The role of topical application of bone morphogenetic protein 7 (BMP7) on bone healing on rabbits (Immunohistochemical study on TGF-β 3 & IGF-1R)

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ABSTRACT

Background: Bone is essentially a highly vascular, living, constantly changing mineralized connective tissue. It is remarkable for its hardness, resilience and regenerative capacity, as well as its characteristic growth mechanisms. This study aimed to:

1. To evaluate the effect of bone morphogenetic protein7 (BMP7) on bone healing in artificially created intrabony defect in rabbits upper diastema, histologically.
2. To study the immunohistochemical expression of TGF-β3 and IGF-1R as bone formation markers in experimental and control groups during bone healing.

Material and method: Forty male rabbits, was used in this study, 8 rabbits for each healing interval (3 days, 1,2 ,4 and 6 weeks). In each rabbit two bone holes were created on the right and left sides of the maxilla.BMP7 was applied to the bone hole in the left side while bone hole in the right left for normal healing. Routine processing and sectioning technique performed for histological evaluation. Immunohistochemical analysis utilized to localize the expression of TGF-β3 and IGF-1R in experimental and control groups for all animals.

Results: Histological findings indicated that bone defect coated with BMP7 illustrated an early bone formation, mineralization and maturation in comparison to control group. Immunohistochemical findings revealed high positive expression for TGF-B3 and IGF-1R in experimental in comparison to control group.

Conclusion: The study concluded that BMP7 protein enhance bone healing and maturation, also it regulate the expression of TGF-B3 and IGF1R in bone.

Key words: Bone, Bone morphogenetic protein7, Transforming growth factor beta3, Insulin growth factor-1receptor.
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INTRODUCTION

Bone is a connective tissue that consists of cells and extracellular matrix. Bone is a dynamic tissue in constant change; maintenance of bone mass throughout life relies on the bone remodeling process, which continually replaces old and damaged bone with new bone. This remodeling is necessary to maintain the structural integrity of the skeleton and allows the maintenance of bone volume [(1)]. Bone morphogenetic proteins (BMPs), they are so named for their osteoinductive properties and regulate differentiation of mesenchymal cells into components of bone, cartilage or adipose tissue. BMP7, also sometimes referred to as osteogenic protein-1, was originally identified as a potent osteogenic factor purified from [(2)].

BMPs play a pivotal part in skeletal morphogenesis and repair, promoting the differentiation of mesenchymal cells into osteoblasts and inducing new bone formation. BMPs are involved in regulating mesenchymal cell differentiation and proliferation by stimulating intracellular signaling pathways [(3,4)].

Transforming Growth Factor –beta (TGFβ) is produced by many cell types, including bone marrow cells, osteoblasts, and stromal cells and is secreted in a latent form that must be activated to mediate its effects. Although several mechanisms of activation *in vivo* have been proposed, the precise mechanism of this process is not known. Both *in vitro* and *in vivo* studies have shown that TGFβ1–3 have complex effects on bone. They stimulate or repress proliferation or formation of osteoblasts and osteocytes, depending on cell types and culture conditions used [(5)]. Insulin-like growth factor-I (IGF-I) signaling through its type 1 receptor generates a complex signaling pathway that stimulates cell proliferation, function, and survival in osteoblasts. IGF-I has a central role in the growth and development of different tissues in the embryo [(6)].The present study was prepared to illustrate the benefits of using a local application of BMP7 used in bone defect and to discuss the sequences of biological events of healing.

MATERIALS AND METHODS

In this experimental study forty adult New Zealand white male rabbits weighing 1.5 –2.5kg were used**.** The animals were divided into five groups and each group contains eight animals. Each group was scheduled to be sacrificed at a different time periods. Which were three days, 1, 2, 4 and 6 weeks. Bone defect of 2-3 mm in

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diameter and 2 mm in depth were created intra-orally at the diastema in maxillary arch at each sides (right and left) approximately about 2-3mm in diameter and 2 mm in depth. Right sides bone defects of each animal , regarded as control group, in which the bone defect left for normal healing, while left sides bone defects treated with bone morphogenetic protein 7 (BMP 7) considered as experimental group. Immunohistochemical of (TGF-β3 and IGF-1R) was done for each interval.

RESULTS

Expression of TGF- β3 findings

At 3 days duration:

A – Control group: Immunohistochemical view of bone defect of 3 days duration revealed primitive new bone formation; this bone is characterized by presence of progenitor cells that are scattered randomly which shows positive expression of TGF-B3 (Figure 1).

B- Experimental group: After 3days bone defect treated with BMP7 shows primitive ostoid tissue is formed in which number of active progenitor cells are seen that illustrate positive reaction to TGFB3. Localization also positive reaction to undifferentiated mesenchymal cells (progenitor cell), endothelial cells of blood vessels mononuclear cell and fat cell with surrounding extracellular matrix in bone marrow (Figure 2).

