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ORIGINAL ARTICLE

Melatonin promotes angiogenesis during repair of bone defects: a radiological and histomorphometric study in rabbit tibiae

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Abstract
Objectives The pineal gland hormone, melatonin, is an immunomodulator and neuroendocrine hormone; it also stimulates monocyte, cytokine and fibroblast proliferations, which influence angiogenesis. The aim of this study was to investigate the effects of melatonin on angiogenesis during bone defect repair by means of radiological and histomorphometric evaluations of bone response to melatonin implants.
Materials and methods Twenty New Zealand rabbits weighing 3,900–4,500 g were used. Twenty melatonin implants were inserted in the proximal metaphyseal area of the animals' right tibia and 20 control areas were located in the left proximal metaphyseal area. Following implantation, the animals were sacrificed in groups of five, after 1, 2, 3 and 4 weeks, respectively. Anteroposterior and lateral radiographs were taken, and radiographic thermal imaging analysis was performed for all groups at different time stages following implant insertion. Samples were sectioned at 5 μm and stained using Hematoxylin–Eosin and Masson's trichrome, supplementing radiographic findings with histomorphometric analysis.
Results After 4 weeks, radiological images showed complete repair of the bone defects. No healed or residual bone alterations attributable to the presence of the melatonin implant were observed. Histomorphometric analysis at 4 weeks showed the presence of a higher density newly formed bone. There were statistically significant differences in the length of cortical formation between the melatonin group and the control group during the first weeks of the study; there were also statistically significant differences in the number of vessels observed in the melatonin groups at the first two study stages.
Conclusion and clinical relevance Melatonin may have potential beneficial effects on bone defect repair.

Keywords Melatonin · Angiogenesis · Bone tissue remodeling · Osteogenesis

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Introduction

Melatonin, or *N*-acetyl 5-methoxytriptamine, is a hormone synthesized and secreted mainly in the pineal gland [1]. Melatonin synthesis does not only take place in the pineal gland but also in other areas such as the eyes, lymphocytes, gut, bone marrow, skin and gonads where it acts either as a paracrine or an autocrine [2]. Because of its diverse activity, melatonin is not a hormone in the strictest sense. Melatonin has significant bone protecting properties [3]. Ladizesky et al. [4] found that melatonin inhibited in vitro the increased calcium uptake in bone samples of rats treated with pharmacologic amounts of methylprednisolone. Numerous studies [5–10] have documented melatonin's importance as a mediator in bone formation. It acts as a promoter of bone formation in vivo by enhancing both the proliferation and differentiation of osteogenic cells [11]. In micromolar concentrations, melatonin stimulates the synthesis of type I collagen fibers in human osteoblasts in vitro [12]. In addition, Roth et al. [13] found that it increased gene expression of bone sialoprotein as well as other proteins and bone markers (including alkaline phosphatase, osteopontin and osteocalcin in preosteoblasts), reducing the osteoblast differentiation period from 21 to 12 days.

Another possible target cell for melatonin is the osteoclast, which reabsorbs existing bone through the generation of free radicals [14]. The generation of oxygen-derived free radicals may play a crucial role in bone resorption occurring in inflammatory conditions. Cyclooxygenase (COX) is the key enzyme that catalyzes the two sequential steps in the biosynthesis of prostaglandins from arachidonic acid. The inducible isoform of COX, namely COX-2, plays a critical role in the inflammatory response [15]. Melatonin, by means of its indirect antioxidant and direct free radical scavenging action, may interfere with osteoclast activity and thereby increase bone mass, mainly through the suppression of bone resorption [16]. Melatonin also seems to cause the inhibition of bone resorption by downregulating nuclear factor B-mediated osteoclast activation [17]. Studies examining the effects of melatonin upon the immune system have considered that abundant reactive oxygen species are produced in situations of bone disease, characterized by an increase in peroxidation products generated by the infiltrating polymorphonuclear cell population [18]. Melatonin influences both antioxidant enzyme activity and cellular messenger RNA (mRNA) levels for these enzymes both under normal physiological conditions and during elevated oxidative stress [19]. In this way, melatonin may play a role in protecting the oral cavity from tissue damage due to oxidative stress and may contribute to the regeneration of alveolar bone through the stimulation of type I collagen fiber production and the modulation of osteoblastic and osteoclastic activities [20].

The repair of bone defects is a natural wound healing process involving the remodeling of bone tissues; a set of complex biochemical events take place in closely orchestrated arrangement. Although these events overlap in time, they may be categorized into separate stages: inflammatory, proliferative, maturation and remodeling phases [21]. The proliferative phase is characterized by angiogenesis, collagen deposition and formation of granulation tissue. Angiogenesis, the growth of new blood capillaries from existing vessels, is the key physiological process and is controlled by signals from proangiogenic molecules [22]. Numerous mediators including growth factors, transcription factor and signaling molecules have been reported to play a major role in inflammation-associated angiogenesis [23]. Growth factors such as vascular endothelial growth factor (VEGF) are considered to be potential angiogenic modulators, and melatonin brings about significant proangiogenic activity by increasing VEGF expression [24]. This study investigates the effects of melatonin on angiogenesis in healing bone defects in rabbit tibiae.

Materials and methods

Animals, surgery and treatment

A total of 20 melatonin implants were placed in the proximal metaphyseal area of the right tibias of 20 albino New Zealand rabbits aged 30–35 weeks and weighing 3,900–4,500 g. All experiments were approved and performed according to the Spanish Government and European Community Guidelines for animal care.

Fifteen minutes before general anesthesia, the animals received an intramuscular injection of an anxiolytic (0.5–1 mg/kg acepromazine maleate). General anesthesia included 5–8 mg/kg ketamine plus chlorbutol administered intravenously, 0.5–1 mg/kg acepromazine maleate as coadjuvant and 0.05 mg/kg atropine.

