ORIGINAL ARTICLE

CONTENTS

Evaluation of immunohistochemical expression of collagen type I in bone defect treated by local application of collagen and beta–tricalcium phosphate in rats

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ABSTRACT

Aims: This study aimed to estimate outcome of combined application of collagen/β-tricalcium phosphate (Coll /β- TCP) on bone defect healing by immunohistochemical localization of collagen type I.

Materials and Methods: A total of 20 adult male rats (albino rats) were used in this study. Four intra bony defects were created in both femurs of each rat, three defects were treated with Coll, β- TCP and combination of Coll /β- TCP while forth defect left to heal spontaneously as control. Scarification of animals was done after 2- and 4-weeks healing periods (10 rats for each). Immunohistochemical localization of collagen type I (COL1) monoclonal antibody was done for all bone specimens.

Results: Immunohistochemical results showed positive expression of (COL1) by bone cells osteoblast (OB), osteocyte (OC), osteoclast (OCL) and bone marrow stromal cells (BMSCs) at 2- and 4-week duration for all examined groups with variable rates of intensity. Mean values of immunoreactive score (IRS) decreased with time for bone cells and for BMSCs except for OC where values increased with time as the highest values were recorded in combination group at 4 weeks.

Conclusions: Combined application of (Coll /β- TCP) was effective in accelerating bone healing process through elevating the IRS of (COL1) noticed by osteoblast, osteocyte and bone marrow stromal cells.

KEY WORDS: bone defect, collagen, β-TCP, collagen type I

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INTRODUCTION

Bones are hard structures that are a component of the endoskeleton of vertebrates. In addition to producing red and white blood cells and storing minerals, they support and shield the body's numerous organs [1]. Bone, being a structural tissue, has the ability to repair itself via a process called "bone remodeling," where in new bone tissue (formation) replaces aged bone tissue (resorption) [2]. Bone deformities and fractures were traditionally treated with autologous or artificial bone grafting techniques. However, these operations are not always advised because of the risks connected to bone replacement surgery, including infection and morbidity. The use of artificial scaffolds, which stimulate osteoblasts to differentiate and generate new bone tissue to replace the damaged areas, is an additional method for bone repair and regeneration [3]. Beta-tricalcium phosphate, denoted as β-TCP, represents a calcium phosphate compound derived from phosphoric acid, characterized by the chemical formula $Ca_3(PO_4)_2$ [4]. β-TCP is a material that is frequently used in clinical orthopedics because it is highly osteoconductive and osteoinductive with lack of histotoxicity [5]. These characteristics, along with its cell-mediated resorption enable the regeneration of entire bone defects [6], also has the ability to modulate osteogenic events that contribute to bone repair and healing including the differentiation of mesenchymal stem cells towards bone-forming cells, the generation of blood vessels, the secretion of growth factors that promote angiogenesis, and the initiation of blood clot formation [7]. The most prevalent macromolecule in the body is collagen (Coll) which is found in different tissues as extracellular matrix. Collagen's intricate structure plays a significant function in the body's various biological processes during the processes of development and regeneration. The process of calcification in bone tissue is one instance [8]. Products containing collagen have been shown to have a significant positive impact on human health, particularly in older adults, clinical trials on the effects of collagen treatment on a variety of conditions, such as rheumatoid arthritis, osteoarthritis, sarcopenia,

dental therapy, wound healing, and skin regeneration [9]. β-TCP/ColI, a biocompatible and bioresorbable composite material, mimics the characteristics of the inorganic component of bone, leading to the generation of newly formed bone tissue [10]. Collagen type I marker (COL1) is referred to as a triple-helical domain because of its composition in the formula of G-X-Y. Here, X and Y can be any amino acid, but glycine (G) is a persistent amino acid. Typically, proline and hydroxyproline use X and Y [11]. Each of these three units will show up more than once as repeated units. Glycine comes in third position is important for confirming the helical structure construction. Later on, this will be contained in a different dimension of the hexagon and quasi-hexagonal formulae to form fibrillar COL1. Therefore, COL1 is normally discovered as a long fibril [12]. Collagen type I constitutes the primary organic element within the mineralized structure of bone matrix and its expression in the bone matrix was found through the use of immunohistochemical staining. The extracellular matrix made up of type I collagen and several noncollagenous proteins must be deposited in order for osteoblastic cells to form bone, which afterward mineralizes by hydroxyapatite crystals formation [13].