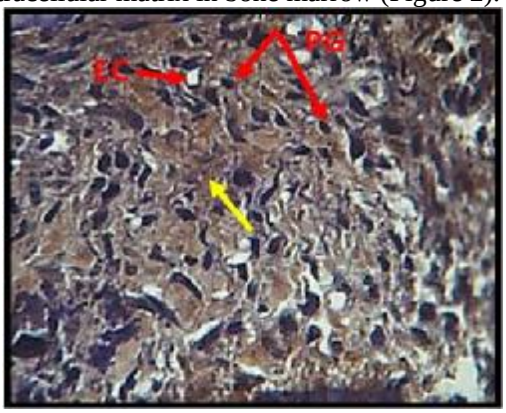

Figure 1:Immunohistochemical expression of TGEB3 of bone defect (control) for 3days duration shows positive expression in progenitor cell (PG) endothelial cell (EC) and in extracellular matrix (arrow) of bone marrow. DAB stain with hematoxylin counter stain X 40.

At 1 Week Duration:

A- Control group: positive localization of TGFB3 in osteoblast cell, osteocyte cell, osteoid tissue, progenitor cell, fat cell, fibroblast cell and endothelial cell (Figure 3).

B- Experimental group: View of bone defect section (experimental) of 1 week duration shows positive localization of TGFB3 in formative osteoblast cell, osteocyte cell, progenitor cell and extracellular matrix of bone marrow (Figure 4).

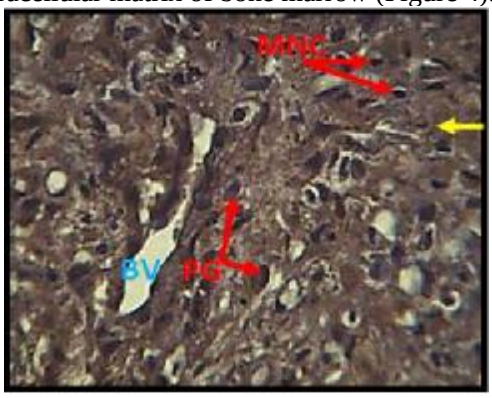

Figure 2: Immunohistochemical view of bone defect of 3 days duration in experimental group revealed positive expression of TGFB3 in progenitor cell (PG), endothelial cell of B.V mononuclear cell (MNC) and in ground substance of bone marrow (arrow). DAB stain with hematoxylin counter stain X 40.

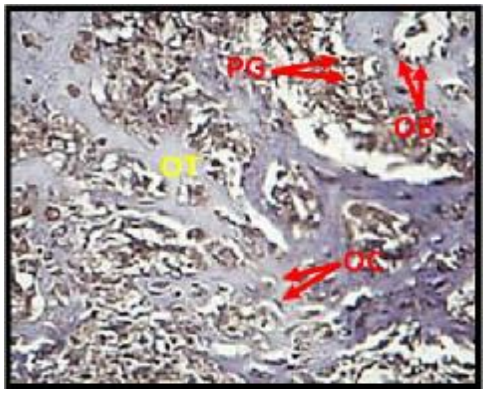

Figure 3: Positive localization of TGFB3 in bone defect of control group at 1 week duration expressed in osteoid tissue (OT), osteocyte cell (OC) in progenitor cell (PG) and in osteoblast cell (OB). DAB stain with hematoxylin counter stain X 20.

At 2 weeks duration

A – Control group: Bone section of 2 weeks duration of control group shows bone trabeculae that are negatively stained enclosing areas of marrow tissue and surrounded by fibrous C.T which are positively stained also positive expression of TGFB3 detected by osteocyte cell and marrow tissue (Figure 5).

B- Experimental group: View of bone defect treated with BMP7 for 2 weeks duration shows formation of bone trabeculae with positive immunohistochemical localization of TGFB3 in osteocyte cell osteoblast cell and osteoclast cell indicated for bone turn over and bone marrow tissue (Figure 6).

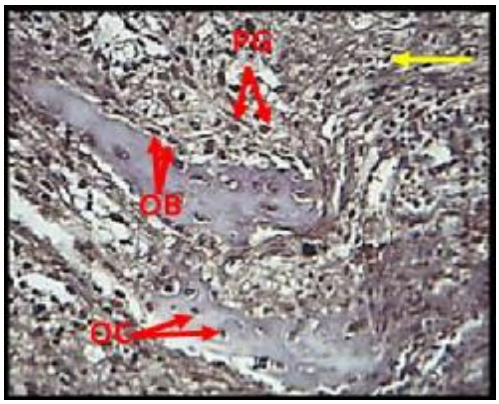

Figure 4: Positive localization of TGF-B3 in formative osteoblast cell (OB), osteocyte cell (OC), progenitor cell (PG) and inflammatory cell (arrow) of experimental group of 1 week duration. DAB stain with hematoxylin counter stain X 20.

