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In vivo Study of the Effect of Local Application of Melatonin and Beta-Tricalcium Phosphate on Healing of Experimentally Induced Bone Defect

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ABSTRACT

Introduction: *Bone healing is a highly complex regenerative process that the musculoskeletal system undergoes in order to restore skeletal integrity. Medical management for bone defects has been fundamentally absorbed in exchanging the lacking bone with bone grafts material.* ***Aim of the study:*** *The study aims to evaluate the effect of the combined local application of melatonin and ß-tricalcium phosphate (β-TCP) on the healing of induced bone defect by histological and histomorphometric analysis.* ***Materials and methods:*** *Total 24 adult male rats were divided into control and experimental groups, induction of femoral holes was done in both sides, whereas combined local application of melatonin and beta-tricalcium phosphate was done in one hole where the other hole was used as a control. The histological and histomorphometric analysis was done for all the specimens after scarification of animals for 1,2 and 4 weeks healing periods.* ***Results:*** *According to the findings of this study, the deposition of bone at operating sites was enhanced and accelerated after application of both melatonin and beta-tricalcium phosphate as mean values of bone trabecular area and number increased more than in control groups, and bone marrow area values decreased. Highest mean values of bone cells (osteoblasts and osteocytes) were detected in experimental groups. Osteoclast values decreased with time in the experimental group. In the control group showed the highest values in 2-weeks.* ***Conclusion:*** *Results of this study showed that the combined application of melatonin and beta-tricalcium phosphate was effective in the acceleration of the bone healing process.*

Keywords: Bone defect, Melatonin, ß-tricalcium phosphate

INTRODUCTION

Bone is a dynamic tissue undergoing remodeling throughout life, and this remodeling requires a balance between deposition of new bone by osteoblasts and resorption of old bone by osteoclasts [1]. Bone remodeling requires the interaction between multiple bone cells (osteoblasts/osteoclasts/osteocytes) to renew, maintain, or adjust the bone strength and/or mineral homeostasis in response to changing environmental influences. There are 4 distinct phases to this process: activation, resorption, reversal, and formation with resorption and formation taking place via osteoclasts and osteoblasts, respectively [2]. Bone defect healing represents complex processes that mimic parts of the embryological development of the bone framework. When there are proper environmental conditions, the bone will regain its initial form after the healing is complete. In order for this to happen, sometimes it is necessary to intervene by means of osteosynthesis materials to create the perfect circumstances for bone regeneration [3]. Appropriate biomaterials used for bone regeneration should be resorbable and gradually replaced by the newly formed bone. Melatonin is an endogenous hormone rhythmically produced in the pineal gland under the control of the suprachiasmatic nucleus (SCN) and the light/dark cycle. The investigation and applications of melatonin in the hard tissues bone and tooth have received great attention [4]. The β-tricalcium phosphate has been commonly conducted in rodents. Kondo, et al., in 2005 investigated the biocompatibility of highly purified and resorbable bone graft substitutes using a rat femur defect model [5]. The β-TCP has a low degradation rate and takes a longer period to be replaced by new bone tissue. When adherent to healthy bone, osteoid production occurs directly in the ceramic contact surface, this osteoid mineralizes and, consequently, bone remodeling occurs [6,7]. Remaining β-TCP particles were identified and it was observed that these areas constituted the focus for the formation of new bone [8].

PATIENTS AND METHODS

Total 24 adult male rats (*Rattus norvegicus*) weighing 250-350 g, age 7-8 months were used in this study. The animals were randomly divided into control and experimental groups (8 animals) for healing periods (1,2, and 4 weeks). Two holes of about 2.5 mm in depth and 2.5 mm in diameter were induced in each rat femur (one hole in right and one in left femur bones) [9]. In the control group, bone defect left to heal spontaneously. Bone defects of experimental groups were filled with a combination of (melatonin (0.5) mg dissolved in 0.1 ml propylene glycol) and β-TCP.