The internal approach was performed in the proximal metaphyseal–diaphyseal area of each tibia, several millimeters below the anterior tibial tuberosity. Spherical surgical drills at low speed with constant irrigation were used to remove bone tissue to form two concave defects approximately 5 mm in diameter per tibia. The first was filled with 1.2 mg lyophylized powder of melatonin (Sigma-Aldrich™–M5250) and the second was used as a control site (Fig. 1).

Amoxicillin (0.1 ml/kg intramuscularly) was administrated at the end of surgery. The animals were given 0.05 mg/kg buprenorphine subcutaneously every 12 h after the operation for 3 days. Within 2–3 days, the animals resumed normal ambulation and did not show signs of pain or distress. Five rabbits per group were sacrificed by means of

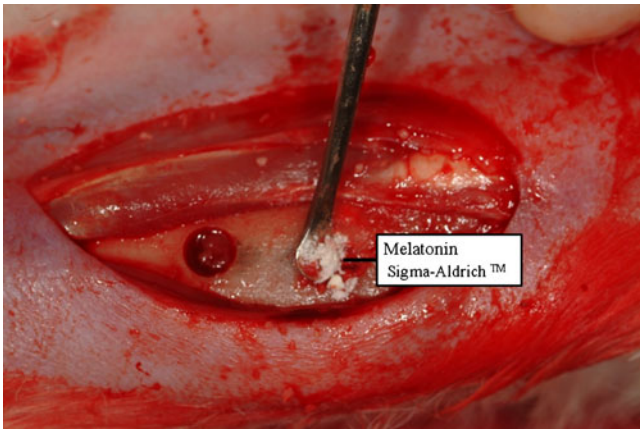

Fig. 1 Two concave defects per tibia were created, approximately 5 mm in diameter. The first was filled with melatonin implant and the second was used as a control site

intracardiac overdoses of thiopental, at 1, 2, 3 and 4 weeks following implantation. The same sedation and anesthesia protocol as for surgery was applied prior to sacrifice.

Radiological study

Two x-rays, anteroposterior and lateral, were taken of the sections of bone containing implants and control defects using the Kodak RVG 6100 Digital Radiography System (Kodak DS, Rochester, NY, USA) with x-rays taken at 32 kV, 40 mA, using an automatic light meter. Radiovisiographs were taken of each transversal tibia section containing implants. All radiographs were taken by the same researcher (Fig. 2).

The images obtained were processed using Image J software, developed by the National Institute of Health (NIH) of the United States, a 3D plug-in with a thermal LUT with a grid size of 128×128, smoothing of 6 and a perspective of 0.2 at a scale of 1:1; color thermal graduation was used to observe changes in radiopacity within each medullar and cortical areas where the defects had been created. The scale was graduated with values from 20 to 140. The value range 20–30 corresponded to air, 31–79 to water and soft tissues, 80–99 to lower density bone and 100–140 to higher density bone (Figs. 3 and 4).

Optical microscopy and morphometric analysis

The surgically acquired samples were fixed in 10% neutral buffered formalin and decalcified by means of immersion in Osteomol®, Merck KbaA (Germany) containing HCl (10%) and CH2O (4%) for 17 days, renewing the solution every 24 h. Subsequently, all samples were embedded in paraffin via the usual method, sectioned at 5 μm and stained using Hematoxylin–Eosin, Masson's trichrome stain. All samples were examined under a Nikon Eclipse 80i microscope (Teknooptik AB, Huddinge, Sweden) equipped with the EasyImage 2000 system (Teknooptik AB) using×1.0 to×4.0 lenses for descriptive evaluation and for taking morphometric measurements. The entire circumference of each section was traced manually to create individual regions of interest (ROI). Five consecutive regions were chosen randomly and the number of vessels in each was detected and recorded under×40 amplification.

To calculate the percentage of covered cavity defect, images were generated using a Leika Z6 APO macroscope connected to a Leika DC 500 (Barcelona, Spain) digital camera and enlarged×23. These were used to digitalize and calibrate images of the defect cavity zone and then interactive measurements of the areas of interest were obtained using Leica Q Win V3 (Barcelona, Spain) image analysis software.

Statistical analysis

The significance of differences between study groups were tested for all parameters using Student's *t*-test, performed using specialized software (SPSS 15.0 for Windows, Chicago, IL, USA), taking $p<0.05$ as significant.

Results

One week

Radiological study

Control bone defects showed radiotransparent concave depressions of round or rectangular morphology depending on the image studied. They had clear and regular outlines showing homogenous densities that clearly defined their boundaries (Figs. 2a and 4a). X-rays of melatonin implant-filled bone defects revealed the characteristics of the implanted material, which could not be identified within the trabecular bone structure in which it was implanted due to its radiological density (Figs. 2b and 4b).

Optical microscopy

The most relevant morphological changes to the bone defect areas in the control group were at the cortical level, where an outer layer of fibrinohematic tissue covered the orifices (Figs. 5a and 6a). At implant sites, an anatomopathological study highlighted the cortical bone; the surgical orifice had reformed with a fine outer layer of fibrinohematic tissue. Optical microscopy of the implant and cortical defect sites revealed the substitution of bone by granulated tissue extending towards the implant and invading the implanted material, which it partially furnished (Figs. 5b and 6b). This