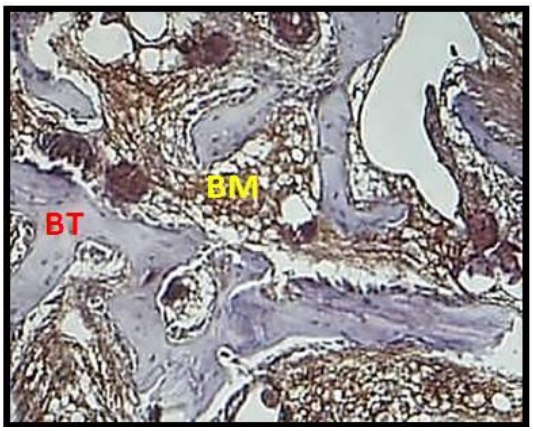

Figure 5: Immunohistochemical view shows negatively stained bone trabeculae (BT) surrounded by positively stained bone marrow tissue (BM). DAB stain with hematoxylin counter stain X 20.

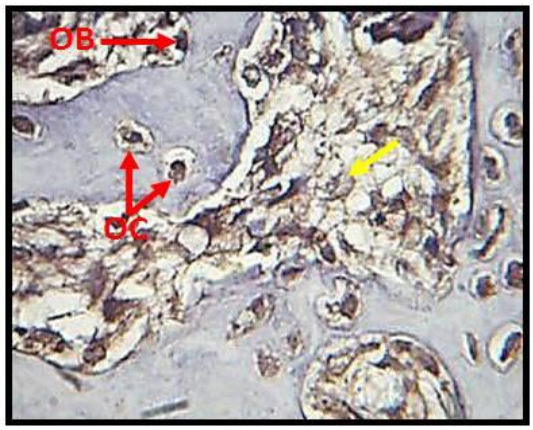

Figure 6: Immunohistochemical localization of TGFB3 on osteoblast cell (OB), osteocyte cell (OC) and bone marrow tissue (arrow) in bone section of experimental group for 2 weeks duration DAD stain with hematoxylin counter stain X 40.

At 4 weeks duration

A – Control group: Immunohistochemical view of bone defect of control group of 4 weeks durations shows formation of immature bone with positively stained osteoblast and osteocyte cell (Figure 7).

B – Experimental group: View of mature bone formation for 4 weeks duration in experimental group shows positive localization of TGF.B3 in osteocyte, cell marrow tissue and Haversian canal (Figure 8).

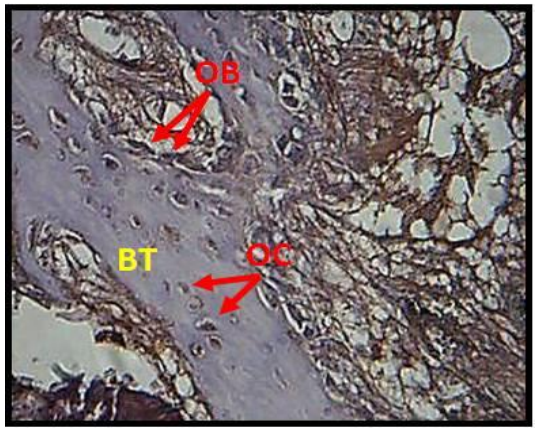

Figure 7: Immature bone formation of 4 weeks healing of bone defect (control) shows positive expression of TGF-B3 on osteocyte cell (OC) , osteoblast (OB) , while it shows negative expression in bone trabculae (BT) DAB stain with hematoxylin counter stain X 40 .

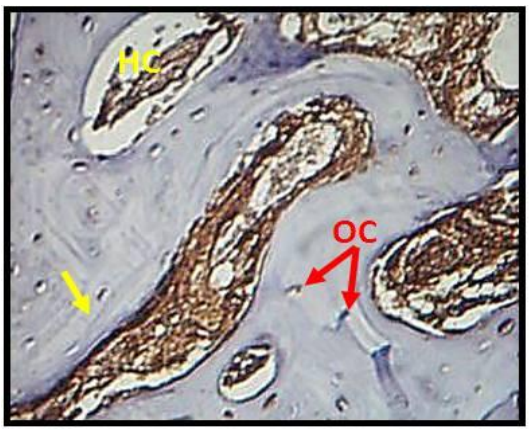

Figure 8: Positive expression of TGFB3 in mature bone for 4 weeks duration of bone defect treated with BMP7 in widely distributed osteocyte cell (OC) inside bone matrix and around Haversian canal (HC) resting line are seen (arrow). DAB stain with hematoxylin counter stain X 40.

At 6weeks duration:

A- Control group: Microphotograph of bone defect (control) for 6 weeks duration shows formation of bone with positive expression of TGFB in osteocyte cells, formative osteoblast cells and Haversian canals (Figure 9).