Scarification of all animals was done for the aforementioned healing periods. Specimens were prepared for histological and histomorphometric analysis of the trabecular area and bone marrow area. Histomorphometric measurements were determined using specific software which analyzes the microscopic images [10].

RESULTS

Histological Findings

Examination of histological sections of the bone of control group of 1-week duration revealed the deposition of osteoid bone by osteoblasts, view of the experimental group showed degradable materials invaded by newly deposited bone matrix osteocytes irregularly distributed inside bone matrix (Figures 1 and 2).

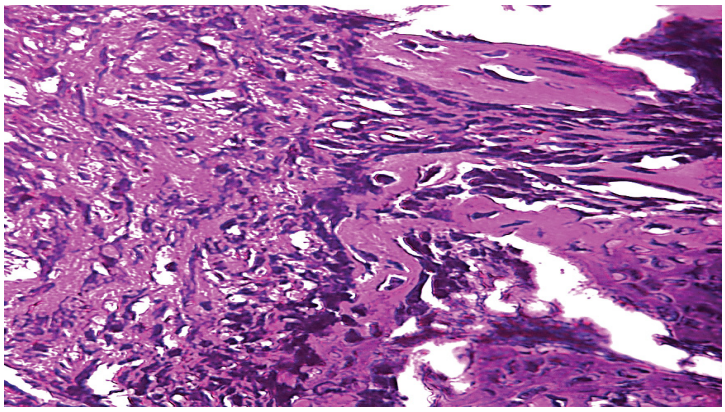

Figure 1 View of the defect site of a control group of 1week duration shows osteoid bone (OST), osteoblasts (OB) and osteocytes (OC)

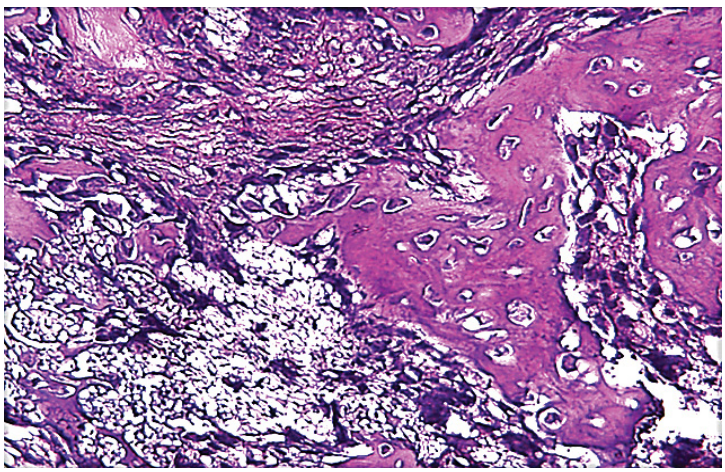

Figure 2 View of an experimental group of melatonin +β TCP after 1 week, shows material (MT) invaded by new bone, osteocytes (OC), osteoblast (OB) are noticed

After 2-weeks the histological examination of sections of control groups showed deposition of bone trabeculae

rimmed by osteoblasts, containing a large number of osteocytes, also reversal line demarcating new and old bone was detected. View of the experimental group shows the bone trabeculae enclosing areas of the applied material, osteocytes seen in bone which is rimmed by osteoblasts (Figures 3 and 4).

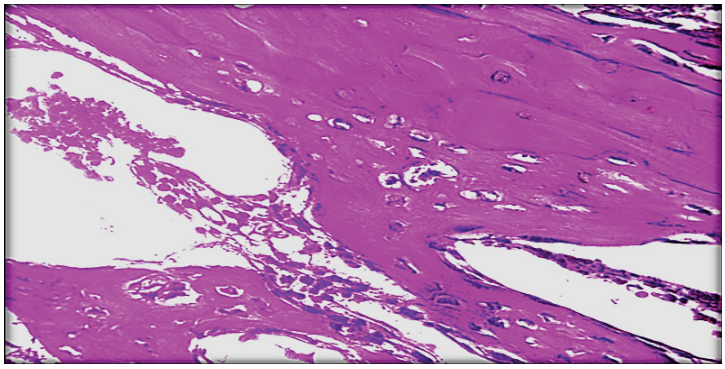

Figure 3 Microphotograph view of the control group after 2-weeks, shows deposition of new bone trabeculae (BT), osteocytes trapped in bone (OC), reversal line (arrow)

