Research Article

Localization of TGF-β3 in intra bony defect treated by local application of chitosan \ β- TCP in rabbits

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Abstract: Background: Bone defect is a lack of bone tissue continuity, bone defects may be caused by trauma, tumor, or infection. The aim of study to evaluate the expression transforming growth factor beta three (TGF-β3) in intra bony defects treated with chitosan and beta tricalcium phosphate and their combination. Materials and methods: A total of thirty two male New Zealand rabbits were weighed about(1.5-2kg) assigned randomly into four groups, each rabbits received bilateral defect in each femur, these bony defects were divided into four groups: Control group (8 bony defects): these bony defect were left to heal normally without treatment, chitosan hydrogel group (8 bony defects), beta tricalcium phosphate group (β-TCP): (8 bony defects , Combination group (8) bony defects): these bony defects were treated with both of chitosan hydrogel and β-TCP powder . All animals had been sacrificed after 2,4 weeks and the specimens were processed routinely for a serial decalcified sections for the immunohistochemical study on TGF-β3. Result: The immunohistochemical findings had shown a higher immunoreactivity of the bone cells of the experimental group than the control groups. Conclusion: The study revealed that local application of chitosan hydrogel and their combination with TCP accelerate bone formation by increased the expression of TGFβ3 in intra bony defect more than that in normal physiological process.

Keywords: Chitosan, β -TCP, Immunohistochemistry, TGF-β3, Rabbit.

Introduction

Bone is a specialized type of connective tissue, its mineralized and provides support to the structure of living body and has significant function in systemic physiology, including mineral metabolism. [1]Critical-sized bone defects are defined as those that the defects not heal spontaneously throughout patient lifetime. Bone tissue engineering, which uses a various of scaffolds, stem cells, and growth factors to treat a variety of bone defects, is becoming more popular. [2]. Chitosan (CS) is a biopolymer, which has been known as a biological material in promoting the healing process of soft and hard connective tissues [3]. Chitosan regard as prospective material for the reason of unique biological properties, included antimicrobial, bioactive and biocompatible with another material, so chitosan based materials expansively used in wide-range of dental application (4). Chitosan application included guided tissue regeneration, prevention dentistry, dentin pulp complex and enamel repair and coating dental implants[5] .Beta-tricalcium phosphate (β-TCP) is an alloplastic biomaterial that is well-known for its stable integration with new bone formation. Its characteristics and biological activities have made it suitable for use in various bone regeneration applications [6].

Beta-tricalcium phosphate (β-TCP) is a promising material for the regeneration of bone tissue as a result of osteoinductivity and osteoconductivity properties, in addition to a high degradation rate of materials. β-TCP, with the chemical formula Ca3(PO4)2, is a favorable bioceramic material for maxillofacial and orthopedic surgeons due to its direct role in bone regeneration through physical features such as porosity, surface roughness, Degradation, and the release of calcium and phosphate ions. Ions releasing regard as essential phenomena throughout increase the local concentration of calcium and phosphate ions into the defected tissues environment which can stimulate the formation of bone minerals on the surface of calcium phosphates ions and increase the expression of osteoblastic differentiation markers such as ALP, COLI, OCN, BMPs, OPN, BSP, and RunX2 [7].

Transforming growth factor beta is the most prominent cytokine that plays an important function in the regeneration of connective tissues, especially the remodeling of bone tissues. TGF-β3 enhances osteoblast differentiation by increasing the mRNA level of osteoblasts and increasing alkaline phosphatase activity [8]. TGFβ-3 is 3–5 times more potent than its more widely studied counterpart TGFβ1 due to a higher affinity for TGF-3 receptors. TGF-s are known to play an important role in the coupling of bone formation and resorption, and can also elicit increased bone formation both in vivo and in vitro [9].Positive localization of TGFβ3 were seen in a stromal cells component which signified by fibroblasts, lymphocytes, plasma cells and endothelial cells [10].Transforming growth factor-β3 also can stimulates adipocyte progenitor proliferation, resulting in a higher number of cells undergoing differentiation in vitro [11]. Both osteoblasts and osteoclasts synthesize and respond to TGF-β3 depend on experimental and physiological condition, also TGF-β3 is abundant in bone matrix and has been shown to regulate the activity of osteoblasts and osteoclasts in vitro [12].

We found no available data on the expression of TGF-β3 in bone healing after treatment with chitosan \ β-TCP. Hence, the present *in vivo* study aimed to study the localization of TGF-β3 in intra-bony defects treated by local application of chitosan \ β- TCP in rabbits.