B- Experimental group: Immunohistochemical view of bone defect treated with BMP7 for 6 weeks duration revealed mature bone formation filling the defect area , the newly bone appear dense with positive expression of TGFB in osteocyte cells, reversal line are seen separated between old bone and new bone with the presence of numerous resting lines. Circular arrangement of positively expressed osteocytes around Haversian canal which positively expressed could be seen in some area, positively expressed osteoblast lined these Haversian canal (Figure 10).

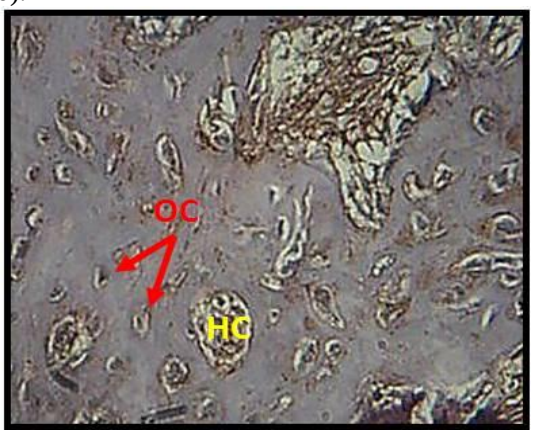

Figure 9:Positive immunohistochemical expression of TGFB3in osteocyte (OC) cell and Haversian canal (HC) in new bone of 6 weeks duration of control group . DAB stain with counter hematoxylin stain X40.

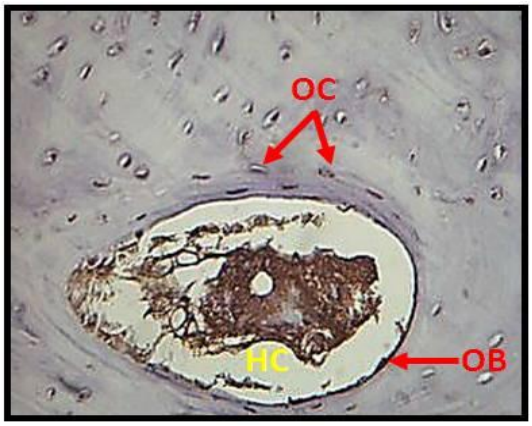

Figure 10: Positive expression of TGFB3 is seen in Haversian canal (HC) positively expressed osteocyte cell (OC) and positive osteoblast cell (OB) in experimental group of 6 weeks duration DAB stain with hematoxylin counter stain X 40.

Expression of IGF-1R findings

At 3 days duration:

A- Control group: Immunohistochemical finding of bone defect at 3 days duration (control) shows positive expression of IGF –IR in bone marrow stromal cell and in extracellular matrix of bone marrow (Figure 11).

B- Experimental group: Immunohistochemical localization of IGF–IR in bone defect treated with BMP7 of 3 days duration shows positive expression of IGF-IR in endothelial cell, progenitor cell and in extracellular matrix of marrow tissue new bone formation were indicated and are negatively expressed (Figure 12).

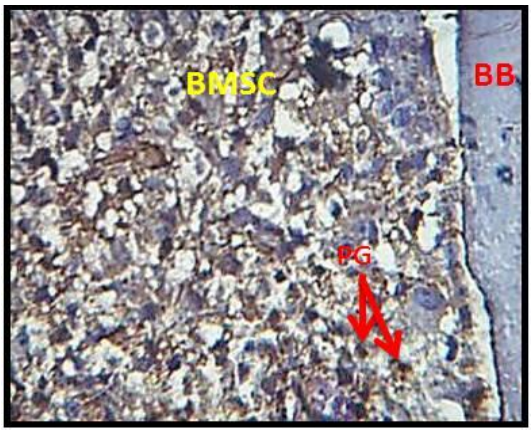

Figure 11:Immunohistochemical view of bone defect of control group at 3 days duration shows positive expression of IGF-IR in bone marrow stromal cells (BMSC) ,progenitor cells (PG), basal bone (BB) shows negative expression . DAB stain with hematoxylin counter stain X 40.

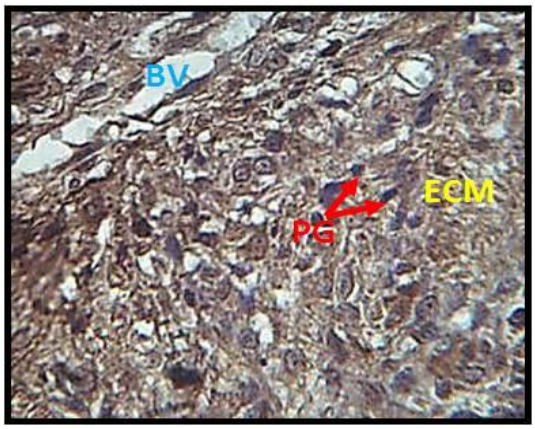

Figure 12 Positive expression of IGF –IR in progenitor cell, B.V and extracellular matrix (ECM) of marrow tissue in bone defect treated with BMP7 of 3 days duration DAB satin with hematoxylin counter stain X 40.