AIM

This study aimed to estimate outcome of combined application of collagen/β- tricalcium phosphate (Coll /β-TCP) on bone defect healing by immunohistochemical localization of collagen type I.

MATERIALS AND METHODS

Twenty adult male albino rats weighing approximately 250–350 g and aged 3–4 months were used in this study. They received injectable ketamine hydrochloride 50 mg intramuscularly (1 ml/kg body weight) plus 2% xylazine (0.2 ml/kg body weight). Surgery was achieved under sterile conditions by making an incision on the skin and underlying fascia. Then, reflection was performed to expose the rat femurs. Intrabony defects approximately 3 mm in depth and 2 mm in width were induced in both femurs of each animal (Fig. 1), with intermittent drilling and constant cooling with normal saline using a micro engine that was set at a rotational speed of 2500 rpm**.** The operation sites were cleaned with normal saline to eliminate debris. Then subdivided into group A (20 defects) as the control group, were left untreated for spontaneous healing. Groups B (20 defects) filled with collagen, group C (20 defects) filled with β-TCP and finally group D (20 defects) were filled with combination of Coll and β-TCP materials in a ratio of 1:1. Animals were sacrificed after 2- and 4-weeks healing periods (10 rats for each) by administering an excessive amount of anesthesia.

IMMUNOHISTOCHEMICAL PREPARATION

Collected bone specimens were fixed for 24 hours using 10% freshly made formalin. Then the process of decalcification was carried out using 10% formic acid for 2-3 days afterward they were embedded in paraffin wax. A microtome was used for section of the blocks for serial slices of 4μm, which were positioned on charged slide. Following immunohistochemical staining, the analysis of COL1 localization by bone cells and bone marrow stromal cells (BMSCs) was done for all bone specimens in 2- and 4-weeks durations.

STATISTICAL ANALYSIS

Descriptive analysis of mean, standard deviation (SD), minimum (Min), and maximum (Max) values of the IRS of COL1 by bone cells (OB, OC, and OCL) and bone marrow stromal cells (BMSCs) at 2- and 4-week duration was done for all investigated groups. For immunohistochemical evaluation of COL1, a x40 objective lens was used as follows: positively stained cells were counted in five representative fields (x40). The approximation of the percentage of positively stained cells was obtained by dividing the total number of stained cells by the total number of cells present, then multiplying this result by 100. The scores were: 0 (no stain), 1 (<25%), 2 (25-50%), 3 (>51-80%) and 4 (>80%) stained cells in two sections and scoring the intensity of stain as: 0 (no clear stain), 1 (mild stain), 2 (moderate stain), 3 (intense stain) and accomplishing the immunoreactive score (IRS), which is the result of multiplying the staining intensity score by the percentage of positive cells (0–4), has a range of 0–12 [14].

ETHICAL APPROVAL

The study was approved by the College of Dentistry/ University of Baghdad's local ethics commission (Ref. number:429. Date 27/12/2021).

RESULTS

IMMUNOHISTOCHEMICAL RESULT FOR EXPRESSION OF COLLAGEN TYPE I AFTER 2- AND 4-WEEKS DURATIONS

TWO WEEKS DURATION

Immunohistochemical localization of COL1 in control groups shows positive expression of COL1 in bone