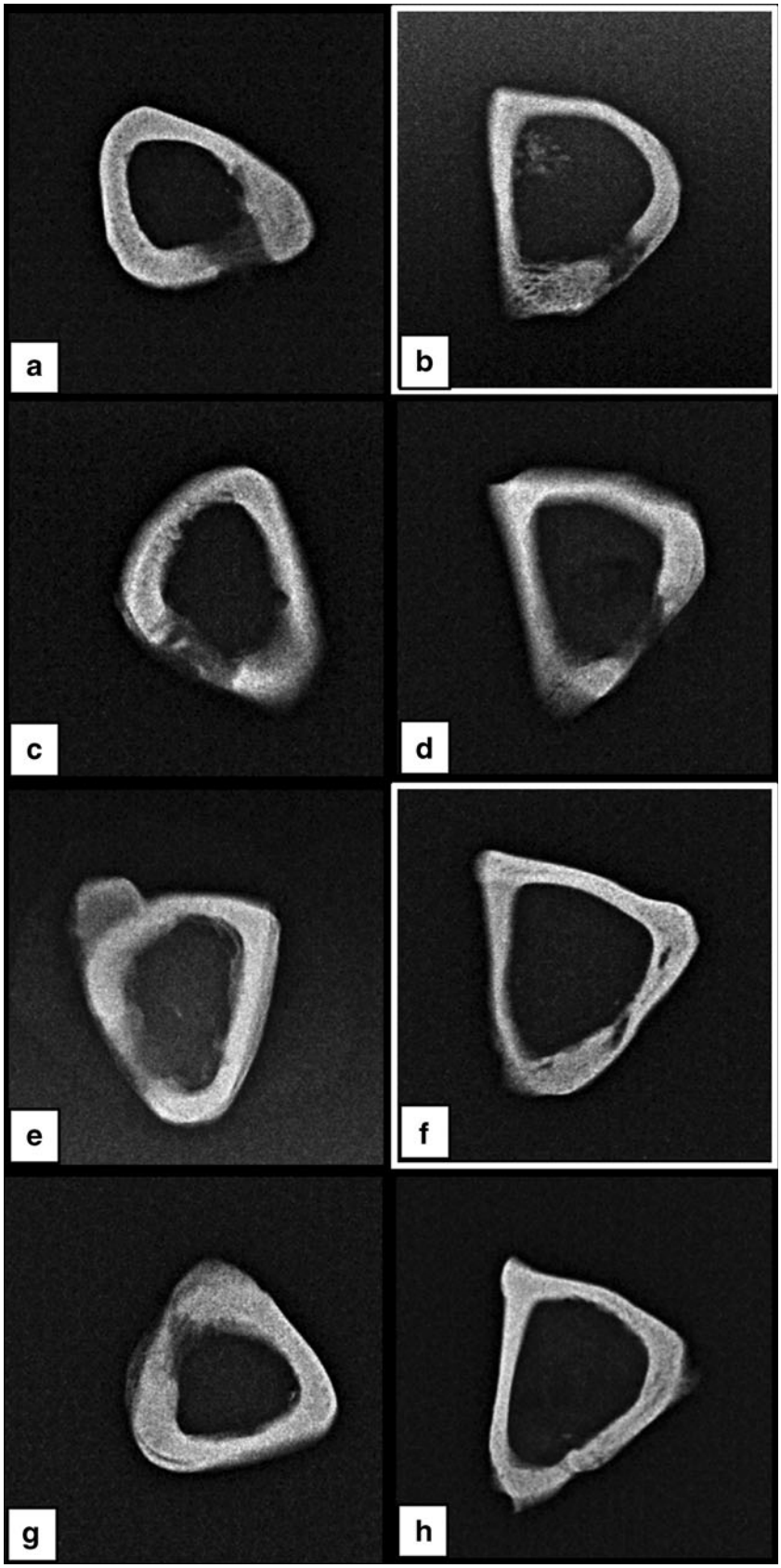

Fig. 2 **a** (Control group) transversal section radiovisiograph of surgical specimen at 1 week. **b** (Melatonin group) transversal section radiovisiograph at 1 week. **c** (Control group) transversal section radiovisiograph at 2 weeks. **d** (Melatonin group) transversal section radiovisiograph at 2 week. **e** (Control group) transversal section radiovisiograph at 3 weeks. **f** (Melatonin group) transversal section radiovisiograph at 3 weeks. **g** (Control group) transversal section radiovisiograph at 4 weeks. **h** (Melatonin group) transversal section radiovisiograph at 4 weeks

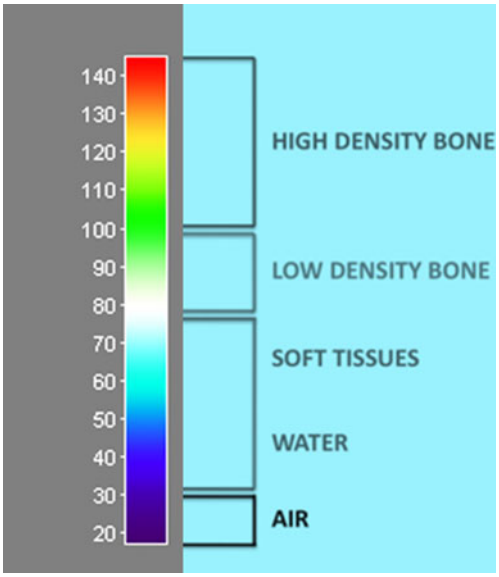

Fig. 3 Color graduated density scale for thermal imaging interpretation from 20 (lower densities) to 140 (higher densities)

tissue was made up of numerous endothelial sprouts and capillary blood vessels as well as abundant mesenchymal cells of irregular morphology with ample cytoplasm and numerous fibroblasts arranged randomly in a matrix of abundant fundamental substance, collagen fibers, macrophages and scattered lymphocytes (Fig. 7).

Two weeks

Radiological study

In the bone defects taken as control sites, some significant differences were observed when compared with the previous evaluation period. X-ray images revealed linear elements representing irregular trabecular lines that did not follow the axes or load forces of adjacent bone trabeculation. Some of these images were framed inside areas of greater radiotransparency, which at this point in time did not show the same concave bone defect morphology as the study sites (Figs. 2c and 4c).

In the melatonin implant-filled bone defects, X-rays highlighted the cortical–osteoblastic line as being completely repaired, albeit with less density than that of the adjacent cortical bone (Figs. 2d and 4d).