At 1week duration:

A- Control group: Bone section view shows positive localization of IGF-IR in progenitor cell, endothelial cell and extracellular matrix of marrow tissue, new bone formation (bone trabeculae) shows negative expression (Figure 13).

B- Experimental group: Positive expression of IGF_IR in progenitor cell, osteocyte cells, formation osteoblast cell rimming the border of new trabeculae (Figures 14).

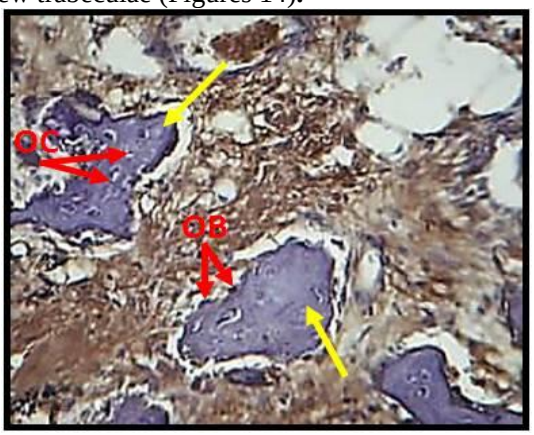

Figure 13: Positive immunohistochemical localization of IGF-IR in formative osteoblast cell (OB), osteocyte cell (OC), bone specules (arrow) shows negative expression in bone defect (control) for 1week duration. DAB stain with hematoxylin counter stain X 20.

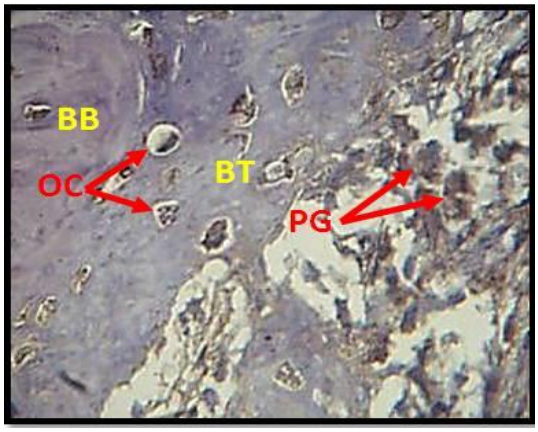

Figure14: Immunohistochemical view shows bone trabeculae (BT) formation coalesce with basal bone (BB) osteocyte cell (OC) trapped inside bone matrix and osteoprogenitor cell (PG) shows positive expression of IGF-IR . DAB stain with hematoxylin counter stain X 40.

At 2weeks duration:

A – Control group: Microscopical evaluation of bone section in control group after 2 weeks of healing shows osteoid tissue formation, negatively expressed by IGF-IR. Osteocyte cell and bone marrow stromal cells are positively expressed (Figure 15).

B – Experimental group: Immunohistochemical view of bone Section at 2 weeks duration of experimental group shows positive expression of IGF-IR by osteoblast cell rimming the border of bone trabeculae and osteocyte cell are embedded inside the bone matrix (Figure 16).

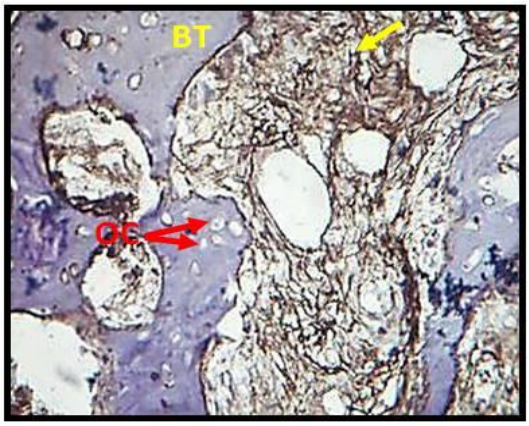

Figure15:Immunohistochemical view of bone defect (control) of weeks duration showing negative expression of IGF-IR in bone trabeculae (BT) , osteocyte cell (OC) and bone marrow stromal cells (arrow) shows positive expression of IGF-IR . DAB stain with hematoxylin counter stain X 20.

