Original Article

Evaluation the Effect of Local Application of Hydroxyapatite/ Beta Tricalcium Phosphate and Hyaluronic Acid on Bone Healing

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Abstract

Background: Bone regeneration is a complicated, well-organized physiological process of bone creation that is, visible during normal fracture healing and is involved in continual remodeling during an adult's life. **Objectives:** The aim of this study was to evaluate the osteogenic potential of hydroxyapatite/beta tricalcium phosphate (HA/β-TCP) and hyaluronic acid (HY) separately or in combination. **Materials and Methods:** Thirty-two males albino rats, were used in this experimental study. Intra bony defect of about (2.5 mm) in width and (2.5 mm) in depth were created in the distal side of both right and left femurs bones of each rat. Four groups of animals were created at random: The control group was left to heal naturally. Experimental group 1 received HY gel and experimental group 2 received HA/β-TCP materials, experimental group 3 received a mixture of HA/β-TCP and HY in the bone defect. Rats were slaughtered after 2 and 4 weeks of recovery. Histological sections were used for the histological evaluation, and Image J software was used for histomorphometry analysis for assessment of osteoclasts, osteoblasts, osteocytes, trabecular number, trabecular area, and bone marrow space area. **Results:** It demonstrated that bone defect dealt with HA/β-TCP and HY demonstrated earlier mineralization, maturation, and bone formation than the control group. **Conclusion:** Results showed that using HA/β-TCP and HY together appeared to be more effective in osteoconduction than using HA/β-TCP alone.

Keywords: Histomorphometric, hyaluronic acid, hydroxyapatite/beta tricalcium phosphate, osteoconduction

Introduction

Bone defect repair is one of the most common regenerative procedures which may be dealt with using bioactive ceramics such as of hydroxyapatite/beta tricalcium phosphate (HA/β-TCP). Owing to their structural and chemical similarities to the inorganic component of bone, HA/β-TCP has attracted interest for applications in biomedical engineering and hard tissue regeneration.[1,2] A HA/β-TCP was developed as a useful scaffold material that is, more efficient than either pure HA or pure β-TCP alone.[3,4] Biomaterials must be immune-free, biocompatible, and biologically stable in the body. Also, they must be osteoinductive and osteoconductive.[5-8] HA/β-TCP are classified as bioceramic materials with remarkable biological features, with their non-reactivity and osteoconductivity being of particular interest.[9] By mixing HA and β-TCP with proper ratio, biphasic calcium phosphate's benefits, such as its high bioactivity (HA) and quick biodegradation (β-TCP) can be controlled in a bodily fluid.[10] Hyaluronic acid (HY) is a naturally occurring substance that plays a significant role in the extracellular matrix of the human body. It has been employed extensively in bone regeneration in recent years, especially in the fields of dentistry and craniofacial surgery.[11] HY has several benefits, such as increasing cell adhesion, proliferation as well as wound healing, acting as a cell-seeding scaffold or a vehicle for bioactive ingredients.[12] HY is currently

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attracting attention as a potential biomaterial for tissue engineering. A new direction of research focuses on the integration of biomaterials and tissue engineering technology.[13]

Materials and Methods

All experimental procedures were 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. Thirty-two males healthy albino rats, aged between 5 and 6 months weight ranged between 250 and 300 g were used in this experimental study. Intra bony defect of about (2.5 mm) in width and (2.5 mm) in depth were created in the distal side of both right and left femurs bones of each rat.[14,15] Animals were randomly divided into four groups as (control group) left their intra bony defect for normal spontaneous healing, whereas group (HY) (experimental group I), the intra bony defect was filled with 0.1 mL HY gel (Hyadent, BioScience GmbH, Dümmer, Germany). In contrast, group HA/*β*-TCP as experimental group II was treated with 0.5 mg of HA/*β*-TCP (Osteon, Genoss, Korea) and was moistened with 0.1 mL sterile saline[16] and combination group (HA/*β*-TCP + HY) (experimental group III) was treated with 0.5 mg of HA/*β*-TCP and was moistened with 0.1 mL HY gel. After 2 and 4 weeks, all animals were euthanized by anesthetic overdose. Cutting the bone 5 mm away from the surgical site was done to prepare the bone specimen. In 10% buffered formalin all tissue samples were fixed and processed in a routine paraffin blocks after complete bone decalcification. For the standard H&E staining process, serial sections of each paraffin-embedded specimen of about 4 µm thick were put on clean glass slides. Using Image J software, osteoblasts, osteocytes, osteoclasts, trabecular number, trabecular area, and bone marrow space area were histomorphometrically evaluated.

