

Local Evaluation of Chitosan and B-Tricalcium Phosphate Alone and Combination in Bone Defect of Rabbit by Histological and Histomorphometric Analysis

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ABSTRACT

Background: Repair of bone tissue is a complex multistep process including proliferation, migration, activation, and differentiation of many type of cells, guided toward the mineralization and remodelling of the defect site. Chitosan and beta-tricalcium phosphate may help to increase the bone minerals contents, mechanical strength and density of bone.

Objectives: The aim of this research was to evaluate the process of bone regeneration in rabbits after surgical osteotomy by using chitosan and beta-tricalcium phosphate separately or in combination of chitosan/beta-tricalcium phosphate. **Materials and methods:** A total of 32 New Zealand rabbits with average weight of 1.5-2 kg were used in this study. Animals were randomly divided into four groups (8 rabbits for each). Intrabony defect of about 3 mm width and 3 mm depth were performed in both right and left femur for each rabbits. Group (CONT) control group was not receive any treatment and leaving it spontaneously healed, group (CH) was received chitosan materials, while group (TCP) was received β -TCP materials and group (CHCT) was received combination of β -TCP and chitosan in bony defect. The animals were sacrificed in two healing intervals 2, and 4 weeks (16 rabbits for each healing).

Processing and sectioning technique performed on all bone specimens for histological and histomorphometric analysis. Histological evaluation were performed by section stained with Hematoxylin and Eosin (H and E) and histomorphometric analysis for assessment of osteoclasts, osteoblasts, osteocytes, trabecular number, trabecular width and bone marrow space area by Image J. software.

Key words: Chitosan, β -tricalcium phosphate, Bone healing

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INTRODUCTION

Critical bone defect is generally caused by aggressive accident, infection, tumor and congenital malformation. Critical bone defects ranged from too huge to be self-restored through heals spontaneously [1]. Bone is complicated process through overlapping three stages: the inflammatory phase, the repair phase, and the remodelling phase. Early phase of bone healing characterized by systematically and local responses to the defect stimuli. Additionally, mesenchymal stromal cell and immune cells participate in cellular interaction to control bone healing [2].

Chitosan is a polymer-derived from chitin which is manufactured by deacetylation of chitin. Chitin is generally

extracted from several types of fungi and exoskeletons of crustaceans [3,4] chitosan has several applications in biomedical field, including wound healing, regulation of hypertension and cholesterol and bilirubin absorption, prevention from dental plaque of skin grafting, hemostasis [5].

On other hand, in spite of advantages uses of chitosan but also has many disadvantages, such as poor mechanical strength, low acid resistance [6]. So to minimize these disadvantages, chitosan should be blended with cross linker polymer to, for example Polyvinyl Alcohol (PVA), cellulose and polyacrylamide [8,9].

PVA regard as a most popular polymer that blended with chitosan to provide mechanical strength due its nontoxic structure and inter molecular hydrogen bonds [10].

Beta tri-calcium regard as most popular bone synthetic due to osteo conductive and ostroinfective properties make it one of most efficient materials to management bone defect in orthopedic and maxillofacial surgery [11].

MATERIALS AND METHODS

Preparation of chitosan-Polyvinyl Alcohol (PVA) hydrogels

- In room temperature prepare 0.2 g of chitosan powder (95% deacetylated) in 10 mL of 0.1 M glacial acetic acid solution and stirrer the mixture overnight.

Prepare 1 g of PVA in 10 mL of ultrapure water and stirrer at 90°C for 2 h.

- The two systems mixed in percentage 1:1 in magnetic stirrer to provide homogeneous structure at room temperature, the mixture poured on lab petri dishes. The system left for 1 h at atmospheric pressure.
- Freeze the hydrogels at -20°C for 20 h then thawed in to 8 hours at room temperature, these cycles repeated about 6 times then wash the hydrogels with deionized water, finally the hydrogel kept in ultra-pure water ready to applied in experimental study [12,13].

Animal preparation: All experimental procedures carried out according to the ethical approval of animal experiments of College of Dentistry, University of Baghdad. All animals Supervision and nursing from the stuff of private animal house. 32 males New Zealand rabbits (6-9) months weight (1.5-2 kg) were used in this experimental study, where intrabony defects were achieved 3 mm in depth and 3 mm in width in both right and left femur Figure 1. All animal euthanized in two and four weeks by giving overdose of anesthesia (16 rabbits for each healing periods). Bone specimen was prepared by cutting the bone about 5 mm away from site of operation Figure 1.

