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Early Healing of Rabbit Bone Fracture Using Beta Tri-Calcium Phosphate and Osteocalcin Antibody

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Abstract

Background Early osteogenesis and bone formation are key for treating bone injuries and bone diseases like osteoporosis. β-Tricalcium phosphate (β-TCP) is a popular synthetic bone graft substitute with excellent osteoconductive properties and absorbability.

Objective To evaluate the histological and immunohistochemical changes by using β-TCP on fracture healing in rabbits.

Methods Experimental study on animal model using 40 male adult albino rabbits, (*Oryctolagus cuniculus*). The control group included 20 rabbits and subdivided into 2 periods; two weeks and four weeks each one used 10 rabbits, and the treated group was also 20 in number and subdivided into 2 periods, two weeks and four weeks, each one used 10 rabbits. General anesthesia was induced with an intramuscular injection of xylazine 20 mg (0.2 ml/kg body weight) and ketamine HCl 50 mg (20 mg/kg body weight). The new bone formation length was measured histologically and by immunohistochemistry.

Results Histological findings for the bone healing process in the treated group with β-TCP showed early bone marrow, trabecular bone, mineralization, and compact bone formation in comparison to the healing process in the control group. The comparative histomorphometric study showed a significant P value in comparison between 2 and 4 weeks the control group P value <0.001 and the treated group with β-TCP, P value <0.011. Immunohistochemical findings expression of anti-osteocalcin revealed that there were significant differences in the treated group with β-TCP between (2 weeks mean+SD 0.43±0.04 and 4 weeks mean+SD 0.5±0.05).

Conclusion β-TCP has a major osteogenesis role in the early bone formation in the treated group as compared with the control group.

Keywords Beta-Tricalcium phosphate, osteocalcin

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List of abbreviations: β-TCP = βeta tricalcium phosphate, OSC = Osteocalcin

Introduction

Early osteogenesis and bone formation are key to treating bone injuries and bone diseases like osteoporosis. Minor bone fractures can recover without surgical intervention, the recovery period can be long and inconvenient [(1)]. β-Tricalcium phosphate (β-TCP) is a popular synthetic bone graft substitute with excellent osteoconductive properties and absorbability. However, its osteoinductive (the ability of a scaffold or implant to promote the differentiation of mesenchymal stem cells down an osteoblastic lineage, ultimately leading to the formation of

mineralized tissue) properties are inferior to those of autologous or allogeneic bone [2]. β-TCP has high porosity and improved osteoconductive (means that bone grows on a surface) properties with a solubility that is approximately 30-fold higher than that of hydroxyapatite, making it more bioabsorbable and convenient for clinical application [3].

Osteocalcin (OSC), known as bone gamma carboxy glutamic acid-containing protein (BGLAP), is a small (49-amino-acid) noncollagenous protein hormone found in bone and dentin, first identified as a calcium-binding protein in chick bone [4]. OSC specifically produced by osteoblasts was demonstrated to inhibit bone formation and function as a hormone, it is recognized as a multifunctional bone-derived hormone that modulates numerous physiological activities and developmental processes.

This study aimed to evaluate the histological and immunohistochemical changes by using β-TCP alone or combined with systemic administration of salmon OSC on fracture healing in rabbits and Study the outcome of the osteoprogenitor cells for quantitative study of osteoblast on different stages of early bone healing using OSC antibody.

Methods

This study was performed at College of Medicine, Al-Nahrain University from November 2021 to August 2022.

Design of study

This was an experimental study on animal model. The total number of male adult albino rabbits (Oryctolagus cuniculus) was (40), and were divided into two groups animals; (20) for each group, the first control group with no treatment, the second was the treated group (experimental group), β-TCP was placed in the hole made by surgical operation for each animal in this group. the (10) animals were sacrificed after 2 weeks and the other (10) animals were sacrificed after (4) weeks.