Materials and Methods

1.Chitosan powder (yellow color)

2- Glacial acetic acid

3-Polyvinyl alcohol (white powder)

4- β -TCP granules

5- Monoclonal antibody "mouse TGF-β3 antibody(sc-166833), from Santacruz USA (65529.1113), Two-step plus poly HRP Anti Rabbit/Mouse IgG Detection kit from Abcam UK (No: E-IR-R213).

All experimental procedures were carried out in accordance with the ethical principles of animal experimentation of the College of Dentistry, University of Baghdad (Ref. 530 on June 30, 2022). This in vivo study was performed at the Department of Oral Diagnostic Sciences, College of Dentistry, University of Baghdad. The rabbits were kept under standardized, separated cages and were fed with standard diet.

Chitosan hydrogel prepared by dissolving 0.5 g of chitosan powder in glacial acetic acid with polyvinyl alcohol and stirring the components in a magnetic stirrer for about 4 hours until providing a hydrogel [13].

Thirty-two New Zealand rabbits were used in this study, aged between 8 – 12 months, their weight ranged between -1.5 and 2 kg. A bilateral intra-bony defect of about 2 mm in width and 3mm in depth was made in both femurs of each rabbit [14]. All the rabbits were divided randomly into 4 groups as follows:

1. Control group (8 rabbits), the bony defect left for spontaneous normal healing.

2. Chitosan hydrogel group(8 rabbits) the bony defect received 0.5 ml of chitosan hydrogel.

3.β-TCP group(8 rabbits), the bony defect received 0.5mg of β-TCP particles.

4-combination group (8 rabbits) the bony defect received 0.25 ml of chitosan hydrogel and 0.25 mg of TCP powder.

The animals were sacrificed in 2, 4 weeks and the specimens were prepared for histological (H&E) stain then decalcification by EDTA after that imunohistochemical detection of TGF-β3 were done for all groups in both healing periods.

Assessment of immunohistochemistry

TGFβ-3 positive reaction was indicated by the presence of cell membrane brown and extracellular matrix cytoplasmic stain, whereas TGF-3 negative reaction was indicated by the absence of immunoreaction [10].

For the quantification of immunohistochemical reactions a positive composite score was obtained by multiplying the reaction intensity score (1-mild, 2-moderate, 3-strong) with the percentage of labelled cells score: 0-25%(1), 25-50% (2),50-75%(3) ,75-100%(4). The scoring was graded as follows:

0-Scores of 0- 4 were defined as negative expression (-)

1 - Scores of 5-8 were defined as weakly positive expression (+)

2 -Scores of 9-12 were defined as moderate positive expression (++)

3 –Scores of more than 12 were defined as strongly positive expression (+ + +) [14] .

Statistical analysis

For bone cells count and for stromal cells that expressed to TGF-β3 was estimated by descriptive statistic that include mean and SD and inferential statistic that include ANOVA and LSD test in all comparison.

Results

Two weeks duration: The control group shows strong positive localization of TGF-β3 in fibrous connective tissue, osteoblasts, and osteocytes (Figure,A). The chitosan hydrogel group shows positive expression of TGF-β3 in osteoblasts, osteocytes, and progenitor cells in bone marrow tissue (Figure 1,B).β-tricalcium phosphate group revealed positive expression of TGF-β3 in osteoblasts and bone marrow stromal cells (Figure 1,C). Combination group showed strong positive localization of TGF-β3 in bone section two weeks postoperatively in osteocytes, osteoblasts and bone marrow stromal cells (Figure 1,D).

Four weeks duration: The control group revealed positive expression of TGF-β3 in bone marrow stromal cells, osteoblast-lined haversian canal, and osteocytes (Fig. 2, A). The hydrogel group revealed strong positive expression seen in osteocytes, osteoblasts, and mesenchymal stromal cells (Fig. 2,B).β-tricalcium phosphate group showed moderate expression of TGF-β3 in the bone section of the group at four weeks duration in osteoblasts and osteocytes. Mature bone had a negative expression of TGFβ3 (Fig. 2,C). Combination group illustrated strong immunohistochemical localization of TGF-β3 in bone marrow stromal cells (BMSC) inside haversian canal and osteoblasts, and moderate expression in osteocytes (Fig. 2, D).