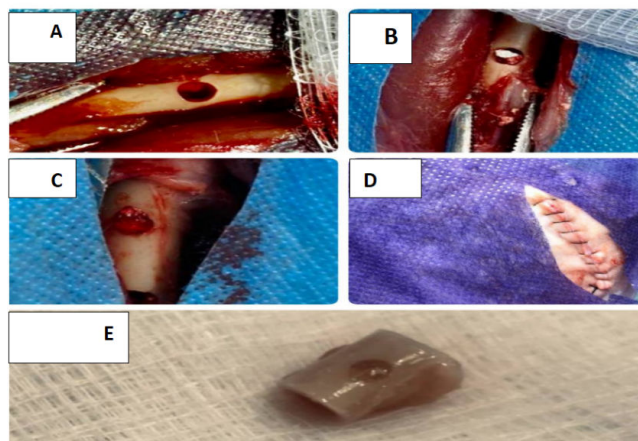


Figure 1: Show surgical procedure including, A) bone defect; B) Application of beta tricalcium phosphate; C) Application of chitosan gel; D) Skin suturing; E) Bone cutting.

Histological preparation: Bony specimens were fixed with 10% freshly prepared formalin for 24 hours, then decalcification process started using 10% EDTA (in two months), embedding done using paraffin wax, bone blocks were sectioned by microtome for serial sections of 4 µm was taken then sited on slide. Staining was done by using Hematoxylin and Eosin (H and E) stain.

Histomorphometric measured by software using Image J program [14].

RESULTS

Histological finding showed that all histological sections displayed respectable bone repair for groups in the study but there was difference in their rate within time consuming of experiment. In Chitosan Hydrogel (CH) group at 2 weeks showed irregular bone trabeculae with irregular distributer osteocytes with rich count of osteoblast when compared with control group (Figures 2 and 3) whereas within time at 4 weeks chitosan group show mature bone trabeculae with regular distribution of osteocyte and recognizable osteons were observed (Figures 4 and 5).

β-tricalcium phosphate groups illustration irregular deposition of osteoid tissues were seen at the site of defect after two weeks duration surrounded by osteoblast and osteoclasts cells also showed osteocyte entrapped, while at 4 weeks indicated maturation of bone by increase trabecular thickness were seen with regular organized osteocytes (Figures 6 and 7).

Histological assessment of after two weeks duration show regular bone trabeculae with entrapped by osteocytes and surrounded by osteoblast, after 4 weeks trabecular area appear more mature and regular distribution of osteocyte were seen (Figures 8 and 9).

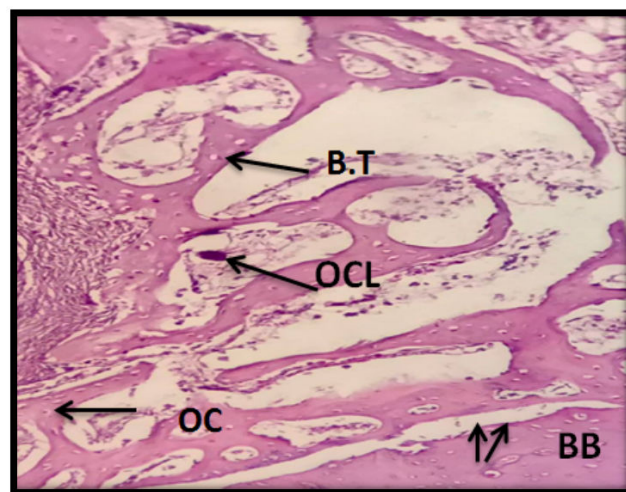


Figure 2: View of 2 weeks control defect area showed a new fine Bone Trabeculae (BT) rimed by Osteoblast (OB) and Osteocyte (OC), woven bone separated from old bone by reversal line (arrows) also showed osteoclast were seen H and E X10.