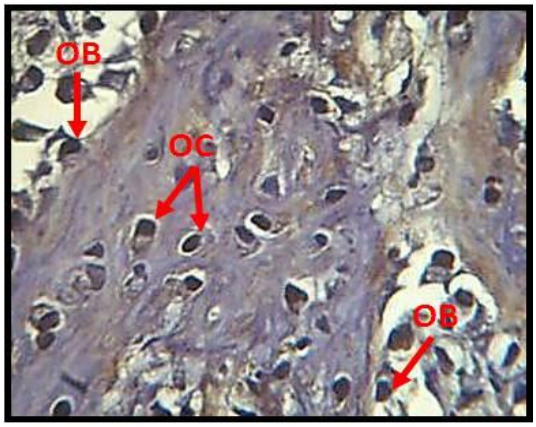

Figure 16: Positive expression of IGF-IR in osteoblast cell (OB) rimming the border of bone trabeculae, osteocyte cell (OC) trapped inside the bone matrix, DAB stain with hematoxylin counter stain X40

At 4 weeks duration:

A- Control group: Microphotograph view of bone defect of control group at 4 weeks duration shows formation of immature bone trabeculae, negatively stained by IGF-IR (Figure 17).

B – Experimental group: Bone section treated by BMP7 at 4 weeks duration shows well developed bone trabeculae, positively stained osteocyte cell embedded inside bone matrix and osteoblast cell present in the border of new bone formation (Figure 18).

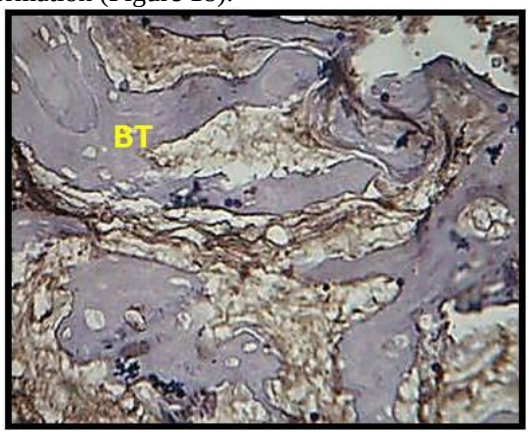

Figure17:Immunohistochemical view of bone defect (control) of 4 weeks duration shows formation of immature bone trabeculae (BT) negatively stained by IGF-IR . DAB stain with hematoxylin counter stain X 20.

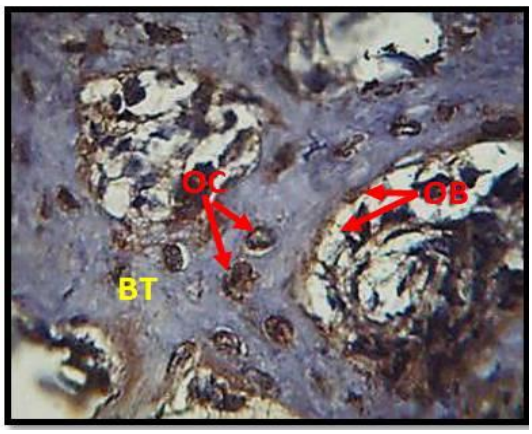

Figure 18: Positive localization of IGF-IR in osteocyte cell (OC) embedded inside matrix of well developed bone trabeculae (BT) , osteoblast cell (OB) in bone defect of 4 weeks duration treated by BMP7. DAB stain with hematoxylin counter stain X 40.

At 6weeks duration:

A- Control group: Bone section of control group at 6 weeks duration shows mature bone formation with positive expression of IGF-IR in osteocyte cells embedded in matrix of bone trabeculae and in Haversian canal (Figure 19).

B- Experimental group: Positive expression of IGF-IR in mature dense bone filling the defect area of 6 weeks duration in osteocyte cells, Haversian canal and many resting lines (Figure 20).

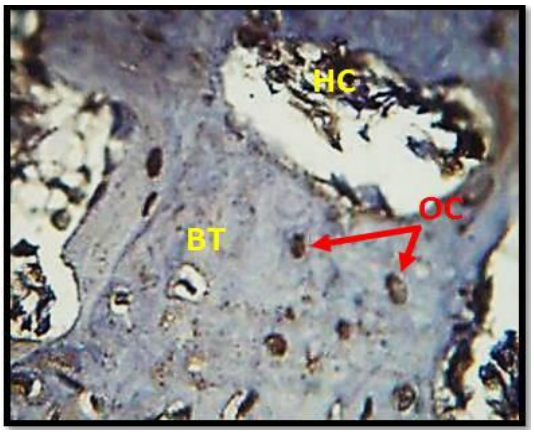

Figure 19: Immunhistochemical view of bone section of control group at 6 weeks duration shows positive expression of IGF-IR in osteocyte cell (OC) and in Haversian canal (HC) while bone trabeculae (BT) show negative expression, DAB stain with hematoxylin counter stain X 40.