Statistical analysis of immunohistochemical findings

Table 1 showed group comparison difference by ANOVA test for mean number of bone marrow stromal cell(BMSC), osteocyte(OC)osteoblasts(OB) and osteoclast (OCL) in each healing duration (two and four weeks). The results showed highly significant difference in osteoblast, bone marrow stromal cell and osteocyte in two weeks. Also showed non-significant differences in osteoclast among groups in two and four weeks duration.In four four-week duration, the results show significant differences among groups in bone marrow stromal cells and osteocytes in addition to highly significant differences among groups in osteoblasts.

Table 2 represents the LSD tests, which were used for group comparison differences of osteoblast, bone marrow stromal cell, osteocyte, and osteoclast numbers in each healing duration. The results revealed highly significant difference in bone marrow stromal cell between control group with chitosan and between chitosan and TCP group also showed significant differences between combination and control group and between TCP and combination in two weeks duration In four weeks the result show highly significant differences between control and chitosan and significant differences chitosan and TCP groups.

Group comparison of osteocytes show highly significant differences between control and chitosan and combination, and between chitosan and significant differences between TCP and combination in two weeks

In a four-week duration, the results show significant differences between control with chitosan and TCP and the combination. In group comparison of osteoblast cell the results show highly significant differences between control with chitosan and TCP and combination groups, and between chitosan and TCP groups and between chitosan and combination groups. Moreover, the result showed significant differences between TCP and combination groups.

In four weeks, the results show highly significant differences between control and chitosan and between control and combination and between chitosan and TCP, and significant differences between chitosan and combination groups. The result showed nonsignificant differences between groups in osteoclast mean value in each healing period.

Table 1: Group comparison difference by ANOVA test for positive expression of TGFβ3 in each healing duration

	Study Group	**BMSC**		**O.cyte**		**o.blast**		**o.clast**	
		P-value	**F value**	**P-value**	**F value**	**P-value**	**F value**	**P-value**	**F value**
Two Week	**Cont. Chitosan Tcp Combination**	0.002 HS	6.144	0.000 HS	27.006	0.000 HS	59.665	0.228 NS	1.552
Four Week	**Cont. Chitosan Tcp Combination**	0.015 S	4.111	0.033 S	3.366	0.000 HS	8.571	0.577 NS	.688

NS= not significant at p>0.05, S=significant at p<0.05, HS= highly significant at <0.01.