Optical microscopy

In this study period, the control group showed noticeable bone repair phenomena around the defects' peripheries and around adjacent bone marrow (Figs. 5c and 6c). An anatomopathological study highlighted the perforation made in the cortical bone in order to perform the melatonin implant, now almost covered by neoformed or immature osseous tissue. Likewise, the implanted melatonin was surrounded by osseous trabeculae, which were more extensive and thicker than those observed in the previous time period, giving the implant zone a reticular appearance (Figs. 5d and 6d).

Three weeks

Radiological study

After 3 weeks, x-ray images of control sites showed similar characteristics to the previous study period (Figs. 2e and 4e). X-rays revealed the external cortex of the artificial osseous lagoons into which the melatonin implant had been introduced as having a calcium density similar to that of the adjacent cortex, making it difficult to identify the surgical orifice. At the level of the cortex, the implant area displayed a decreased radiological density with respect to the previous group as well as a more oval shape with a lower calcium density within. Well-defined borders could not be distinguished radiologically (Figs. 2f and 4f).

Optical microscopy

In the control group, gap junctions between newly formed cortical bone and the adjacent cortices were observed, accompanied by extensive bone regeneration phenomena, which spread beyond the lower edge of the adjacent cortex (Figs. 5e and 6e). An anatomopathological study of the melatonin group showed complete bone reparation of the cortex at the implant orifices, manifested as well-organized trabecular bone with an increase in osseous remodeling. We also observed, albeit to a lesser degree, the formation of osseous trabeculae as well as a marked increase in hematopoietic and adipose bone marrow at the center, which had partially replaced the granulated tissues (Figs. 5f and 6f).

Four weeks

Radiological study

X-rays of the control bone defects at the end of the experiment showed characteristics similar to those described for the previous group with one or more rectilinear lines that could be observed traversing the bone perpendicularly (Figs. 2g and 4g). X-rays of the implanted material produced images showing complete repair of the osseous defects. Trabeculae reaching the implant were greater in number and density than those in the previous time period, giving the implanted area a slightly reticular appearance. No healed or residual bone alterations attributable to the presence of the implant were observed. Nor were any osseous

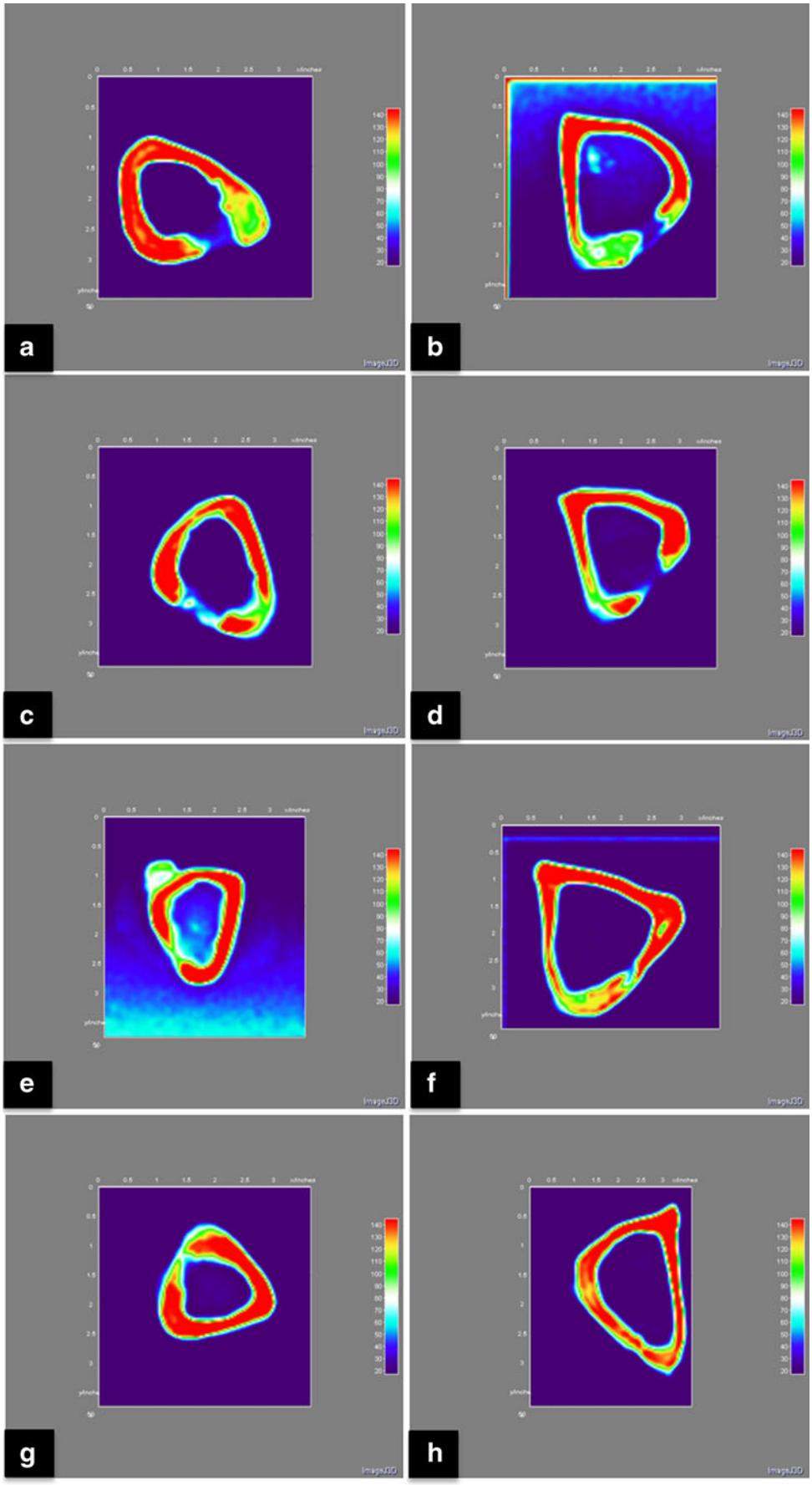

Fig. 4 Colour thermal graduation was used to observe variations related with bone formation and bone defect closure. As we can observe the comparison between controls (*images at left side*) and test (*images at right side*) clearly showed a different rate of defect closure, improved by melatonin effect