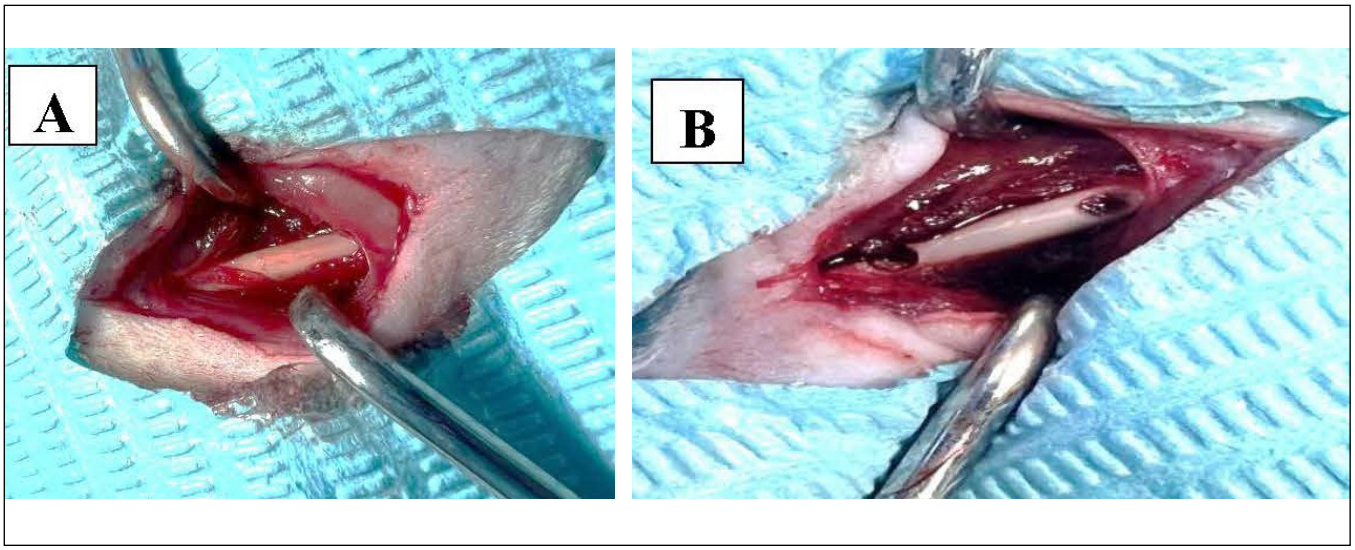

Fig. 1. Intrabony defects in femurs of animal: A) exposure of rat femur; B) intrabony defects prepared.

marrow stromal cell, osteoblasts, osteocytes and osteoclasts, bone trabeculae are negatively stained (Fig. 2). Microphotograph view of defect area of collagen group shows positive expression of COL1 monoclonal antibody by bone marrow stromal cells, osteoblasts, osteocytes, osteoclasts (Fig. 3). In β-tricalcium phosphate group immunohistochemical localization of COL1 monoclonal antibody noticed by osteoblasts, osteocytes, and BMSCs, whereas bone trabeculae are negatively stained (Fig. 4). Immunohistochemical positive localization of COL1 in bone section of combination group shown in osteocytes, osteoblasts, osteoclasts and bone marrow stromal cells (Fig. 5).

FOUR WEEKS DURATION

Immunohistochemical expression of COL1 in bone section of control group at 4 weeks duration shows positive expression in osteoblasts, osteocytes and bone marrow stromal cells, negative expression noticed in bone trabeculae (Fig. 6). For collagen group immunohistochemical localization of COL1 at 4 weeks duration shows positive expression in osteocytes, while bone is negatively stained (Fig. 7). Immunohistochemical expression of COL1 in bone section of β-TCP group after 4 weeks duration seen in osteoblasts, osteocytes and bone marrow stromal cells. Mature bone shows negative expression (Fig. 8). Immunohistochemical localization of COL1 at 4 weeks duration of combination group (β-TCP +Collagen) showed positive expression in osteocytes, osteoblasts and bone marrow stromal cells (Fig. 9).

STATISTICAL ANALYSIS OF IMMUNOHISTOCHEMICAL RESULTS

DESCRIPTIVE DATA ANALYSIS FOR PERCENTAGES OF POSITIVELY STAINED CELLS FOR COL1 IN STUDIED GROUPS AND DURATIONS

The descriptive data of mean, SD. Min., and Max. values of the immunoreactive score (IRS) expression of COL1 by bone cells (osteoblasts, osteocytes, osteoclast) and bone marrow stromal cells at 2- and 4-week durations for all studied groups are illustrated in table 1 and figures 10A-D. Mean values of IRS decreased with time for bone cells and BMSCs except for OC where mean values of IRS increased with time as the highest values were recorded in combination group at 4 weeks. The highest mean values of IRS noticed in combination groups for OB and BMSCs and for OC at 2 and 4 weeks respectively.

GROUP COMPARISON FOR BONE CELLS AND BMSCS IN HEALING DURATIONS (2 AND 4 WEEKS)

The group comparison of IRS and COL1 for bone cells (OB, OC, and OCL) and BMSCs are illustrated in table 2. An ANOVA test was conducted, and the results showed that all groups had a highly significant difference ($P<0.01$) for osteoblasts, osteocytes, osteoclasts and BMSCs after 2 and 4 weeks.