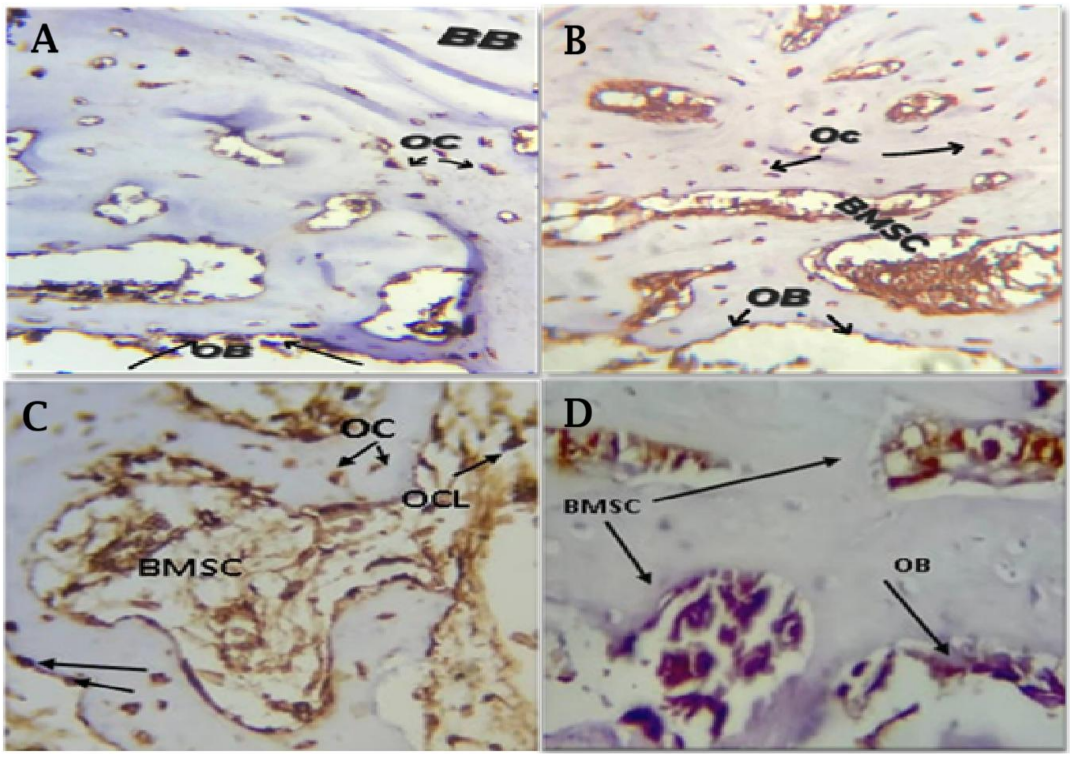

Figure 1: View of 2 weeks duration showing Positive localization of TGF-β3 in: A:control group, B: Chitosan group, C:TCP group, D:Combination group. OB: Osteoblasts, OC: Osteocytes, OCL: Osteoclasts, BMSC: Bone marrow stromal cells X40

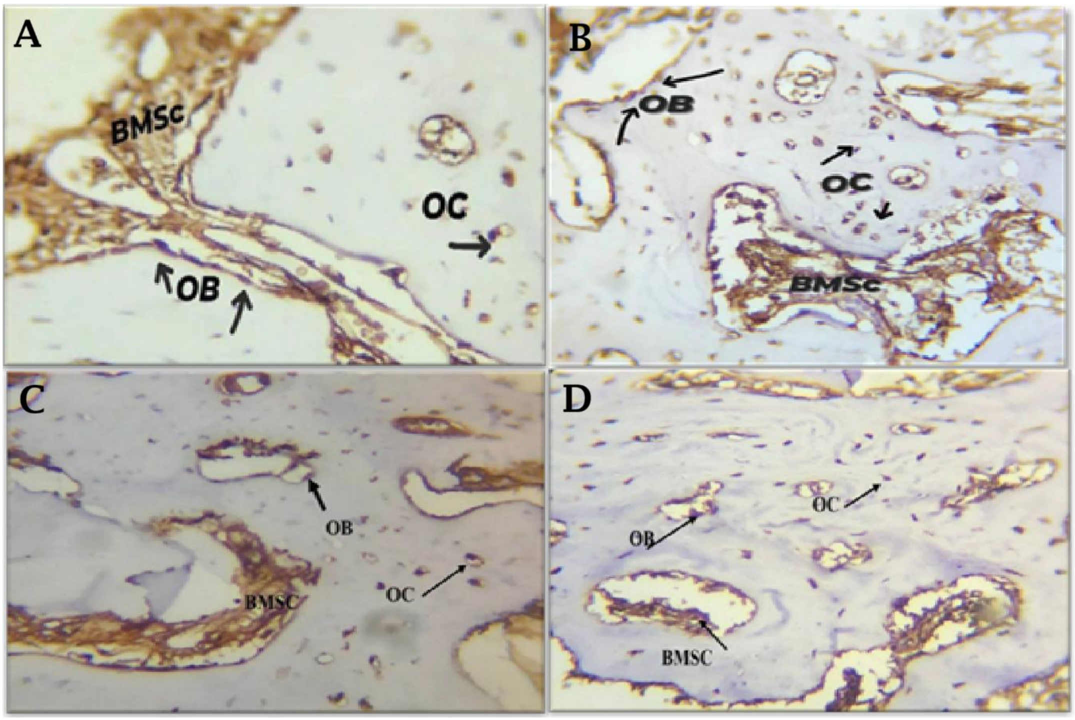

Figure 2: View of 4 weeks duration showing Positive localization of TGF-β3 in: A: control group, B:Chitosan group, C:TCP group, D:Combination group. OB: Osteoblasts, OC: Osteocytes, OCL: Osteoclasts, BMSC: Bone marrow stromal cells X40

Table 2: The LSD tests to confirm the differences occurred between groups after two and four weeks.