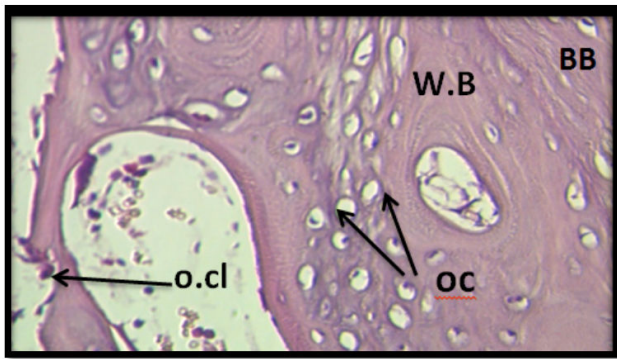


Figure 3: Micrograph of control group of 4 weeks showed Osteocytes (OC) entrapped in woven bone (WB) rimmed by Osteoblast (OB) and osteoclasts cells also are present (OCL) H and E X40.

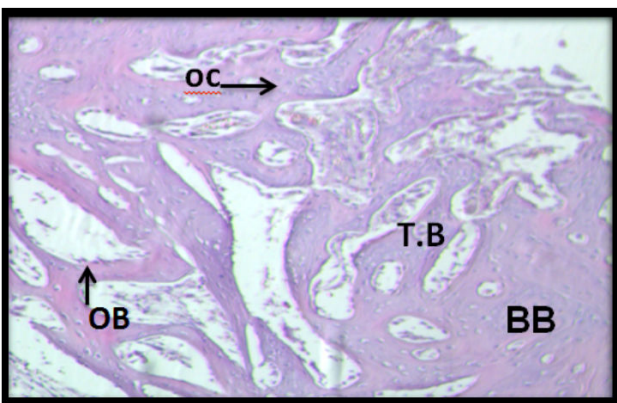


Figure 4: View of 2 weeks defect area treated with chitosan shows new bone Trabeculae (T.B) separated from Basal Bone (BB) by reversal line (arrows) H and E X10.

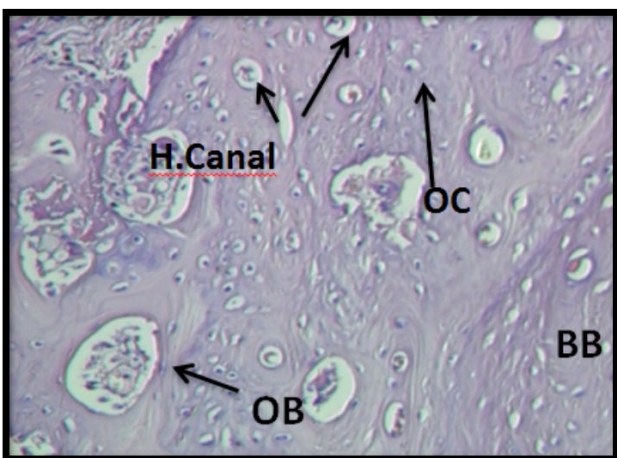


Figure 5: Micrograph of chitosan group of four weeks duration showed mature bone trabeculae which entrapped with regularly arranged Osteocyte (OC), Osteoblasts (OB) seen lined Haversian Canal (HC) H and E X40.

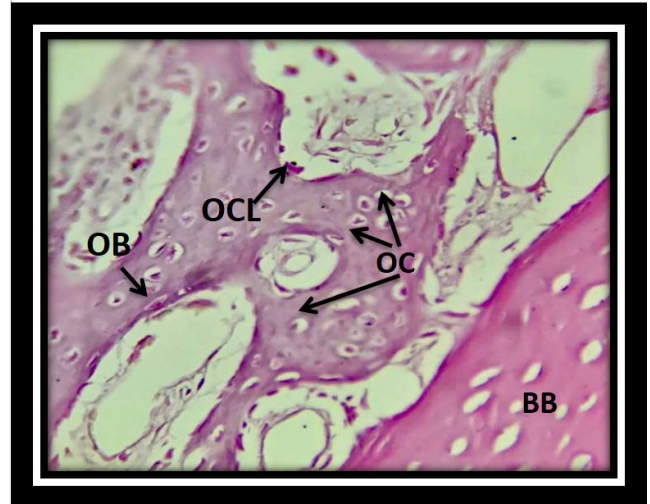


Figure 6: Micrograph of beta tricalcium phosphate showed woven bone entrapped by Osteocytes (OC), and rimmed by Osteoblasts (OB), and also show Osteoclasts (OCL) H and E X40.