DISCUSSION

The process of bone healing is intricate and dynamic, involving a carefully planned sequence of biological

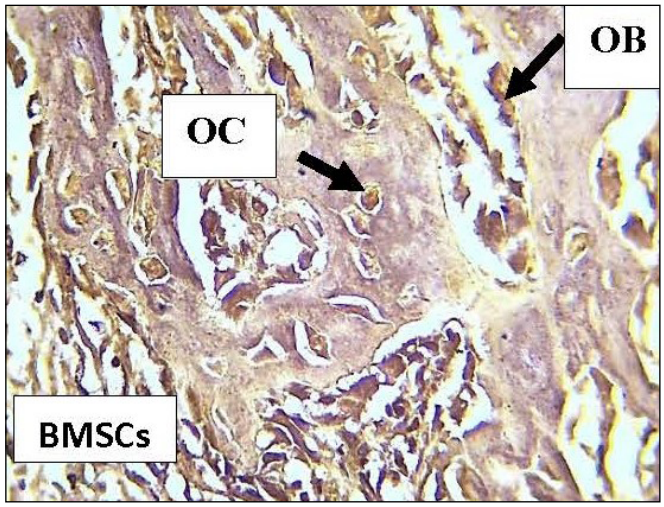

Fig. 2. View of control group after 2 weeks shows positive localization of COL1 1in bone marrow stromal cells (BMSCs), osteoblasts (OB), osteocytes (OC). DAB stain with hematoxylin counter stain (x40).

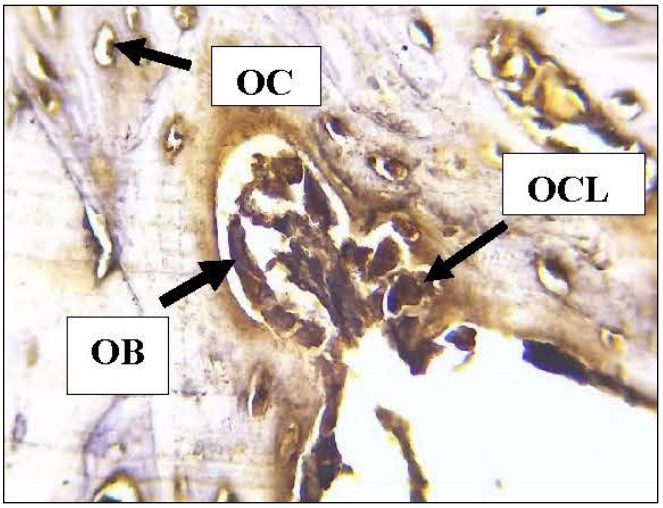

Fig. 4. Microphotograph view of β-TCP group at 2 weeks duration shows positive expression of COL1 in osteocytes (OC), osteoblast (OB) and osteoclasts (OCL). DAB stain with hematoxylin counter stain (x40).

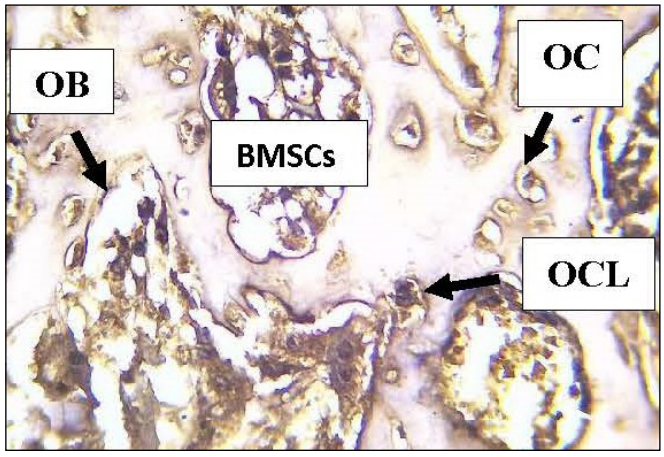

Fig. 3. Defect area of collagen group after 2 weeks shows positive staining of COL1 in bone marrow stromal cells (BMSC) osteocytes (OC), osteoblasts (OB) and osteoclasts (OCL). DAB stain with hematoxylin counter stain (x40).