Statistical analysis

SPSS version 25 (Statistical Package for Social Sciences) was used to analyze the data. The statistical analysis used three degrees of significance: Non-significant (NS) $P > 0.05$, Significant (S) $0.05 \geq P > 0.01$ *, Highly significant (HS) $P \leq 0.01$**.

Ethical approval

The Declaration of Helsinki's ethical principles were followed during study performance. Before collecting the sample, the patients' verbal and analytical consent was obtained. There was agreement. The study protocol was reviewed and approved by a local ethics committee according to document number 295 on January 2023 by the College of Dentistry, University of Baghdad.

Results

According to the histological findings, all histological sections for the study groups showed signs of bone repair, although the rates of repair varied within the experiment's time-consuming. When compared with control group [Figures 1 and 2], HY group showed new trabecular bone which demarcated from basal bone by reversal

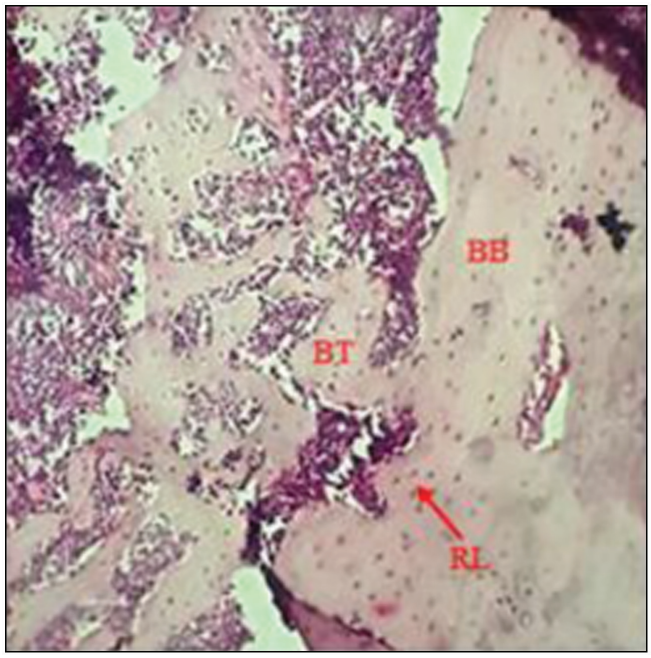

Figure 1: View of control group after 2 weeks shows basal bone (BB), new bone trabeculae (BT) surrounded by (OB). H&EX10

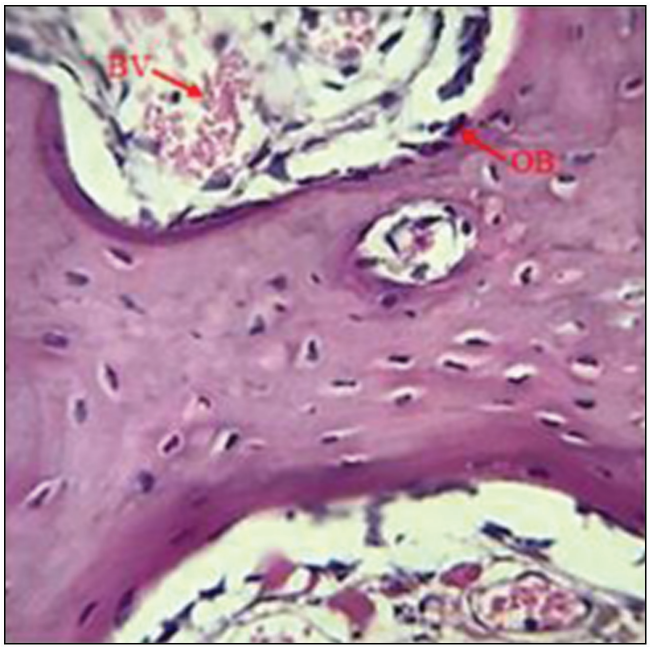

Figure 2: High magnified view of control group after 4 weeks showed (OC) filled the new trabecular bone (TB) and (OB) at periphery. H&E X40