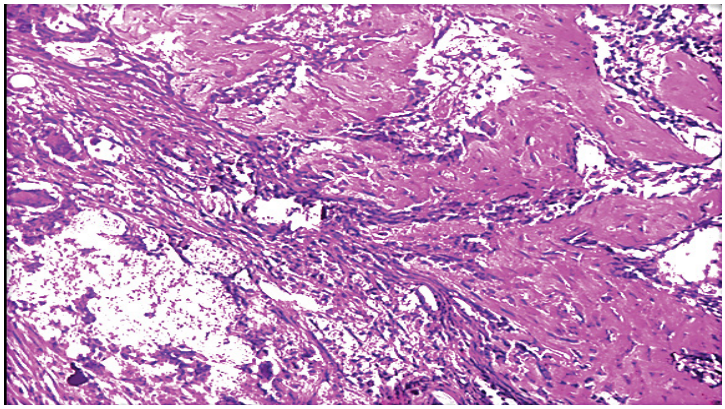

Figure 4 View of 2-weeks of MT2 group shows new bone trabeculae (BT) enclosing numerous osteocytes (OC), osteoblasts (arrow), melatonin and β-TCP materials (MT)

Microphotograph view of the operating site of the control group after 4-weeks shows dense bone filling defect site with small osteocytes trapped in bone. Mature dense bone is shown in view of experimental group after 4-weeks, osteoblasts at peripheries and a large number of osteocytes regularly distributed inside bone (Figures 5 and 6).

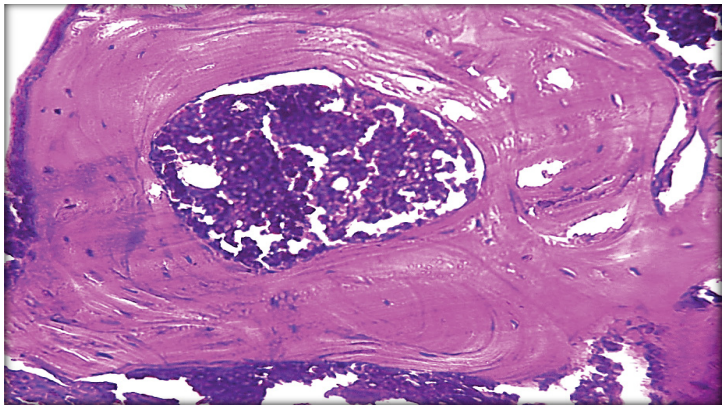

Figure 5 View of defect site of the control group after 4-weeks duration shows dense bone and osteocytes (OC)

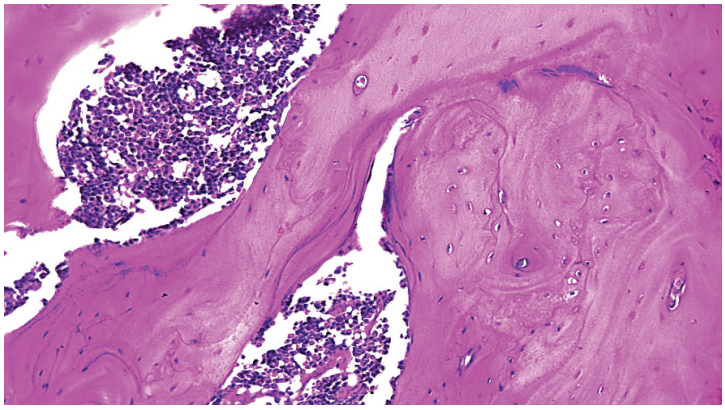

Figure 6 View of 4-weeks duration of a combination of melatonin +β-TCP group shows dense mature bone, osteocytes (OC)

Histomorphometric Analysis

Histomorphometric analysis of bone trabecular area and number showed that mean values were higher in the experimental group as compared to controls and those values increased with time, whereas bone marrow area showed a decrease in mean values as lowest values were recorded in experimental groups after 4-weeks (Table1) (Figures 7-9).