Study Group			BMSC		O.cyte		o.blast		o.clast	
	(I)	(J)	Mean Difference (I-J)	P-value	Mean Difference (I-J)	P-value	Mean Difference (I-J)	P-value	Mean Difference (I-J)	P-value
Two Week	Cont.	Chitosan	-7.125	0.002	-5.000	0.000	-8.000	0.000	0.2619	0.264
		Tcp	0.250	0.906	-1.125	0.066	-3.875	0.000	0.0952	0.681
		Combination	-5.500	0.014	-2.625	0.000	-5.250	0.000	0.4286	0.056
	Chitosan	Tcp	7.375	0.002	3.875	0.000	4.125	0.000	-0.1667	0.489
		Combination	1.625	0.447	2.375	0.000	2.750	0.000	0.1667	0.460
	Tcp	Combination	-5.750	0.011	1.500	0.017	1.375	0.032	0.3333	0.147
Four Week	Cont.	Chitosan	-4.875	0.002	-1.500	0.025	2.875	0.000	0.2000	0.310
		Tcp	-1.625	0.267	-1.625	0.016	1.125	0.061	0.2000	0.271
		Combination	-2.875	0.055	-1.750	0.010	1.625	0.009	0.2000	0.271
	Chitosan	Tcp	3.250	0.031	-0.125	0.844	-1.750	0.005	0.0000	1.000
		Combination	2.000	0.174	-0.250	0.695	-1.250	0.039	0.0000	1.000
	Tcp	Combination	-1.250	0.391	0.125	0.844	0.500	0.393	0.0000	1.000

Discussion

TGF-β is regarded as a regulatory protein that is identified to play a significant role in the process of bone healing and remodeling [15]. All experimental and control groups show a high mean value of strong positive expression after two weeks of scarification, as stained the osteoblast-lined bone trabeculae, bone marrow stromal cells, and osteocytes, while in four weeks, the expression decreases to moderate expression in osteoblasts, bone marrow mesenchymal stem cells, and osteocytes.

The present study agrees with Augustine et al [16] that chitosan-based membranes play a crucial role in wound healing by mimicking the extracellular matrix, enhancing biocompatibility, and providing antibacterial activity. These membranes, often loaded with bioactive agents such as polyvinyl alcohol, to improve mechanical stability, degradation, and vascularization, making them effective for challenging wounds like diabetic and burn wounds. Moreover, the present study is in agreement with Zhou et al [17] who show the low molecular weight of chitosan increases expression of BMP-2, which is regarded as a member of the transforming growth factor-β (TGF-β) superfamily of multifunctional cytokines which has a significant role in induction of bone regeneration and bone metabolism. Moreover, they show that the low molecular weight of chitosan may enhance the process of interaction between the ligand of BMP-2 and its receptor to stimulate osteoblast differentiation.

In the group treated with β-tricalcium phosphate (β-TCP), TGF-β3 showed strongly positive expression and a high mean value when compared to the control group. The immunostaining of TGF-β became moderately expressed after four weeks. This may be due to the biocompatibility of β-TCP and its role in the inflammatory reaction when the particles of TCP induce the secretion by inflammatory cell and bone-forming cells. The present study agrees with Xu et al [18], who implanted macroporous β-TCP scaffolds with prior in vivo prevascularization, significantly enhanced vascular invasion and bone consolidation compared to non-prevascularized scaffolds. This prevascularization approach upregulated the expression of BMP2 and VEGF, accelerating vascular formation and bone regeneration.

In the group treated with the combination of chitosan and β-TCP the sections show a higher mean value of strong positive expression in osteoprogenitor cells, osteocyte and osteoblasts, while with progression of time, TGF-β, the expression begins to be moderate positive expression in bone cell. Increase expression of TGF-β in defect treated with the combination of chitosan and β-TCP may be due to role of chitosan as adhesion of bone cells in the site of defect due to gel consistency which facilitation the adherence of cell on scaffold to begin hypersecretion of cytokines. The result matched with Busilacchi et al [19]) who used chitosan scaffold with plate contain adipose derived stromal cell contain platelet lysate, which is responsible to the bulk of growth factors associated with bone regeneration included TGF β, PDGF, FGF and IGF. Their result showed high expression of these bone biomarkers associated with chitosan scaffold.

This finding agrees with Zhao et al[20], who used scaffold of chitosan combined with Gadolinium phosphate enhance bone regeneration and matrix mineralization through upregulation of phosphorylated smad1/5/8 signaling pathway by western blotting test. Also agree with Yi et al who[21] utilize a membrane based on chitosan and culture it on mesenchymal stromal cell to evaluate the anti-inflammatory cytokines. In vitro, the study showed decreased expression of anti-inflammatory IL-6,18 and TNF while the study showed significantly increase expression of TGFβ in the group based on chitosan when compared with control group by QPCR test.

