stimulates osteointegration of dental implants. J Pineal Res 2008; 45:174–179.
20. Ducy P, Schinke T Karsenty G. The osteoblast: a sophisticated fibroblast under central surveillance. Science 2000; 289:1501–1504.
21. Nakade O, Koyama H, Ariji H, Yajima A, Kaku T. Melatonin stimulates proliferation and type I collagen synthesis in human bone cells in vitro. J Pineal Res 1999; 27:106–110.
22. Koyama H, Nakade O, Takada Y, Kaku T, Lau KHW. Melatonin at pharmacologic doses increases bone mass by suppressing resorption through down-regulation of the RANKL-mediated osteoclast formation and activation. J Bone Miner Res 2002; 17:1219–1229.
23. Cardinali DP, Ladizesky MG, Boggio V, Cutrera RA, Mautalen C. Melatonin effects on bone: experimental facts and clinical perspectives. J Pineal Res 2003; 43:81–87.
24. Roth JA, Kim BG, Song F, Lin W, Cho M. Melatonin promotes osteoblast differentiation and bone formation. J Biol Chem 1999; 274:22041–22047.
25. Satomura K, Tobiume S, Tokuyama R, et al. Melatonin at pharmacological doses enhances human osteoblastic differentiation in vitro and promotes mouse cortical bone formation in vivo. J Pineal Res 2007; 42:231–239.
26. Boyce BF, Schwarz EM, Xing L. Osteoclast precursors: cytokine-stimulated immunomodulators of inflammatory bone disease. Curr Opin Rheumatol 2006; 18:427–432.
27. Ladizesky MG, Cutrera RA, Boggio V, et al. Effect of melatonin on bone metabolism in ovariectomized rats. Life Sci 2001; 70:557–565.
28. Suzuki N, Hattori A. Melatonin suppresses osteoclastic and osteoblastic activities in the scales of goldfish. J Pineal Res 2002; 33:253–258.
29. Radio NM, Doctor JS, Witt-Enderby PA. Melatonin enhances alkaline phosphatase activity in differentiating human adult mesenchymal stem cells grown in osteogenic medium via MT2 melatonin receptors and the MEK/ERK (1/2) signaling cascade. J Pineal Res 2006; 40:332–342.
30. Witt-Enderby PA, Radio NM, Doctor JS, Davis VL. Therapeutic treatments potentially mediated by melatonin receptors: potential clinical uses in the prevention of osteoporosis, cancer and as an adjuvant therapy. J Pineal Res 2006; 41:297–305.
31. Donath K, Breuner G. A method for the study of undecalcified bones and teeth with attached soft tissues. J Oral Path 1982; 11:318–326.
32. Laczkó J, Levai G. A simple differential staining method for semi-thin sections of ossifying cartilage and bone tissues embedded in epoxy resin. Mikroskopie 1975; 31S:1–4.
33. Hedner E, Linde A, Nilsson A. Systemically and locally administered growth hormone stimulates bone healing in combination with osteopromotive membranes: an experimental study in rats. J Bone Miner Res 1996; 11:1952–1960.
34. Andreassen TT, Jørgensen PH, Flyvbjerg A, Orskov H, Oxlund H. Growth hormone stimulates bone formation and strength of cortical bone in aged rats. J Bone Miner Res 1995; 10:1057–1067.
35. Kireev RA, Tresguerres ACF, Castillo C, et al. Effect of exogenous administration of melatonin and growth hormone on pro-antioxidant functions of the liver in aging male rats. J Pineal Res 2007; 42:64–70.
36. Gibbs FP, Vriend J. The half-life of melatonin elimination from rat plasma. Endocrinology 1981; 109:1796–1798.
37. Drange MR, Melmed S. IGFs in evaluation of acromegaly. In: Roberts CT, Rosenfeld RG, eds. The IGF System: molecular biology, physiology and clinical applications. Totowa, NJ: Humana Press, 1999:702.
38. Biltz RM, Pellegrino ED. The chemical anatomy of bone: I. A comparative study of bone composition in sixteen vertebrates. J Bone Joint Surg Am. 1969; 51:456–466.
39. Lai CC, Singer L, Armstrong WD. Bone composition and phosphatase activity in magnesium deficiency in rats. J Bone Joint Surg Am. 1975; 57:516–522.
40. Boskey AL, Rimnac CM, Bansal M, Federman M, Lian J, Boyan BD. Effect of short-term hypomagnesemia on the chemical and mechanical properties of rat bone. J Orthop Res 1992; 10:774–783.
41. Shiga K, Hara H, Suzuki T, Nishimukai M, Konishi A, Aoyama Y. Massive large bowel resection decreases bone strength and magnesium content but not calcium content of the femur in rats. Nutrition 2001; 17:397–402.