Table 1 Descriptive statistics of control and experimental groups at different healing periods for the bone trabecular area, trabecular number, and bone marrow area

Variables	Duration	Control Group						Combination Group					
		N	Mean	S.D	S.E	Min	Max	N	Mean	S.D	S.E	Min	Max
Trabecular area	2-weeks	8	0.112	0.007	0.002	0.106	0.119	8	0.152	0.008	0.002	0.145	0.159
	4-weeks	8	0.12	0.005	0.001	0.116	0.124	8	0.176	0.01	0.006	0.161	0.191
Trabecular No.	2-weeks	8	5.12	1.5	0.54	3.83	6.42	8	13.25	0.7	0.25	9.21	12.79
	4-weeks	8	7.5	1.8	0.65	5.95	9.05	8	15.5	1.7	0.62	13.48	17.02
Bone marrow area	2-weeks	8	0.142	0.011	0.003	0.132	0.0151	8	0.063	0.01	0.003	0.054	0.071
	4-weeks	8	0.12	0.009	0.003	0.112	0.128	8	0.041	0.004	0.002	0.035	0.047

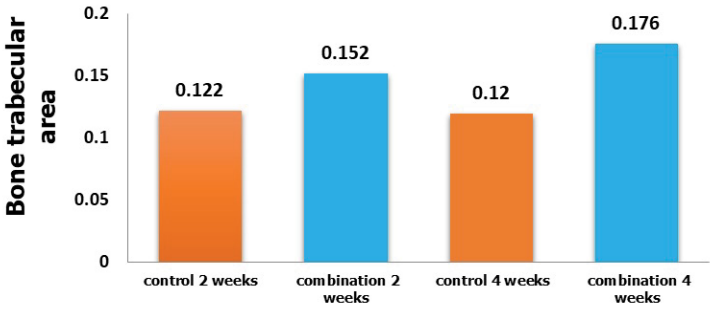

Figure 7 Comparison of mean values of the trabecular area in studied groups of different duration

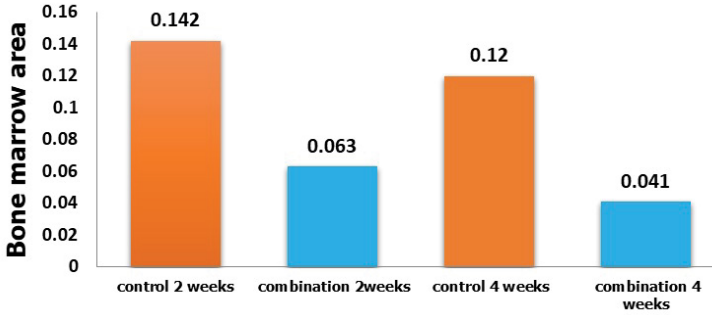

Figure 8 Comparison of mean values of the trabecular number in studied groups in different durations

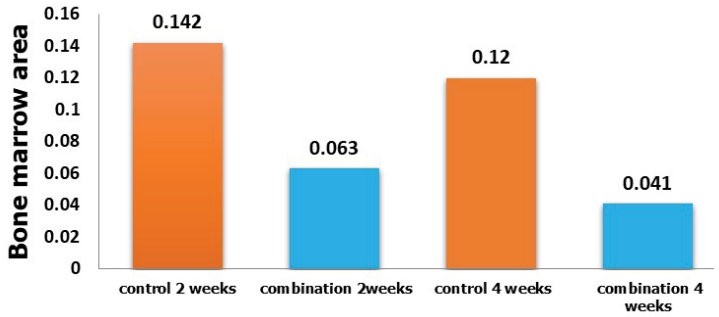

Figure 9 Comparison of mean values of bone marrow area in studied groups in different durations

Bone Cell

Regarding bone cells mean values of osteoblasts and osteocytes increased with time and were less in control groups as compared to experimental groups in different durations, the highest values were detected at 4-weeks. After 1-week the mean values of osteoclasts were higher in the experimental groups. The control group recorded the highest value in 2-weeks duration as compared to experimental groups. At 4-weeks duration period, the mean values of osteoclast cells decreased in control and experimental groups as illustrated in (Figures 10-12).