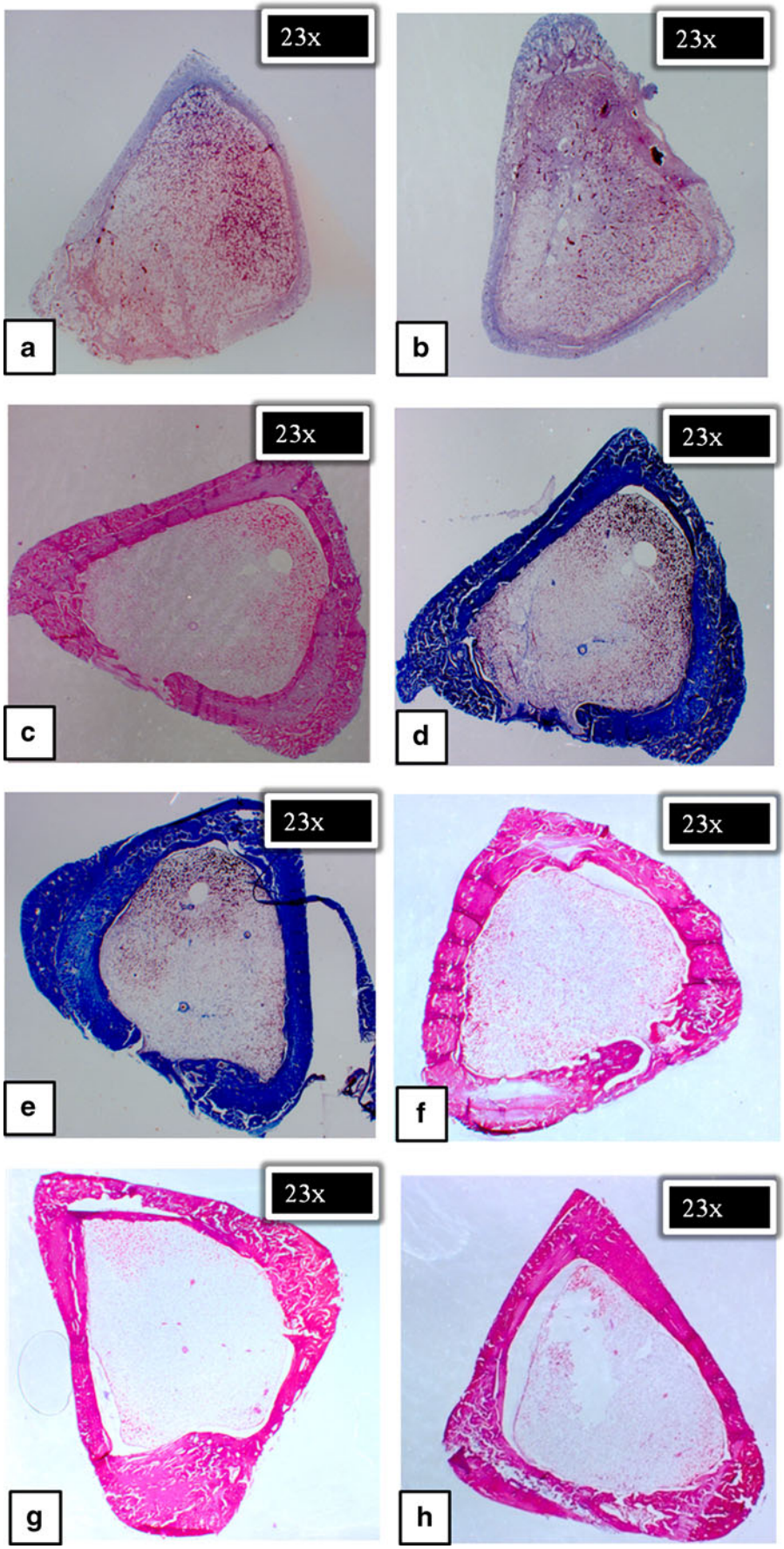

Fig. 5 **a** (Control group) panoramic image at 1 week **b** (Melatonin group) panoramic image at 1 week. **c** (Control group) panoramic image at 2 weeks. **d** (Melatonin group) panoramic image at 2 weeks. **e** (Control group) panoramic image at 3 weeks. **f** (Melatonin group) panoramic image at 3 weeks. **g** (Control group) panoramic image at 4 weeks. **h** (Melatonin group) panoramic image at 4 weeks. **a** T.M. 23×, **b** T.M. 23×, **c** H.E. 23×, **d** T.M. 23×, **e** T.M. 23×, **f** H.E. 23×, **g** H.E. 23×, **h** H.E. 23×