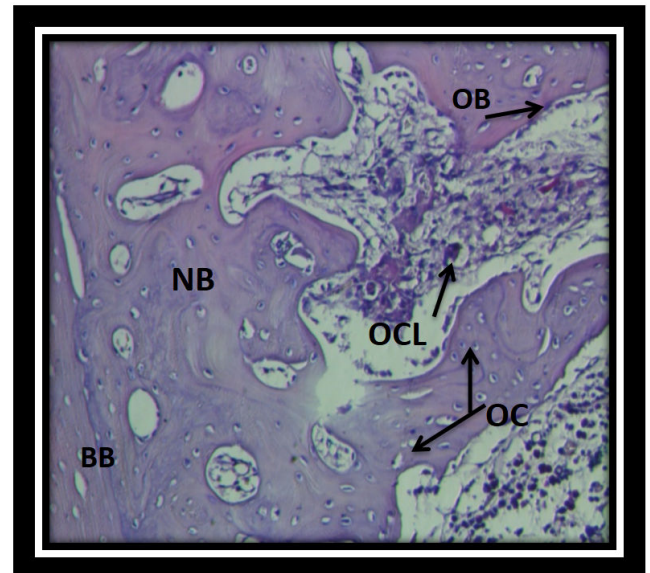


Figure 7: Micrograph of TCP group after four weeks revealed regular distribution of Osteocytes (OC) in New Bone (NB) rimmed by Osteoblasts (OB) at borders of bone also Osteoclast (OCL) were seen H and EX10.

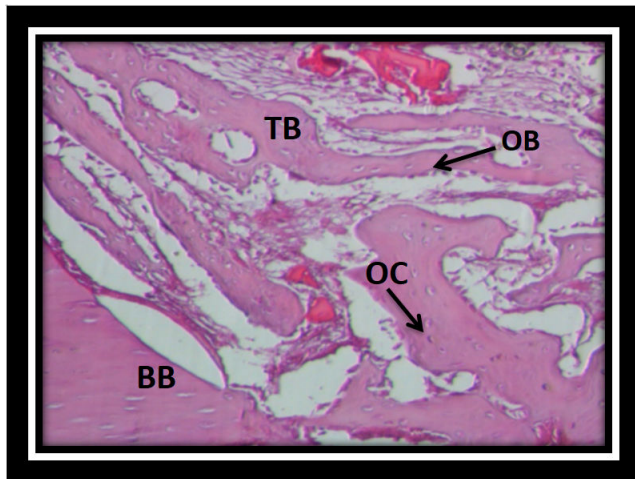


Figure 8: Micrograph of combination group of two weeks the bone defect area treated with chitosan hydrogel and β -tricalcium phosphate after 2 weeks showed new Trabecular Bone (BT) also, reversal line that separated the old bone from new one H and E X10.

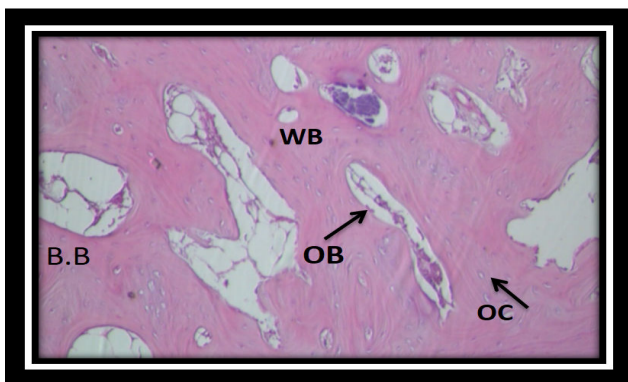


Figure 9: Micrograph of combination group after of 4 weeks duration of combination group showed mature bone contains Osteocytes (OC, Osteoblast (OB cells H and EX40).