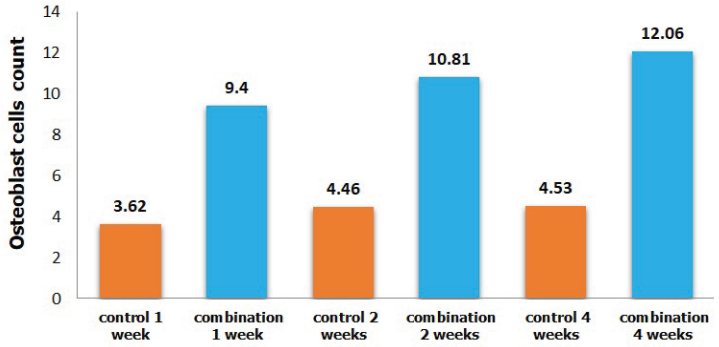

Figure 10 Comparison of mean values of osteoblasts in studied groups in different durations

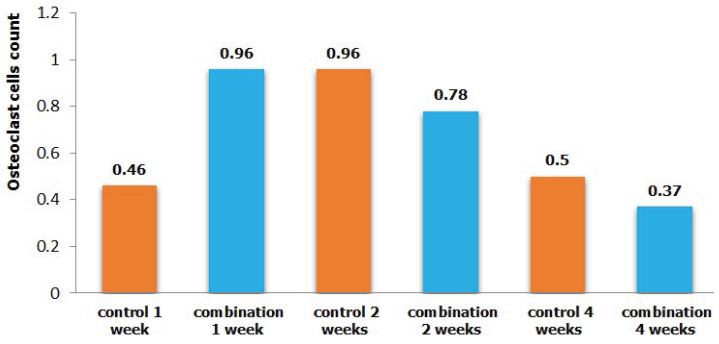

Figure 11 Comparison of mean values of osteoclasts in studied groups in different durations

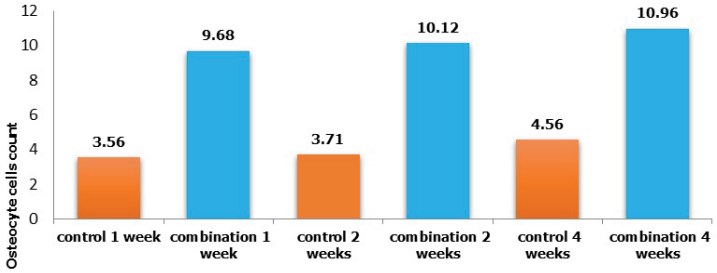

Figure 12 Comparison of mean values of osteocytes in studied groups in different durations

DISCUSSION

During bone healing cellular proliferation has been reported to reach its peak during the first-week post-injury followed by the maturation of cellular components and endochondral ossification which occurs near the end of the second week. In third week post-injury, the osteoblastic activity has been shown to gradually decrease and woven bone was replaced by lamellar bone [11]. Evaluation of bone healing in spontaneously hypertensive rats femurs 7 days post-surgery, showed the defect area filled by delicate and intertwined bone trabeculae. The neoformed trabeculae contained large osteocytes and were surrounded by cuboid osteoblasts in all specimens, which disagreed with histological findings of the present study at 7 days duration which showed matrix deposition filled with large numerous osteocytes and rimmed by osteoblasts, no obvious trabecular formation could be seen [12]. The histological examination of bone sections showed deposition of osteoid bone in all studied groups after 1-week and immature bone spicules rimmed by osteoblasts were observed more clearly in experimental groups in agreement with findings of Calvo-Guirado, et al., in 2015, who stated that significant osteoid matrix synthesis and mineralization accelerated was by early cell differentiation [13].