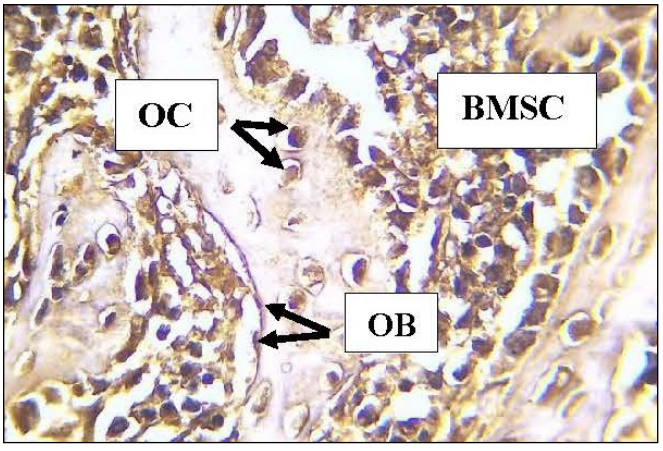

Fig. 5. View of combination group at 2 weeks duration shows positive expression of COL1 in osteocytes (OC), osteoblasts (OB) and bone marrow stromal cells. DAB stain with hematoxylin counter stain (x40).

events such as cellular recruitment, proliferation, and differentiation [15]. In most cases, bone tissue heals on its own unless there are complex circumstances such as large bone defects. There are three stages to bone healing: inflammatory, reparative and remodeling [16]. Osteoblasts produce type 1 collagen during the reparative stage of bone healing [17]. It has been demonstrated that this collagen subtype, which predominates in the extracellular matrix of bone, is essential to osseous repair and is connected to osteoblast maturation [18]. Findings of this study showed that after two weeks that the expression of COL1 was higher in experimental groups than in control groups specifically with the combination group exhibiting the highest expression which might suggest that collagen and β-TCP have a role in regulating healing response, promoting the formation of new bone, besides differentiation and proliferation of bone cells, supported by a previous study conducted by Tsuruoka N et al. [19], who found the expression of COL1 was high in the early stages of bone healing with oral administration of collagen tripeptide at fracture model rats, expression decreased with time, this indicates that all groups accomplished a satisfactory healing process at varying rates. The current study showed that high mean values of IRS for COL1 expression by BMSCs detected in the combination group after 2 weeks may be attributed to biocompatibility and bioresorbable properties of applied materials with properties similar to the inorganic phase of bone. Osteoblasts, which are essential for mineralization of bone matrix, were considered to originate mainly from stromal cells in the bone marrow [20]. In this study the recorded mean values of IRS for COL1 expression by osteocyte increased with time

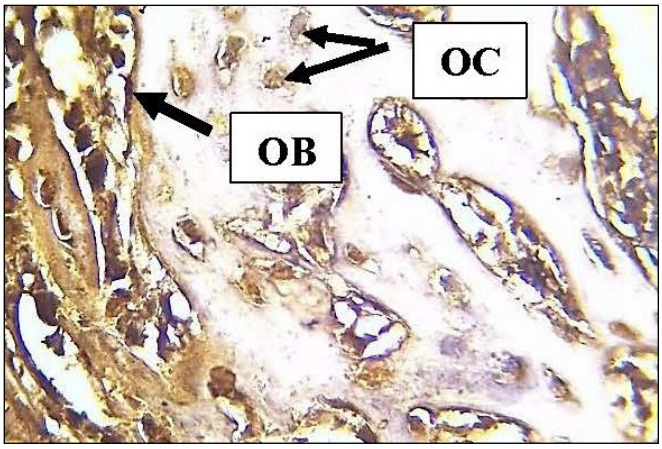

Fig. 6. View of control group at 4 weeks shows positive expression of COL1 in osteoblasts (OB), osteocytes (OC). DAB stain with hematoxylin counter stain (x40).

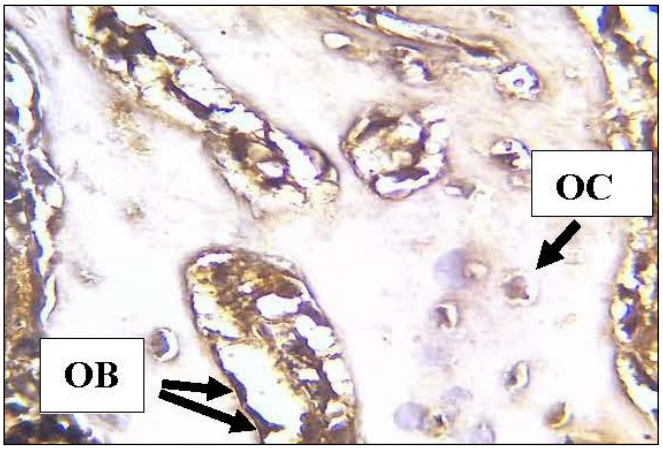

Fig. 8. View of β-TCP group at 4 weeks duration show positive expression of COL1 in osteoblasts (OB), osteocytes (OC). DAB stain with hematoxylin counter stain (x40).