line. These bone trabeculae contain large size osteocytes (OC) with abundant count of osteoblast (OB), whereas within time at 4 weeks' (HY) group showed mature bone trabeculae with regular distribution of osteocyte, and recognizable osteons (OST) were observed [Figures 3 and 4]. Histological section of bone defect treated with HA/*β*-TCP 2 weeks postoperatively showed newly formed bone trabeculae surrounded by bone marrow, and including numerous large osteocytes, osteoblasts seen at peripheries of trabecular bone whereas at 4 weeks indicated maturation of bone by an increase in trabecular thickness were seen with regular organized osteocytes around haversian canal [Figures 5 and 6]. Histological finding of HA/*β*-TCP and HY after 2 weeks' duration

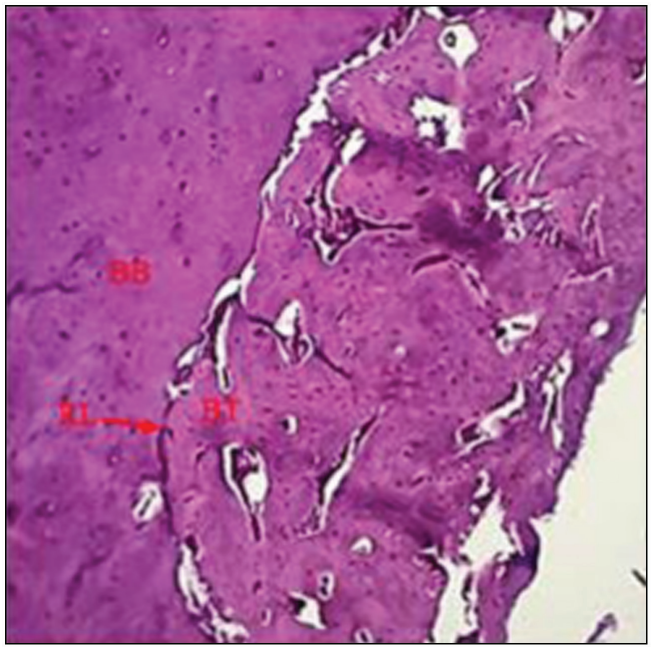

Figure 3: View of 2 weeks' defect area treated with HY shows new bone trabeculae (BT) separated from basal bone(BB) by reversal line.H&E X10

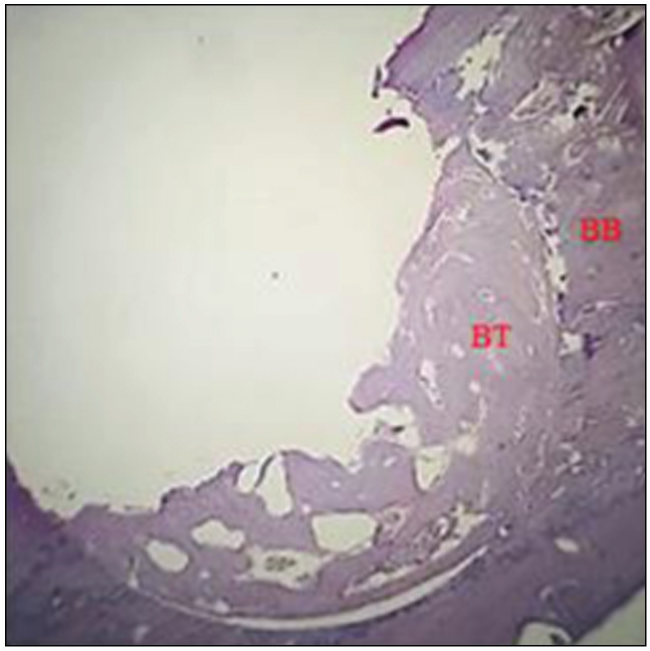

Figure 5: View for the bone defect area treated with HA\β-TCP after 2 weeks, showed new bone trabeculae (BT) separated from basal bone(BB). H&E X4