Oana, et al., in 2008, reported that after 2-weeks early processes of bone healing in the area of the experimental defect was noticed with thin bone trabeculae in agreement with the findings of this study where bone trabeculae enclosing marrow tissue were illustrated by a histological examination of bone sections after 2-weeks [14]. At 4-weeks more areas of bone formation were observed with thin and small bony trabeculae as reported by Amera in 2015, while the present results showed dense mature bone at defect sites in all studied groups after 4-weeks [15].

According to the results of this study, the effect of combined application of β-TCP and melatonin showed progressing increase in bone deposition and maturation as compared to control groups. Besides the deposition of bone, which seemed to replace the degradable materials at defective area could be due to their osteoinductive capabilities. In general histological findings of this study might be explained according to Gomez, et al., in 2015 who stated that relying on the surrounding or available cells that might eventually produce the required local bone regeneration through supplementing potential molecular deficiency in the stimulation of local cell differentiation in the osteoprogenitor line (such as BMP or other growth factor local deliveries) so according to the results of this study, this may be enhanced by local application of melatonin and β-TCP in combination to reduce the time required to promote graft consolidation, and improving the bone maturation [16].

It has been suggested that the bone repair process occurs within the degree of bone maturing progression and that the observation period progression does not cause area size increase, but rather in the bone trabeculae maturation degree [17]. Results of the present study showed that studied parameters showed increasing mean values in experimental groups where the highest recorded values of trabecular area and number (TA, TN) were with the experimental group at 4-weeks duration while concerning bone marrow area (BMA) the mean values decreased indicating increased density and maturity of bone that almost filled defected sites as marrow area decreased.

As indicated by Chu, et al., in 2017 melatonin improved the bone trabecular microstructure of elderly rat including the trabecular number and trabecular thickness, and decreased trabecular spacing in elderly rats also agrees with the present results regarding mean values of TA, TN, and BMA which correspond to marrow spacing [18].

Gao, et al., reported that when the β-TCP composite was used, the histological, histomorphometric, and biomechanical evaluations showed significantly better bone healing in terms of quality and quantity of the new bone formation which agrees with histomorphometric results of this study [19].

Bone cells

A study conducted by Shyng, et al., in 2001, showed that a small flattened and inactive osteoblasts were detected overlying the immature bone surfaces in the histological observations of calvarial defects at 3-week period, his findings seem to disagree with our findings concerning the number of osteoblasts as mean values increased during the transition from the 1, 2 to 4-weeks of healing intervals among all groups [20].

It could be explained by the direct action of applied materials on the differentiating and maturation of osteoblasts accelerating rate of matrix deposition and its corresponding calcification, where osteocytes were embedded which also showed increasing mean values with time among groups.

The stimulatory effects of melatonin on the proliferation and differentiation of osteoblasts influences the release of growth hormone and promotes bone formation by suppressing osteoclast activity; stimulating the formation of a mineralized matrix and Type I collagen; increasing osteoblast alkaline phosphatase activity through the increased gene expression of Type I collagen, osteopontin, bone sialoprotein and osteocalcin as reported by [21,22].

Osteoconductive properties of β-TCP scaffolds affect the signaling pathways through which they induce the differentiation of stem cells into osteoblastic cells creating an environment permissive for the proliferation of bone cells, which fills the bone defect [9].

As previous studies found that the use of each of melatonin and β-TCP alone had a beneficial effect in bone healing [9,13], so the administration of the combination of them had a synergistic effect in the healing of bone defect.

CONCLUSION

The obtained results regarding the studied parameters where the mean values were higher in experimental groups than in controls indicating that bone healing and maturation was obviously accelerated after combined local application of melatonin and beta-tricalcium phosphate.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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