Surgical operation

General anesthesia was induced with an intramuscular injection of xylazine 20 mg (0.2 ml/kg body weight) and ketamine HCl 50 mg (20 mg/kg body weight). The site chosen for operation was the midshaft femoral bone of the lower limb left side. Initial intermittent drilling was done by round bur with a speed 1500 rpm and vigorous irrigation by normal saline, a depth of 2 mm and 3 mm in diameter. the diameter was checked by Vernia then application of β-TCP. Each rabbit had systemic administration of antibiotics, Ceftriaxone vial, 500 mg with 5 ml solvent ampoule, 0.5 ml/kg intramuscular injection, administrated once daily for five days. Afterward, the animals were sacrificed according to each group's healing period. The animals were euthanized by directly giving an intracardiac injection of pentobarbital an anesthetic drug with an overdose (50 mg/kg) of this medication at (2, 4 weeks) healing intervals.

Tissue preparation

Embedded tissue section in 10% formalin. Subsequently, bone decalcification was accomplished using 10% nitric acid (HNO_3) and then the paraffin blocks were sagittally sectioned at a thickness of 5 µm that were ready to make tissue slides for evaluation of the morphometry, histology, and immunohistochemical study for anti-OSC antibody expression of bone formation statistical analysis were evaluated by ImageJ (image processing software) and a quantitative result of expression of anti-OSC AB.

Results

Histological findings

Control group (2 weeks): The cross-section of the callus was composed of fibrous tissue rich with fibroblasts in addition to numerous newly formed blood networks (Figure 1).

Control group (4 weeks): Histological cross sections showed that the hole of cortical bone was partially filled with uncomplete ossified

bone trabecula that revealed numerous osteoids (Figure 2).

Treated group with β-TCP (2 weeks): Histological sections showed that the gap revealed furthermore trabecular formation in addition to the remaining fibrovascular callus that revealed numerous patches of intramembranous ossification which led to furthermore newly formed small sizes of trabecula (Figure 3).

Treated group with β-TCP (4 weeks): Histological cross sections showed normal cortical bone and a partially filled hole with an active ossification center and numerous incomplete ossified trabeculae, the bone spaces filled with the remnant of fibrous osteogenic tissue and without remodeling of endosteum and periosteum (Figure 4).

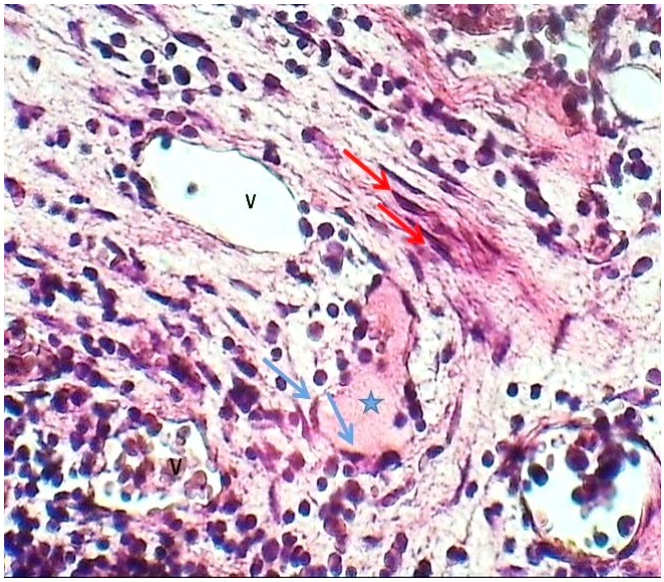

Figure 1. Cross section of callus (control group after 2 weeks) shows active fibrous tissue with marked eosinophilic site referred to as ossification (Red arrows), small ossified trabecula (Asterisk) lined by osteoblasts (Blue arrows) and newly formed blood network lined by endothelium (V). H&E stain. 400x