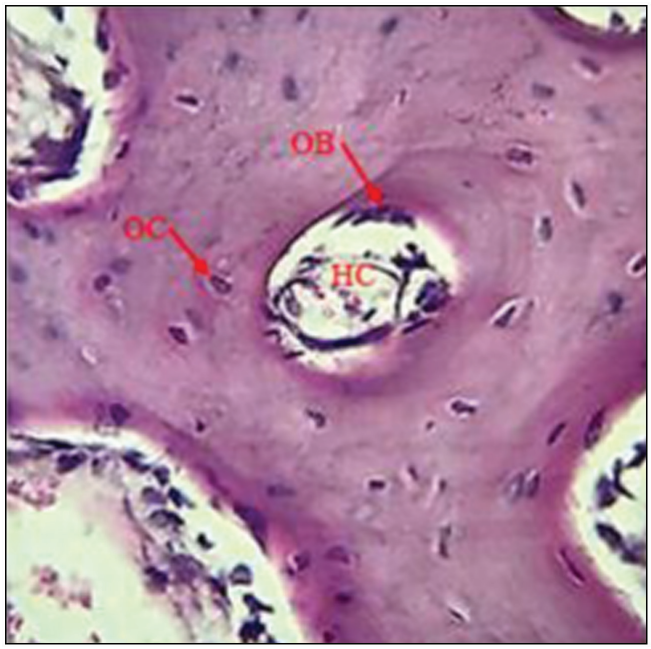

Figure 4: Magnifying view of defect area treated with HY after 4 weeks showed osteon which lined with regularly arranged (OC). (OB) seen lined haversian canal (HC) H&E X40

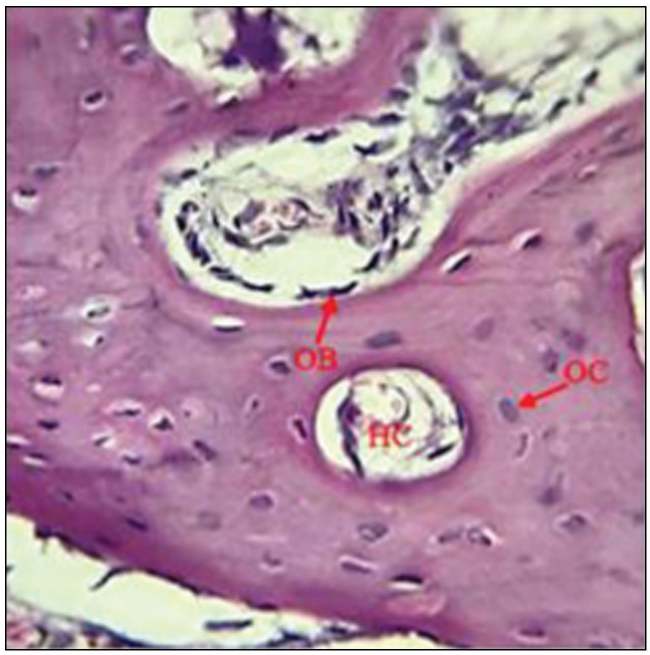

Figure 6: View of 4weeks duration of HA\β-TCP group showed regular arranged (OC) around haversian canal (HC). (OB) seen rimming (HC). H&EX40

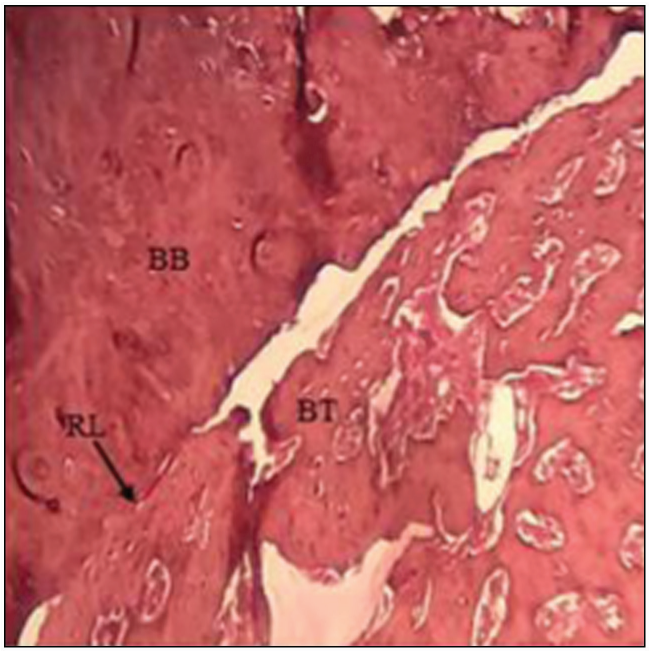

Figure 7: View of defect area treated with HA\β-TCP + HY after 2 weeks showed new bone trabeclea(BT) separated from basal bone(BB) by reversal line H&E X10