The increased bone formation rate observed in the experimental group was consistent with Kamil [22] Histological examination revealed a highly significant difference across all parameters between the experimental and control groups at the two-week mark. The experimental group showed a more prominent distribution of osteocyte cells and osteoblasts, along with a notably larger bone trabecular area in those treated with whey protein. On the other hand, this finding disagree with Tsai et al[23] who showed a negative effect of chitosan on TGF-β expression by downregulating of signaling pathway.

Moreover, the findings of this study are supported by Alsaeed's research, which utilized both H&E and Masson's Trichrome staining. That study demonstrated enhanced bone formation at both the two-week and four-week intervals and reported a significant increase in bone marrow and trabecular areas in the experimental group.[24].

There were no previous studies specifically investigating the localization of TGF-β3 in intra-bony defects treated with a combination of chitosan and β-TCP, making direct comparisons with our findings unavailable. However, the experimental treatment, particularly the combination group, demonstrated a notable role in promoting bone healing, showing the highest mean expression of TGF β3 in both osteocytes and osteoclasts at 2 and 4 weeks, indicating enhanced bone formation and active remodeling. These results suggest that the combined treatment not only accelerates new bone formation but also facilitates the natural bone remodeling process(25)(26).

Conclusion

Immunohistochemical findings of TGF-β3 revealed positive localization of TGF-beta in all groups in bone cell included osteoblast, osteocyte , osteoclast and BMSC especially in 2 weeks intervals with high score associated with chitosan hydrogel and combination while in four weeks shown decrease in expression with continuous of time with higher mean value in combination and chitosan than that of β-TCP and control.

Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

Z.Aj.A. conceived of the presented idea, developed the theory of research, carried out the laboratory animal model experiments and processed the experimental data. wrote the manuscript in consultation with N.MH.A., wrote the manuscript with input from all authors. supervised the findings of this work. N.MH.A., supervised the work and, performed the analysis, drafted the manuscript and designed the figures. All authors discussed the results and contributed to the final manuscript. The research, analysis, and manuscript were improved by all authors, who also offered constructive criticism.

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Informed consent

Informed consent was obtained from all individuals, or their guardians included in this study.

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تحديد موقع عامل النمو المتحول الثالث في العيب العظمي الداخلي المعالج بالتطبيق الموضعي للكيتوزان وبيتا ثلاثي كالسيوم الفوسفيت في الأرانب

زينب عبد الجبار المشهدي , ندى محمد حسن الغبان

المستخلص:

يعد العيب العظمي نقصًا في استمرارية نسيج العظم، وقد تنشأ العيوب العظمية بسبب الصدمة، الورم، أو العدوى. الهدف من الدراسة هو تقييم التعبير عن عامل النمو المحول بيتا TGF-3) β3(في العيوب العظمية الداخلية المعالجة بمادة الكيتوسان وفوسفات الكالسيوم الثلاثي البيتا ومزيجهما. المواد والطرق: شملت الدراسة إجمالي اثنين وثلاثين أرنبًا من نوع نيوزيلندي الذكر، بوزن يتراوح بين (1.5-2 كجم)، تم توزيعهم عشوائيًا على أربعة مجموعات، حيث تم إحداث عيب عظمي ثنائي الجانب في كل عظم فخذ من الأرانب. تم تقسيم هذه العيوب العظمية إلى أربع مجموعات: المجموعة الضابطة (8 عيوب عظمية): تُركت هذه العيوب العظمية لتشفى بشكل طبيعي دون علاج، مجموعة الكيتوسان الهلامي (8 عيوب عظمية)، مجموعة فوسفات الكالسيوم الثلاثي البيتا (β-TCP): (8 عيوب عظمية)، مجموعة المزيج (8 عيوب عظمية): تم علاج هذه العيوب العظمية باستخدام الكيتوسان الهلامي ومسحوق β-TCP معًا. تم التضحية بجميع الحيوانات بعد 2 و 4 أسابيع، وتم تجهيز العينات بشكل روتيني لأقسام منزوعة التمعدن للدراسة المناعية الكيميائية على TGF-β3. النتائج: أظهرت النتائج المناعية الكيميائية زيادة في التفاعل المناعي لخلايا العظم في المجموعات التجريبية مقارنة بالمجموعة الضابطة. الاستنتاج: كشفت الدراسة أن التطبيق الموضعي للكيتوسان الهلامي ومزيجه مع فوسفات الكالسيوم الثلاثي البيتا يعزز تكوين العظم من خلال زيادة التعبير عن TGF-β3 في العيوب العظمية أكثر من العمليات الفسيولوجية الطبيعية.