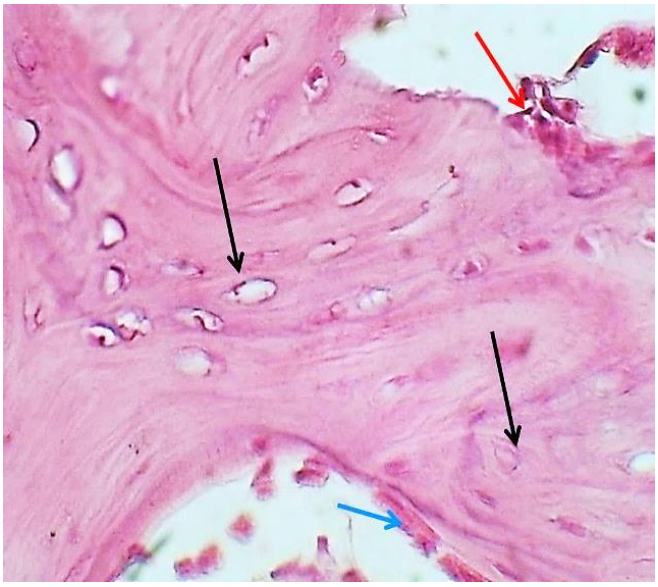

Figure 2. Cross section of trabecula (control group after 4 weeks) shows osteoclast (Red arrow), osteoblast (blue arrow) and osteocyte (Black arrows). H&E stain. 400x

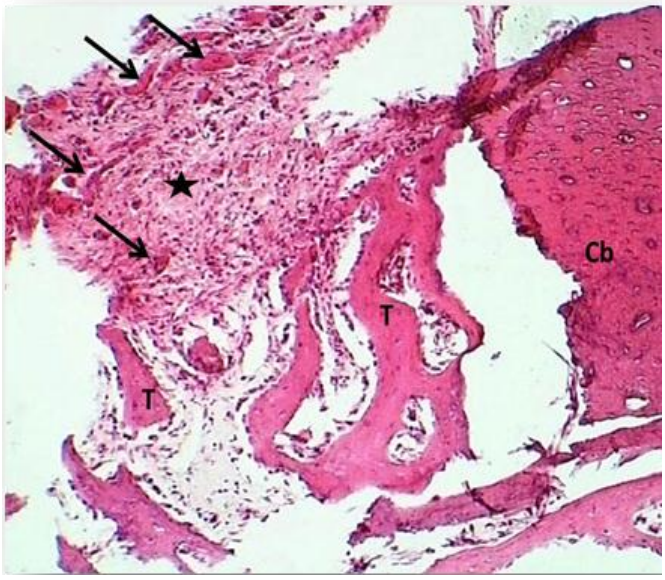

Figure 3. Cross section of fibrocartilaginous callus (treated group with β-TCP after 2 weeks) shows: Appropriate increase in the site of callus that formed at the fractured site (Asterisk), edge of cortical bone (Cb), active fibrous tissue with marked eosinophilic color referred to intramembranous ossification (Black arrows), small ossified trabecula (T) lined by osteoblasts. H&E stain. 40x

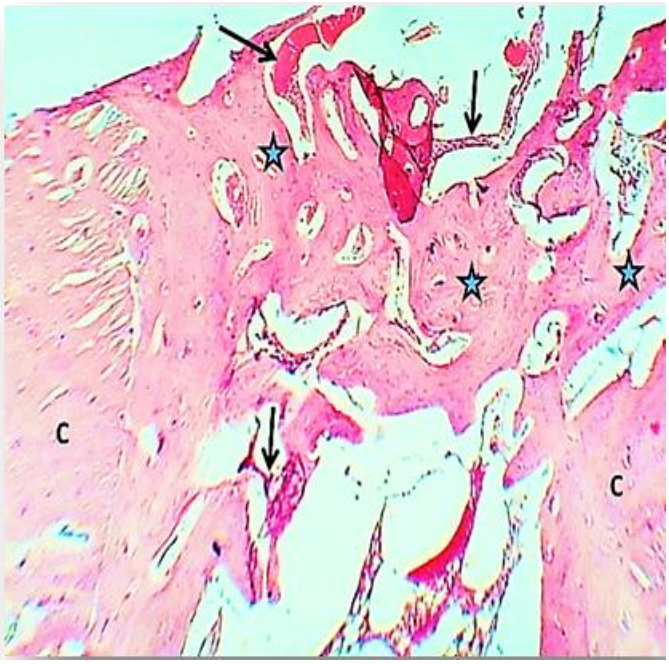

Figure 4. Cross section of femur (treated group with β-TCP after 4 weeks) shows: normal cortical bone (C,), partially filled whole with numerous incomplete ossified trabecula (Asterisks) the spaces filled with remnant of fibrous osteogenic tissue (Arrows).H&E stain.40x

Comparative statistical analysis results between the control and experimental group for 2 weeks and 4 weeks

The statistics for the new bone formation were estimated in all groups in different durations of 2 and 4 weeks. In the comparison of new bone formation between the control group and treated group by unpaired t-test, the new bone formation mean showed highly significant differences in all groups with different duration (control Mean±SD at 2 weeks 0.56±0.27 µm, at 4 weeks 1.66±0.76 µm) (β-TCP Mean±SD at 2 weeks 1.62±0.58 µm, at 4 weeks 2.61±0.94 µm) (Table 1).