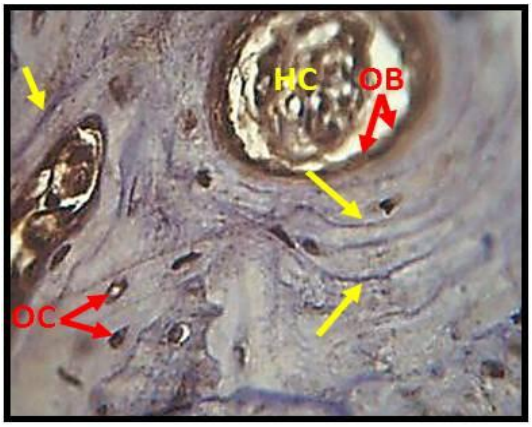

Figure 20: Immunohistochemical view of dense new bone formation filling bone defect of 6 weeks duration treated by BMP7 revealed positive expression of IGF-IR in osteocyte cell (OC), osteoblast cell (OB) surrounded the Haversian canal (HC) and many resting lines (arrows). DAB stain with hematoxylin counter stain X 40.

Statistical analysis

Bone cells

Figure (21) illustrated graphically line chart of Bone cell's mean values of (osteoblast, and osteocyte), associated with different Periods of times in each group. Majority of bone cell's number (osteoblast, and osteocyte) according to different periods are registered by experimental groups (TGF-B3group, and then followed by IGF-IR group), and then finally followed with low count cells number with controlled groups.

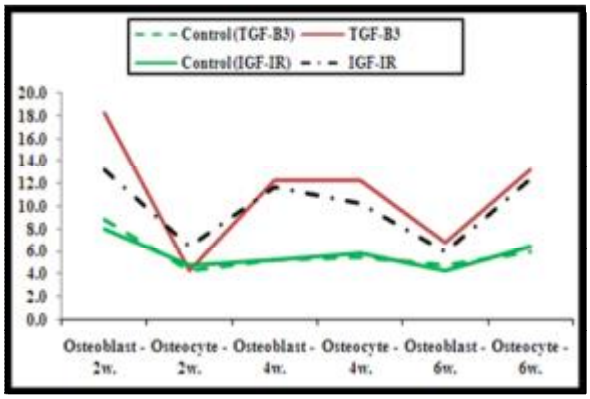

Figure 21: Line chart of Bone cell's mean values of (osteoblast, and osteocyte), in TGF-B3 and IGF-1R associated with different Periods of times in each group.

Table (1) represents comparisons significant by (t- test) among all pairs in contrasts of experimental and controlled groups at different periods associated with bone cells (osteoblast and osteocyte).

The results shows that most of contrasts are accounted significant differences in at least at P<0.05, except with osteocyte – 2 weeks and Osteoblast - 6weeks either for TGF-B3 or for IGF-IR contrasts.

It seems to be needs to continuing testing of comparisons by using (LSD) method, and that were illustrated in the next Tables (2), and (3).

The results shows that all probable contrasts with respect of different periods had no significant differences at P>0.05.

The results shows that all probable contrasts with respect of different groups had significant differences in at least at P<0.05.Bone cell's types had increasing numbers with respect of osteoblast compared with osteocyte, and experimental (BMP7) groups are accounted twice number of bone cells compared with control groups, and finally the last two periods (4 and 6 weeks) had registered significant different.

Table 1: Comparison significant by (t- test) among all pairs in contrasts of experimental and control groups at different periods associated with Bone cells (osteoblasts and osteocytes)

Groups	Bone cells/periods	Levene's Test for Equality of Variances		t-test for Equality of Means			C.S.
		F- test	Sig.	t- test	d.f.	Sig.	
TGF-B3 Control & exprimental	Osteoblast - 2w.	4.101	0.089	-3.601	6	0.011	S
	Osteocyte - 2w.	0.000	1.000	0.000	6	1.000	NS
	Osteoblast - 4w.	3.000	0.134	-7.867	6	0.000	HS
	Osteocyte - 4w.	0.500	0.506	-8.399	6	0.000	HS
	Osteoblast - 6w.	4.000	0.092	-1.656	6	0.149	NS
	Osteocyte - 6w.	0.300	0.604	-8.490	6	0.000	HS
IGF-IR Control & exprimental	Osteoblast - 2w.	4.500	0.078	-6.148	6	0.001	HS
	Osteocyte - 2w.	12.500	0.012	-1.364	4	0.245	NS
	Osteoblast - 4w.	0.600	0.468	-4.645	6	0.004	HS
	Osteocyte - 4w.	0.045	0.839	-3.959	6	0.007	HS
	Osteoblast - 6w.	0.167	0.697	-1.849	6	0.114	NS
	Osteocyte - 6w.	0.429	0.537	-7.348	6	0.000	HS

(*)HS: Highly Sig. at P<0.01; S: Sig. at P<0.05; NS: Non Sig. at P>0.05

Table 2: Multiple Comparisons by (LSD Method) among all pairs of different periods in compact form

Periods		Mean Diff.	P-value	C.S. (*)
2 w.	4 w.	-0.031	0.970	NS
	6 w.	1.000	0.225	NS
4 w.	6 w.	1.030	0.211	NS