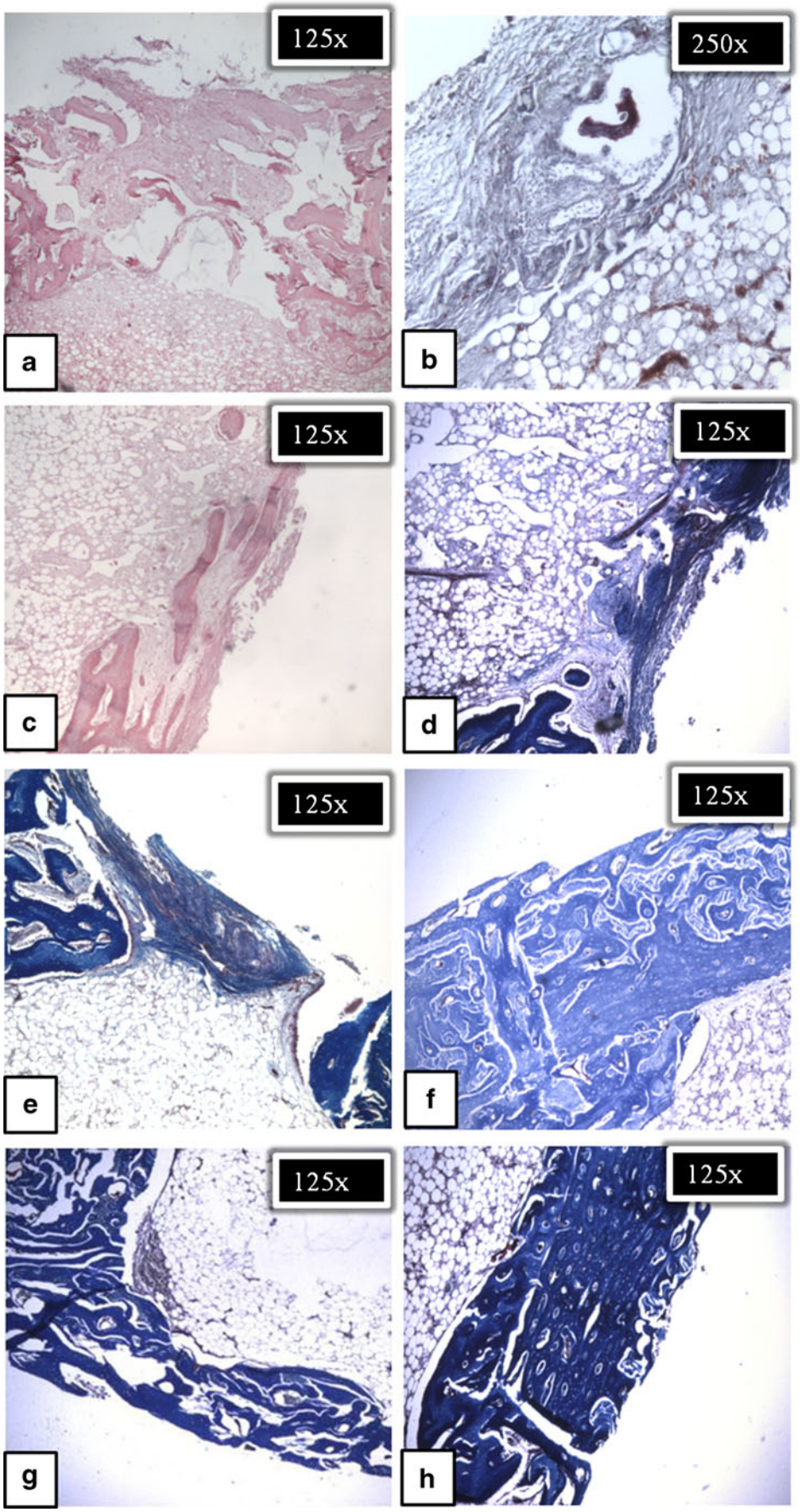

Fig. 6 **a** (Control group) microscopy detail at 1 week: young granulation tissue. **b** (Melatonin group) microscopy detail at 1 week: in the cortical bone, the surgical orifice had reformed with a fine outer layer of fibrinohematic tissue—increase in angiogenesis. **c** (Melatonin group) microscopy detail at 2 weeks: the perforation made in the cortical bone in order to perform the melatonin implant was almost completed by neoformed or immature osseous tissue. **e** (Control group) microscopy detail at 3 weeks: gap junction between newly formed cortical bone and adjacent cortex. **f** (Melatonin group) microscopy details at 3 weeks: trabecular bone formation in the vicinity and in continuity with the cortex: complete bone reparation of the cortex at the implant orifice, manifested as well-organized trabecular bone with an increase in osseous remodeling. **g** (Control group) microscopy details at 4 weeks: osseous remodeling of osseous trabeculae was observed in the control group, which was more pronounced in the proximity of the cortex. **h** (Melatonin group) microscopy detail at 4 weeks: complete bone remodeling was observed at the implant sites; anatomopathological study found the presence of mature osseous bone in the cortex at implant insertion sites, which could not be differentiated from the adjacent cortex. **a** H.E 125×, **b** T.M 250×, **c** T.M 125×, **e** T.M. 125×, **f** T.M. 125×, **g** T. M. 125×, **h** T.M. 125×

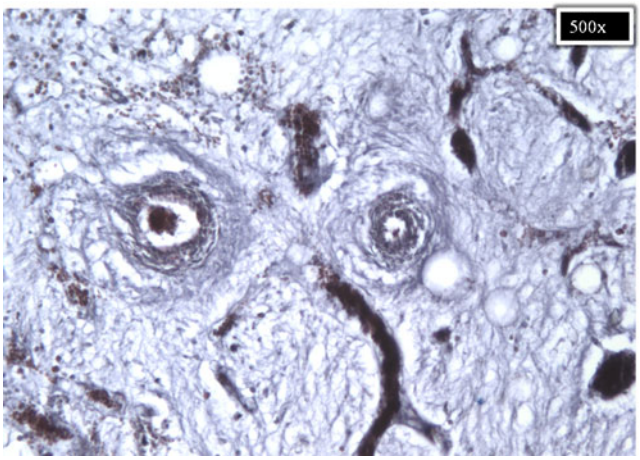

Fig. 7 Microscopy detail: the melatonin group showed a significant increase in the number of blood vessels in the first week of healing. T.M. 500×

malformations or structural changes to bone development observed during the entire study period (Figs. 2h and 4h).