Table 1. Differences in new bone formation length at the site of fracture between each pair of the study groups at 2 and 4 weeks by unpaired t-test

New bone formation (µm)		Control N=10	TCP N=10	P value
2 weeks	Mean±SD	0.56±0.27	1.62±0.58	<0.001
4 weeks	Mean±SD	1.66±0.76	2.61±0.94	0.024

Immunohistochemical study

Expression of anti-OSC antibody Control group after 2 weeks: Bone section at the fracture site after two weeks for the control group showed positive immunostaining reactivity in the bone marrow stromal cells and wide distribution in the connective tissue (Figure 5).

Control group after 4 weeks: Immunohistochemical expression of anti-OSC antibody at the fracture site after 4 weeks for

the control group showed positive reactivity in the bone marrow stromal cells, osteocytes cells, osteoblasts cells with wide formation of trabecular bone (Figure 6).

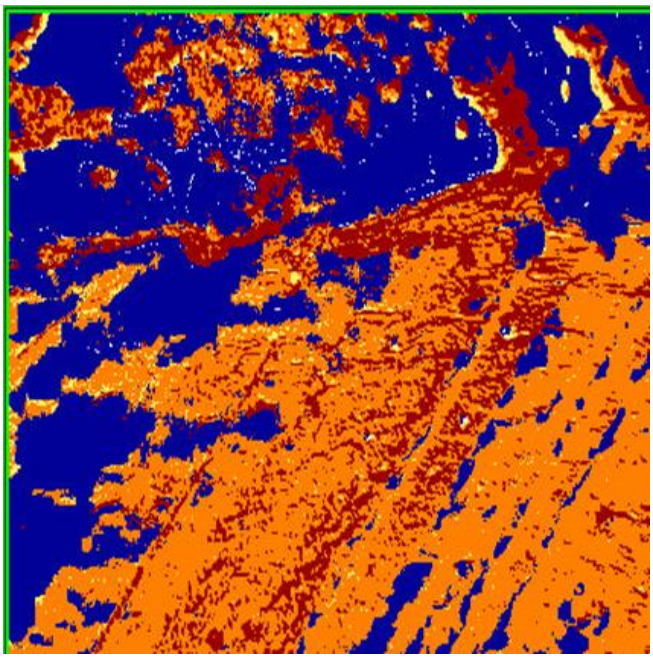

Figure 5. Markup image analyzed by Aperio imagescope software, control group after 2 week; brown color=strong positive, orange color=positive, yellow color=week positive, blue color=negative

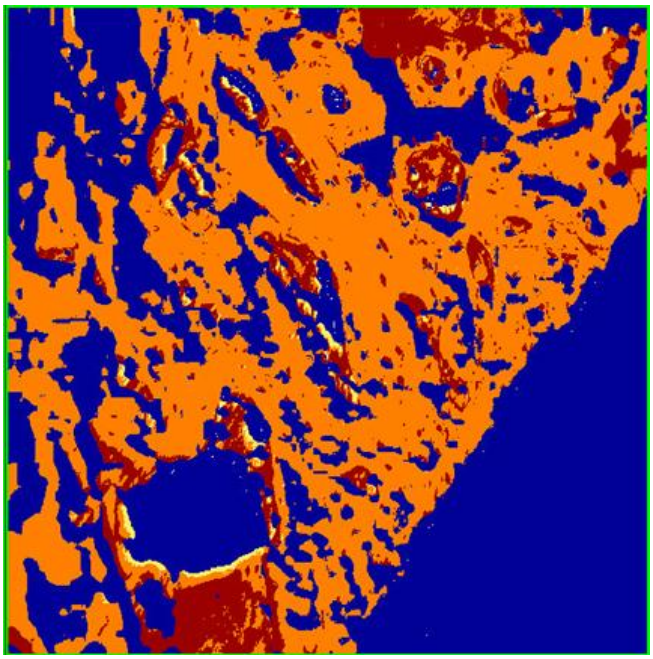

Figure 6. Markup image analyzed by Aperio imagescope software, control group after 4 week; brown color=strong positive, orange color=positive, yellow color=week positive, blue color=negative