Statistical analysis: According to the tables below that illustration descriptive statistic of the mean differences and stander error values of trabecular area, bone marrow area, formative bone cell, bone resorping cell, and bone entrapped cell in defected area at 2 and 4 weeks of healing duration for histomorphometric analysis. According to the LSD test Table 1 illustrated highly significant differences of osteocytes between both Chitosan (CH) and Control (CONT) and between Combination (CHCT) and Control (CONT). Table 2 displayed highly significant differences in osteoblast mean value in both Chitosan (CH) and Control (CONT) groups and between Combination (CHCT) and control in 2 weeks duration. Also high significant differences between TCP and Combination (CHCT) in two weeks and significant differences between Chitosan group (CH) and TCP in two weeks duration.

Table 1: Multiple differences in osteocyte mean value in multiple groups in the study by LSD test for the two healing periods.

	Study Group		Mean Difference (I-J)	Std. Error	P-value
	(I)	(J)			
2 w	Cont.	Chitosan	-4.8750-*	1.16784	0
		TCP	-.5000-	1.16784	0.672
		Combination	-5.8750-*	1.16784	0
	Chitosan	TCP	4.3750*	1.16784	0.001
		Combination	-1.0000-	1.16784	0.399
	TCP	Combination	-5.3750-*	1.16784	0
4 w	Cont.	Chitosan	-3.8750-	2.59786	0.147
		TCP	0.25	2.59786	0.924
		Combination	-6.5000-*	2.59786	0.018
	Chitosan	TCP	4.125	2.59786	0.124
		Combination	-2.6250-	2.59786	0.321
	TCP	Combination	-6.7500-*	2.59786	0.015

Table 2: Illustrate descriptive statistic of osteoblasts cell for each healing periods.

	Study Group		Mean Difference (I-J)	Std. Error	P-value
	(I)	(J)			
2 w	Cont.	Chitosan	-4.2500-*	0.65503	0
		TCP	-.7500-	0.65503	0.262
		Combination	-3.8036-*	0.67802	0
	Chitosan	TCP	3.5000*	0.65503	0
		Combination	0.4464	0.67802	0.516
	TCP	Combination	-3.0536-*	0.67802	0
4 w	Cont.	Chitosan	-1.2500-*	0.48985	0.016
		TCP	-2.6250-*	0.48985	0
		Combination	-2.5000-*	0.48985	0
	Chitosan	TCP	-1.3750-*	0.48985	0.009
		Combination	-1.2500-*	0.48985	0.016
	TCP	Combination	0.125	0.48985	0.8

Table 3 demonstrate significant differences in osteoclasts mean value between Chitosan (CH) group and control groups in 2 weeks. Table 4 exhibited high significant differences in trabecular area between Combination (CHCT) group and control groups, also high significant differences in both chitosan group and TCP group at two

week duration while in 4 weeks show high significant differences between TCP group and Combination (CHCT) group.

Table 3: Show descriptive statistic of osteoclast cell for each healing periods.

	Study Group		Mean Difference (I-J)	Std. Error	P-value
	(I)	(J)			
2 w	Cont.	Chitosan	.7500*	0.307	0.022
		TCP	0.5	0.307	0.116
		Combination	0.575	0.35004	0.113
	Chitosan	TCP	-.2500-	0.307	0.423
		Combination	-.1750-	0.35004	0.621
	TCP	Combination	0.075	0.35004	0.832
4 w	Cont.	Chitosan	0.1429	0.13148	0.292
		Tcp	0.1429	0.12492	0.269
		Combination	0.1429	0.15495	0.369
	Chitosan	TCP	0	0.13597	1
		Combination	0	0.16398	1
	TCP	Combination	0	0.15878	1

Table 4: Illustrate descriptive statistic of the trabecular area in 2, 4 weeks of healing periods.

	Study Group		Mean Difference (I-J)	Std. Error	P-value
	(I)	(J)			
2 w	Cont.	Chitosan	-.0593-*	0.01055	0
		TCP	-.0150-	0.01055	0.166

4 w	Chitosan	Combination	-.0355*	0.01055	0.002
		TCP	.0443*	0.01055	0
		Combination	.0238*	0.01055	0.032
	Tcp	Combination	-.0205-	0.01055	0.062
		Chitosan	-.0870*	0.00719	0
		TCP	-.0350*	0.00719	0
	Cont.	Combination	-.0910*	0.00719	0
		Chitosan	.0520*	0.00719	0
		TCP	-.0040-	0.00719	0.583
	Chitosan	Combination	-.0560*	0.00719	0
		TCP			
		Combination			

Table 5 revealed High significant differences between Combination (CHCT) and Control (CONT) and significant differences between Chitosan (CH) group and Control

group (CONT), similarly shown significant differences between TCP and combination groups at two week duration.