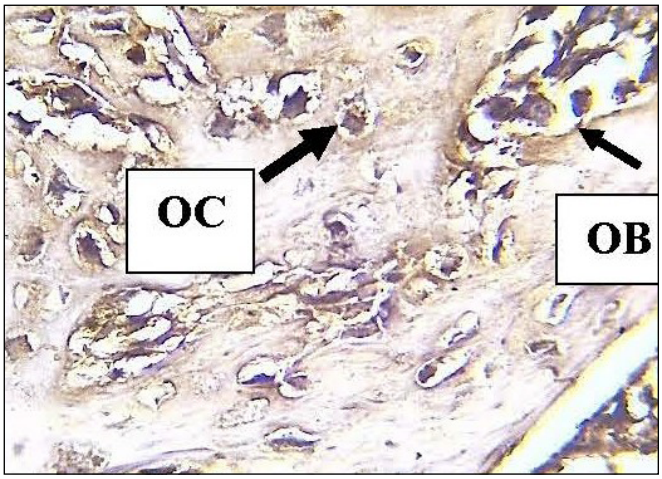

Fig 7. View of collagen group at 4 weeks duration shows positive expression of COL1 in osteocytes (OC), osteoblast (OB). DAB stain with hematoxylin counter stain (x40).

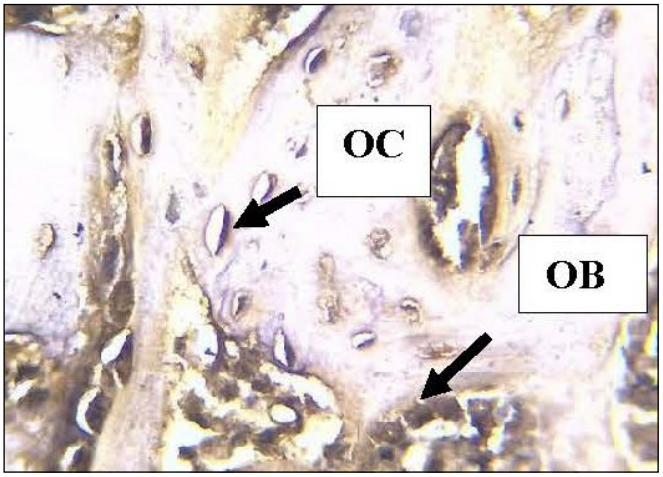

Fig. 9. View of combination group after 4 weeks duration shows positive localization of COL1 in osteoblast (OB) and osteocyte (OC). DAB stain with hematoxylin counter stain (x40).

and highest values recorded in combination group at 4 weeks and this agree with Kusumawati I et al. [22], who evaluated the effect of amelogenin as implant coating material in rabbit tibia on osteointegration through immunohistochemical localization of COL1 and reported that expression of COL1 was noticed during the first stages of extracellular matrix biosynthesis and proliferation. Following differentiation, osteoblasts generate a variety of proteins, including alkaline phosphates, osteocalcin and type I collagen, which, after producing newly formed bone, differentiate under an osteocyte phenotype, consequently, the bone marker COL1, which is linked to osteocyte differentiation. However, statistical analysis showed that the mean value of IRS for positive COL1 expression was highest in osteoblast in all groups examined for 2 and 4 weeks, this agreed with Kresnoadi U et al. [23], who demonstrated that COL1 expression appears strongly by osteoblast sited at bone trabeculae at 7 and 14 days in alveolar bone of rabbits during open flap debridement treated by 10% propolis carbonated hydroxyapatite. Highly significant difference ($P<0.01$) among all groups at 2- and 4-weeks durations was recorded for osteoblasts, osteocytes, osteoclasts and BMSC in this study in line with findings of Kresnoadi U et al.[23] where significant differences in expression of COL1 between control and experimental groups treated with mangosteen peel extract combined with demineralized freeze-dried bovine bone xenograft as a mean of tooth extraction socket preservation at 7 and 30 days.