Treated group with β-TCP after 2 weeks: Immunohistochemical expression of anti-OSC antibody for the bone section at the fracture site after two weeks for the Treated group with β-TCP showed positive expression reactivity in the newly formed bone marrow and wide distribution of the trabecular bone (Figure 7).

Treated group with β-TCP after 4 weeks: Immunohistochemical expression of anti-osteocalcin antibody at the fracture site after four weeks for the Treated group with β-TCP showed positive reactivity in the trabecular bone, osteocytes cells, osteoblasts cells and osteon (Figure 8).

Expression of anti-OSC antibody between control and experimental groups in 2 weeks duration: The expression of anti-OSC antibody in control group (2 weeks) with a mean value 0.27±0.06 and treated group with β-TCP with a mean value 0.43±0.04 and there was a statistical difference in the mean of two groups (P value =<0.001) (Table 2).

Expression of anti-OSC antibody between control and experimental groups in 4 weeks duration: The expression of anti-OSC antibody in the control group (4 weeks) with a mean value 0.29±0.06 and treated group with β-TCP with a mean value 0.5±0.05 and there was a statistical difference in the mean of two groups (P value =<0.001) (Table 2).

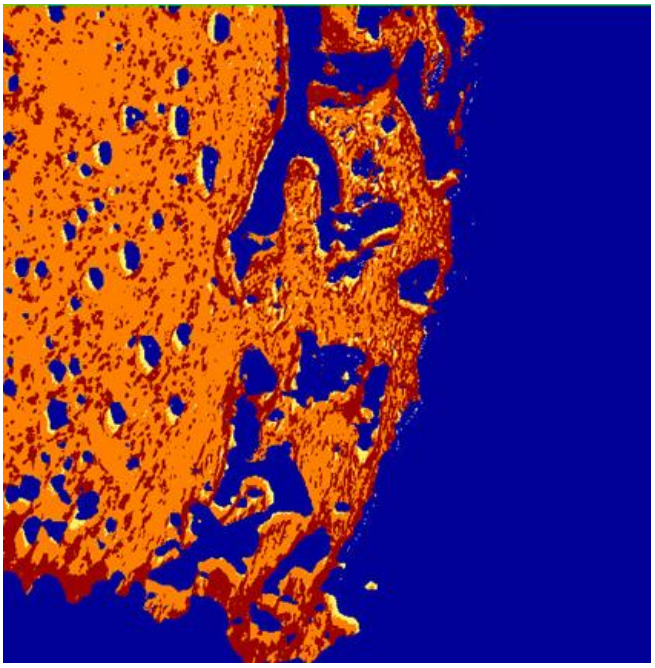

Figure 7. Markup image analyzed by Aperio imagescope software, treated group after 2 week; brown color=strong positive, orange color=positive, yellow color=week positive, blue color=negative