(*) HS: Highly Sig. at P<0.01; S: Sig. at P<0.05

Table 3: Multiple Comparisons by (LSD Method) among all pairs of different groups in compact form

Groups		M.D.	P_{-value}	C.S.
Control- TGF-B3	TGF-B3	-5.42	0.000	HS
	Control- IGF-IR	0.00	1.000	NS
	IGF-IR	-4.29	0.000	HS
Expr. TGF-B3	Control- IGF-IR	5.42	0.000	HS
	IGF-IR	1.13	0.237	NS
Control- TGF-B3	IGF-IR	-4.29	0.000	HS

(*) **HS: Highly Sig. at P<0.01**; **S: Sig. at P<0.05**

DISCUSSION

TGF-β plays very important roles in embryogenesis, development and normal tissue homeostasis .A broad range of biological processes, including cell proliferation, cell survival, cell differentiation, cell migration, and matrix synthesis by inducing the production of ECM molecules such as fibronectin, collagen and proteoglycan are regulated by TGF-β**(7,8).**In the present study TGF-β shows positive expression in control and experimental groups in bone cellsin all healing interval periods, this result in agreement with Jaafar and Warwar [9,10] who found positive expression in bone cells in same periods**.** Osteoblast cells number was obtained as a highest in BMP7 group compared to control group which showed lowest number of osteoblasts. Mean value of osteoblast cell number was higher in 2 weeks of BMP7 group but in 4 and 6 weeks showed a markedly decrease in number. Cell counting revealed that most of surviving osteoblasts cells are settled on the bony defect site because later in 6 weeks duration, there is more formation and maturation of woven bone so most osteoblast forming bone become entrapted within the matrix and called osteocytes cell [11].The present study was in agreement with Zhang *et al*. [12] they found that BMP-7 stimulates the transformation of mesenchymal cells into osteoblasts, stimulating bone formation in both remodeling and repairing processes andare potent physiological inducers of osteoblast differentiation and angiogenesis. The present results was confirmed with the study done by Nevee*t al.* [13] BMP-7 has been show to be able to induce immature cells to differentiate into osteoblasts .Osteoclast precursors circulate amid the monocyte macrophage population and differentiate into preosteoclasts that fuse to form giant bone resorbing mature osteoclasts. The transforming growth factor β /BMPs have widely recognized roles in bone formation during mammalian development and exhibit versatile functions in the body. For instance, a BMP morphogen gradient is established in a multicellular embryo, and BMP drives the differentiation of ectodermal cells and mediates dorsal patterning to establish dorsal-ventral axis. Disruptions of TGF-β/BMP signaling have been implicated in multiple bone diseases including tumor metastasis, brachydactyly type A2, and osteoarthritis [14]. This study was in agreement with Mohamed *et al.* [15] who explained on a fact of early enhancement and recruitment of the fibroblasts and osteoprogenitor cells to be differentiated into osteoblasts (bone formative cells) and enhancement of osteiod tissue formation which need more supplements and more blood vessels. Osteocytes formation was happened by entraption of osteoblasts within their matrix; more osteoblasts resulted in more number of osteocytes and as a result of more and faster building of bone matrix. In the present results, IGF are positively expressed in both control and experimental groups and in different period intervals and in different levels according to osteoblast activity in osteoid formation. Positive expression of IGF –IR in bone marrow stromal cell and in extracellular matrix of bone marrow, endothelial cells, fat cell, progenitor cells, progenitor cell, osteocyte cells and osteoblast cell while bone trabeculae was negatively expressed by IGF-IR ,this results in agreement with Yosif [16] who found IGF positively expressed in both control and experimental implants treated with platelet rich fibrin matrix and in different intervals period (3 days 1, 2 and 6 weeks) and in different levelsaccording to osteoblast activity in osteoid formation.The present study was in agreement with DiGirolamo *et al.* [17] they study The effects of IGF-1 on bone have been well documented. IGF-1 has been shown to induce proliferation of osteoblast- like cells and is an important survival factor for many mammalian cell types, including osteoblasts. IGF-1 production increases during the initial phases of fetal rat calvarial osteoblast differentiation andwith matrix synthesis and mineralization that may account for the ability of IGF-1 to augment synthesis of type I collagen and inhibit collagen degradation in differentiated fetal rat osteoblasts. The results of the present study

record positive expression of IGF in experimental group at all periods and this was in agreement with Arpornmaeklong *et al.* [18]. They found that insulin-like growth factors increase osteoblast proliferation and have a significant role in stimulating the function of mature osteoblasts. Insulin growth factor -1 enhances the mitogenic action as well as the differentiation activity of BMPs. In an in vivo bone–implant integration model, combined delivery of IGF-I and BMP from coated titanium screws significantly improved bone formation compared to BMP alone. The IGF/BMP combination seems to enhance both in vitro and in vivo osteogenesis [19].

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