CONCLUSIONS

Combined application of Coll/β- TCP was effective in accelerating bone healing process through elevating the IRS of COL1 noticed by osteoblast, osteocyte and bone marrow stromal cells.

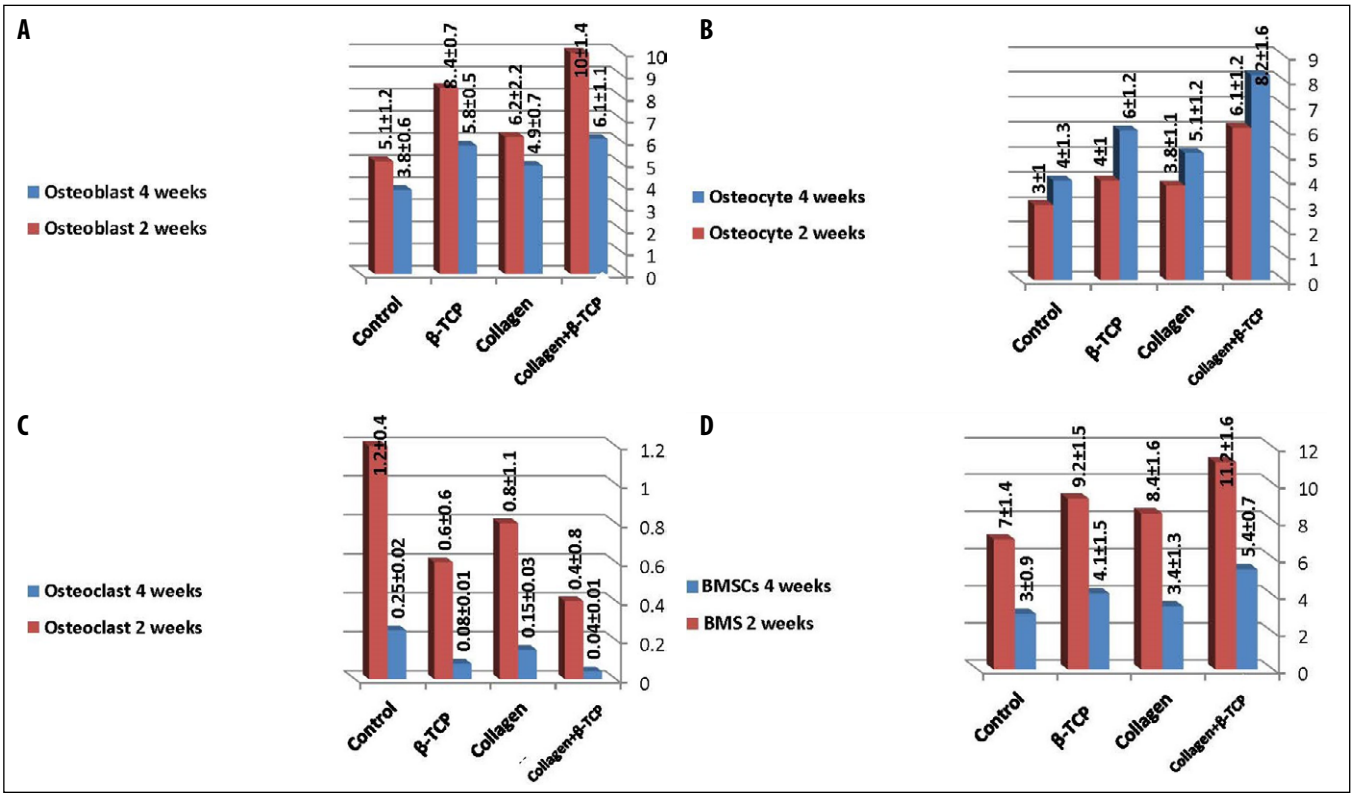

Fig. 10. A. Mean values of IRS for COL1 in OB after 2- and 4-weeks healing durations.
B. Mean value of IRS for COL1 in OC after 2- and 4-weeks healing durations.
C. Mean value of IRS for COL1 in OCL after 2- and 4-weeks healing durations.
D. Mean values of IRS for COL1 in BMSCs after 2- and 4-weeks healing intervals.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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A – Work concept and design, B – Data collection and analysis, C – Responsibility for statistical analysis, D – Writing the article, E – Critical review, F – Final approval of the article

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