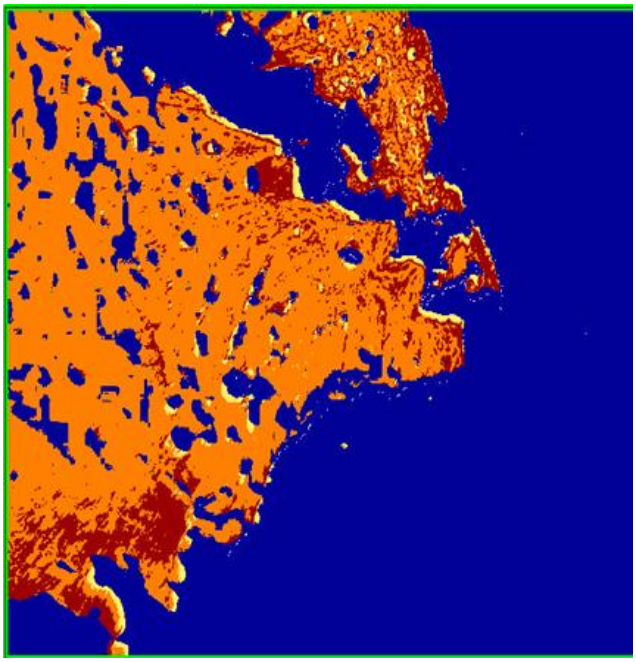

Figure 8. Markup image analyzed by Aperio imagescope software, treated group after 4 week; brown color=strong positive, orange color=positive, yellow color=week positive, blue color=negative

Table 2. Comparison of anti-OSC antibody positivity between each pair of the study groups at 2 and 4 weeks by unpaired t-test

OSC positivity		Control N=10	TCP N=10	P value
2 weeks	Mean±SD	0.27±0.06	0.43±0.04	<0.001
4 weeks	Mean±SD	0.29±0.06	0.5±0.05	<0.001

Discussion

The present study evaluated early bone fractured healing in the rabbit femur model after the application of bone grafting material β-TCP. Experimental study for histological features and changes in the new bone formation area demonstrated that the treated group at the fractured femur bone site showed a remarkable difference in the treated group with β-TCP compared to control group. This result is confirmed by [5] who found that the histological sections of the control group showed the osseous defect filled with bony connective tissue and with non-connected, immature bone trabeculae.

In this study, the treated group with β-TCP enabled new bone to be formed in a femur bone defect at a lower rate than other experimental treated groups. The presence of newly formed mineralized bone, lined at the surfaces with osteoid, demonstrated that active bone formation was still in process of healing after two weeks duration. The presence of both immature woven bone and lamellar bone along with active osteoblasts and osteoclasts suggests that bone remodeling was still going on in the grafted sites. Overall, the histological picture suggested that immature woven bone was formed in the grafted site,

which was later remodeled into the lamellar bone after four weeks' durations [6].

In the present study treated group with only β-TCP histological section showed immature bone, the gap revealed with the trabecular formation in addition to the remaining fibrovascular callus that revealed numerous patches of intramembranous ossification which led to newly formed small sizes of trabecula which is composed of osteocytes within lacunae, lined by osteoblasts and osteoclasts after two weeks durations, with widespread areas of mature trabecular bone was seen with signs of bone remodeling after four weeks duration in agreement with the above study. The findings stated by [7] evaluated the histological characteristics of β-TCP in the human femur and find a considerable amount of new bone formation and observed osteoclast-like giant cells at the defect site. Most OSC secreted by osteoblasts is incorporated into the organic matrix that will later ossify into bone, however, a small fraction is secreted into the circulation. For this reason, OSC is widely considered a bone formation marker and Osteocalcin concentration correlates with direct measurement of bone formation [8]. In this study, the early osteogenesis process was promoted, so as the number of osteoblasts in the periphery of trabecular bone augmented, led to an illustrated increase in the expression of OSC with the early bone formation which become at peak levels at 2 weeks, was revealed in the treated group with β-TCP highly significant than the control group during 2nd weeks and 4th weeks of the treated period, which showed a significant increase in osteocalcin expression, that agree with [9] who said that bone mineralization process takes place from day 7 to day 14.

In conclusions, β-TCP was more active as an osteoconductive and osteoinductive material that enhanced osteogenesis. β-TCP were more effective in the acceleration of the early bone healing process and enhancement of bone regeneration. The bone marrow formation was obviously observed in the control group β-TCP group (2 and 4 weeks). The authors recommend that quantitative histomorphometric study for bone cells (osteoprogenitor cells, osteoblast, osteoclast, and osteocyte). Immunohistochemical investigations of other biochemical bone markers such as collagen II, alkaline phosphatase, and transforming growth factor may be performed to verify the outcomes of this study.

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Author contribution

Both authors contributed directly to the creation of this paper and approved the final version that was submitted. Also, the study was conceptualized, designed, and interpreted by both authors. Similarly, the final manuscript has been read and approved by both authors.

Conflict of interest

The authors declare no conflict of interest.

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