Table 5: Show group differences for bone marrow area between the two and four weeks of healing duration.

	Study Group		Mean Difference (I-J)	Std. Error	P-value
	(I)	(J)			
2 w	Cont.	Chitosan	.0221*	0.00481	0
		TCP	.0138*	0.00481	0.008
		Combination	.0290*	0.00497	0
	Chitosan	TCP	-.0084-	0.00481	0.093
		Combination	0.0068	0.00497	0.18
	TCP	Combination	.0152*	0.00497	0.005
4 w	Cont.	Chitosan	-.6453*	0.23216	0.013
		TCP	-.2375-	0.16955	0.18
		Combination	-.3896*	0.16109	0.028
	Chitosan	TCP	0.4078	0.24226	0.112
		Combination	0.2557	0.23642	0.296
	TCP	Combination	-.1521-	0.17533	0.398

DISCUSSION

After 14 days of bone defect histological outcome showed woven bone deposition where the defect are sited, all groups in experimental study provide good healing characteristic with differences rates among them. Microscopic examination after two weeks after treatment with Chitosan (CH) enhanced osteoblast proliferation through increased number of osteoblast significantly when compared with control group this result agree with [15,16] where their finding higher number of osteoblasts at attachment and elevation secretion of ALP when cultured bone marrow stromal cell into plate contain chitosan when compare to control group due to high deacetylation of chitosan which provide favourable environment to osteoblast attachment and spreading and extracellular matrix protein secretion.

Increase thickness of bone trabeculae and reduction in bone marrow area mostly related to increase osteoblasts differentiation and reduction the osteoclastogenesis through inhibition to osteoclast activity within progressing time from 2 to 4 weeks. This agree with [17,18]. When drilled tibia of rat after 4 week duration show significantly faster bone healing in group treated with chitosan powder when compare to control groups and inhibition to osteoclastogenesis process, moreover show more woven bone and no fibrous tissue in group treated with chitosan.

Histological finding of β -TCP on bone healing revealed increase in osteoblast activity as compared to the control group. This result agrees with [19] augmented tibia of New Zealand rabbits with β -TCP alone who showed β -TCP as optimal materials for inducing significantly provide osteogenesis and reducing osteoclast cell activity.

After 28 days there were observed increase thickness of trabecular and decrease in bone marrow area when compared with control group, this finding proved with [20]. Who filled the femur of rat by β -TCP who showed new bone formation in experimental group higher when compared to control one [21].

Reveals β -TCP encourage regeneration of by enhance mineralization within progression of time into 4 weeks after scarification which explain that by the interconnecting multi pores system which encourage vascular system to growth to produce nutrition and growth factor to the bone formative cell, moreover biodegradation of β -TCP usually coordinate with bone formation process as a result great amount of water content which enable materials to dissolved in body fluid which resorbed by osteoclast cells.

In combination group there was high count of osteoblast cells after 2 weeks duration when compared with control group, also bone trabecular areas were higher in combination than any of the other groups. This agree with [22] who showed scaffold of β -TCP-chitosan that associated with pulverized human bone which provide excellent mechanical properties and induced significantly higher ALP Within time progression when applied β -TCP and chitosan membrane with different proportions to membrane to guide tissues regeneration in critical sized defect at base of skull of rabbits, who show superior bone tissue regeneration when compared to control group. After 4 weeks noticed increase trabecular area thicknesses compare to control group. This agree with [23] who utilize combination of chitosan and tricalcium phosphate associated with polymehymethacrylate bone cement injected into skull cap of rat which resulted superior osteogenic activity and bone regeneration capacity when compare polymehymethacrylate bone cement alone.

CONCLUSION

Bone defect treated with chitosan/ β -TCP alone or as combination scaffold increase the rate of bone regeneration through enhancement of osteoblast proliferation and decrease osteoclast activity and obtain the process of mineralization process of bone matrix superiorly than control group.

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