Optical microscopy

Osseous remodeling of osseous trabeculae was observed in the control group, which was more pronounced in the proximity of the cortex (Figs. 5g and 6g). Complete bone remodeling was observed at the implant sites. An anatomopathological study revealed the presence of mature osseous bone in the cortex of the implant insertion site, so that it was not differentiable from the adjacent cortex (Figs. 5h and 6h).

Morphometric analysis

The results for the different histomorphometric parameters are presented in Table 1, showing the comparison of Mean–SD between blood vessel numbers in the control and melatonin groups. Table 2 shows Mean–SD values for length of cortical formation, comparing the control and melatonin treatment groups. Morphometric measurements revealed increasing amounts of mineralized bone as time went on, with significant differences between the evolution periods. With regard to blood vessel numbers, it was observed that in the first 2 weeks after surgery, the melatonin group showed a significantly increased number of blood vessels ($p<0.05$). In the same way, the length of the cortical formation in the melatonin group underwent statistically significant increases during the first ($p<0.001$) and second ($p<0.05$) weeks.

Table 1 Comparison of Mean–SD values for number of blood vessels in the control and melatonin treatment groups (*t*-test) for each evaluation period

	No. of blood vessels	
	Control	Melatonin group
1 week	4.11±1.71	9.52±2.16*
2 weeks	4.21±1.74	8.43±1.34*
3 weeks	4.16±1.24	7.14±1.23
4 weeks	4.09±1.21	7.11±2.05

*$p<0.05$

Table 2 Comparison of Mean–SD values for cortical formation length control and melatonin treatment groups (*t*-test) for each evaluation period

	Length of cortical formation	
	Control	Melatonin group
1 week	63.7±3.7%	90.1±0.7%**
2 weeks	84.8±3.7%	92.3±1.9%*
3 weeks	95.7±2.3%	96.5±2.1%
4 weeks	98.8±1.1%	99.1±0.7%

*$p<0.05$; **$p<0.001$)

Discussion

Previous studies have investigated the effect of melatonin hormone on angiogenesis in wound healing in animal models. The pineal gland hormone, melatonin, is a well-known neuroendocrine hormone. In addition to its effect as an immunomodulator, it also has a positive effect on monocyte, cytokine and fibroblast proliferation, which influence angiogenesis [23].

Bone healing is a complex process including cell proliferation, angiogenesis, blood circulation and matrix remodeling, of which angiogenesis is the most crucial; it is regulated by several growth factors including VEGF and inducible nitric oxide synthase (iNOS). Ganguly et al. [21] tested the angiogenic potential of melatonin in a gastric ulcer model. An application of melatonin was found to accelerate the healing process by affecting COX-2-mediated prostaglandin (PG) synthesis, expression of hypoxia inducible factor and activation of the iNOS system and so microcirculation restoration.

The present study found that melatonin implants induced angiogenesis significantly during the first 4 weeks. This indicates that melatonin maintains capillary homeostasis under normal conditions, as tissue was made up of numerous endothelial sprouts and capillary blood vessels. It also included abundant mesenchymal cells of irregular morphology with ample cytoplasm and numerous fibroblasts arranged randomly in a matrix of abundant fundamental substance, collagen fibers, macrophages and scattered lymphocytes. In the control group, it was observed that angiogenesis was enhanced time-dependently during the natural healing process, whereas melatonin accelerated the healing process, pointing to its strong angiogenic potential.

Others studies [24] have reported the effect of melatonin on the expression of the most potent angiogenic protein

VEGF, demonstrating that melatonin accelerates the process of normal wound healing by interfering in the modulation of the key biological processes involved in driving wound healing responses including inflammation, angiogenesis, collagen synthesis, orientation and maturation.

In the melatonin group, the angiogenic process was accelerated during the first weeks, a finding that agrees with Soybir et al. [23], who reported an increase in the number of blood vessels resulting from melatonin applications to wounds in rats. Our results also suggest that the regeneration of new bone was dependent on the blood supply available from marrow tissue in the existing peripheral bone. The importance of blood vessels in bone formation was noted as early as 1763, when it was observed that bone relies on arteries for the supply of mineral elements. Recently, the importance of angiogenesis in processes of osteogenesis and bone repair has been explored more fully and the penetration of marrow by new vessels has been found to allow the migration of angiogenic and osteogenic cells into secluded spaces [25–28]. Another study has also examined the role of angiogenesis in bone augmentation, pointing to its importance in guiding bone augmentation and bone formation, although it did not readily explain the correlation between angiogenesis and bone formation [22].

Angiogenesis is a multistep process, with its origins in the existing blood vessels in bone. When a wound occurs, factors responsible for the temporary action of blood vessel formation are released. Growth factors such as fibroblast growth factors have been shown to be potent angiogenic agents, and it has been suggested that cellular adhesion molecules may be important as mediators of angiogenesis [11].

Yamada et al. [22] have shown that angiogenesis precedes osteogenesis; angiogenesis can enhance bone regeneration as the formation of trabeculae would appear to be intimately associated with blood vessels. In this way, these authors' findings are of biological significance and support other studies of angiogenesis including our own that expand our understanding of how bone formation takes place. In the present study, the commencement of neovascularization and a significant increase in the number of vessels in the melatonin group were observed in the first two study stages, accompanied by an increase in the length of cortical formation. Early cell differentiation considerably accelerated the synthesis and mineralization of the osteoid matrix. According to other studies [20], melatonin would appear to contribute to bone neoformation as it stimulates preosteoblast differentiation, transported from bone marrow via the vascular system.

The action of melatonin on bone tissue is of clinical interest as it may be possible to apply melatonin as a biomimetic agent during endosseous dental implant surgery. Some reports have demonstrated the effect of melatonin on bone defects, augmenting cortical bone width and length formation. Calvo et al. [7] carried out radiographic and optical microscopy observation of the outcomes of topical applications of melatonin for accelerating bone formation associated with implants in a canine model. Four weeks after implant insertion, melatonin with collagenized porcine bone was seen to increase osteointegration and reduce crestal bone resorption significantly. Subsequently, a further study by Calvo et al. [8] using a rabbit metaphyseal defect model corroborated the results of the former study with regard to the of cortical bone perimeter of the tibiae, where melatonin induced new bone growth 88.35±1.56% versus 60.20±1.67% for control implants without melatonin; melatonin regenerated the width and length of cortical bone around implants in rabbit tibiae more quickly than around the control implants.

Comparing the present study with these earlier ones, although all observed the presence of a higher density of newly formed bone after 4 weeks, in the present study there were statistically significant differences in the length of cortical formation between the melatonin group and the control group in the first study stages' weeks, a finding that differs from the research carried out by Calvo et al [8] in which significant differences were found at all stages. Our results suggest that accelerated bone healing would appear to be more significant in the short term. The rapid increase in bone formation resulting from melatonin treatment suggests that the indoleamine acts on the remodeling process at two different levels simultaneously.

The process of wound healing has not yet been fully clarified. Biologically, it takes place in three stages: inflammatory, proliferative and remodeling phases. Macrophages and T-cells assume an important regulatory role at all stages. Platelets and fibroblasts as well as macrophages and neutrophils are important cellular elements of wound healing, engendering influential factors that affect healing processes such as migration and proliferation. In addition, some specific proteins produced by the macrophages such as growth factors, proteases, chemoattractans and inhibitory factors also play roles in the wound healing process. Immune system cells and their products including cytokines and growth factors, stimulate wound healing, especially during the proliferative and angiogenesis phases. Fibroblasts serve as the source of endothelial cells that generate neovascularization during the angiogenesis process. During the first week of the present study, fibroblasts were denser and endothelial cell proliferation was higher in the melatonin group than the control group.

Angiogenesis as a part of the wound healing process is another important phenomenon that is not yet fully understood. The study of this process is impeded because there is as yet no quantitative model for measuring vascularization in the wound continuously. Consequently, the effects of

many factors on angiogenesis remain unknown. In a study by Takechi et al. [11] the increase in total blood vessel length observed at 20 days indicated that fibroblast growth factors (FGFs) and transforming growth factor beta (TGF-β) assume the main role as the progenitors of endothelial cells in angiogenesis. The present study observed the effects of exogenous melatonin on the angiogenesis phases of wound healing with significant positive results. VEGF is essential for bone formation and repair during the osteogenesis process as it directly attracts endothelial cells and osteoclasts and enhances osteoblast differentiation [28].

Bone marrow-derived mesenchymal stem cells have demonstrated their potential for regenerative medicine in which oxygen tension plays a pivotal role. In vivo, a hypoxic environment can lead to apoptosis; in vitro, hypoxia can increase proliferation rates and enhance differentiation along the different mesenchymal lineages. Hypoxia also modulates the paracrine activity of mesenchymal stem cells, causing upregulation of various secretable factors, among which are important angiogenic factors such as VEGF. Hypoxia appears to regulate the increased production of soluble factors including VEFG via a nuclear factor kappa B (NF-κB) dependent mechanism, which suggests that hypoxia might also regulate immunoregulatory properties. The role of hypoxia in critical aspects of bone marrow-derived mesenchymal stem cells' (MSCs) behavior is, therefore, relevant to researchers in the field of tissue engineering and regenerative medicine in terms of survival, proliferation, differentiation and overall behavior [29].

Melatonin protects MSCs from apoptosis and promotes secretion of proangiogenic/mitogenic factors. In the present study, the increase in the number of surviving MSCs after grafting was associated with enhanced angiogenesis and cell proliferation. A previous study reported that melatonin strongly enhanced the release of angiogenic and mitogenic factors via mesenchymal stem cells. In addition, melatonin stimulated MSC expression of angiogenic and mitogenic factors and (FGFs) involved in cell protection and repair. Mias et al. [30] confirmed the relevance of the increase in paracrine activity of melatonin-treated MSCs by means of an analysis of renal function following ischemia–reperfusion.

It seems that melatonin promotes angiogenesis via increased VEGF expression. However, Dai et al. [31] reported a contrary observation during neoplastic growth in cancer patients in which melatonin blocked angiogenesis by attenuating VEGF secretion, suggesting that melatonin has an anti-angiogenic property during cancer proliferation prevention.

Other contradictory results have been reported. Melatonin is the major secretary product of the pineal gland and is considered an important natural onconstatic agent. The anti-cancer activity of melatonin is due to its immunomodulatory, antiproliferative and antioxidative effects. Due to the wide spectrum of melatonin's actions, the mechanisms that may be involved in its ability to counteract tumor growth are varied [32]. There are no direct data available as to melatonin's possible influence on angiogenesis, which is a major biological mechanism responsible for tumor growth and dissemination. However, melatonin suppresses HIF-1 transcriptional activity, leading to a decrease in VEGF expression, so melatonin could play a pivotal role in tumor suppression via inhibition of hypoxia-inducible factor-1 (HIF-1)-mediated angiogenesis [33]. The influence of melatonin on angiogenesis in cultured human umbilical vein endothelial cells shows that high concentrations of melatonin have antiproliferative and apoptotic effects. The results of a study by Cui et al. [34] suggest that melatonin's action may depend on the cell type, the functional state of the cell or other factors such as drug concentration and melatonin concentration, which may be crucial. Furthermore, the antitumor effects of melatonin would seem to be exerted at multiple levels [35].

Conclusions

Melatonin may have potential beneficial effects on bone defect repair. Because of its versatility in reducing inflammation, enhancing angiogenesis and bone cell proliferation, the application of melatonin for guided bone regeneration can improve clinical outcomes.

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