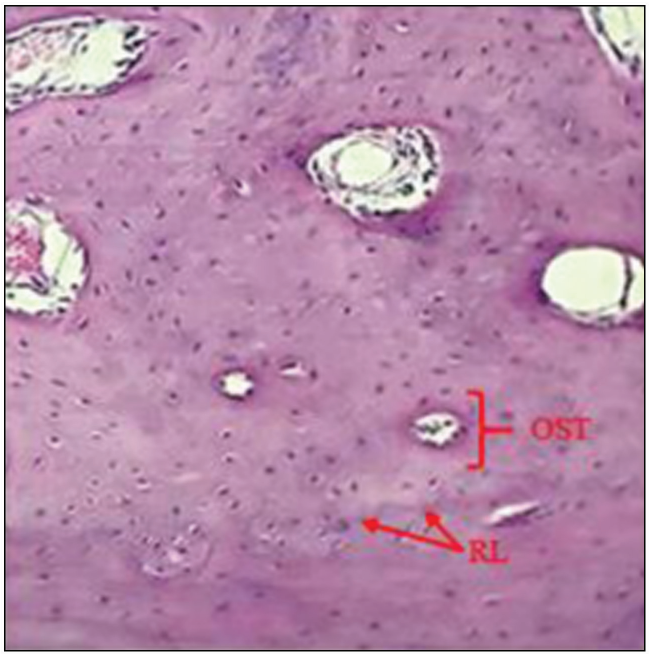

Figure 8: View of (HA\β-TCP + HY) group after 4 weeks showed osteon(OST) which lined with regularly arranged (OC). (OB) seen lined haversian canal, (RL)separating between old and new bone. H&E X20

showed regular bone trabeculae with entrapped by osteocytes and surrounded by osteoblast, after 4 weeks' trabecular area appear more mature and showed osteon (OST) with regular distribution of osteocyte around haversian canal were seen [Figures 7 and 8].

Histomorphometric analysis

According to Tables 1-6 that illustrate a descriptive statistical analysis to bone cells mean number which contains osteoclast, osteoblast, osteocyte, trabecular number, trabecular area and bone marrow area in defected area at 2 and 4 weeks' interval for histomorphometric analysis.

The results given in Table 1 show a highly significant difference in osteocyte among groups in both healing durations. The higher mean values of osteocyte were seen in combination group (HA/*β*-TCP + HY) in both two and four weeks healing durations.

The results given in Table 2 show a highly significant difference in osteoblast among groups in both healing durations. The higher mean values of osteoblast were seen in group HA/*β*-TCP in 2 weeks healing duration, and in combination group at 4 weeks healing durations.

The results given in Table 3 revealed no significant difference in osteoclast among groups in two-study healing duration. The higher mean values of osteoclasts were seen among group HA/*β*-TCP in 2 weeks healing duration and among group control in four weeks healing durations.

Table 4 shows non-significant difference among groups in both healing durations.

Table 5 demonstrates higher mean values of trabecular area were seen among combination group in two and four weeks healing period.

Table 6 shows a significant difference among groups in two weeks healing duration and a highly significant difference among group in four weeks healing period. The higher mean values were seen in group control in both healing durations.

Discussion

With varying rates of healing, all groups in the experimental investigation exhibited good healing characteristics. After two weeks of treatment with HY, the defect area's microscopic inspection revealed more and thicker bone trabeculae than the control one. HY promoted mesenchymal cell differentiation, which improved the production of new bones in the healing of bone wounds. These findings agree with,[17] who investigated the effects of administering a high-molecular HY preparation on bone wound healing following bone marrow ablation in order to study the osteoinductive function of HY.

Also those findings agree with,[18] who found that 2 weeks after tooth extraction in rats, the formation of new bone trabeculae was markedly increased in HY-treated sockets compared with that in control one. Increased osteoblast differentiation and decreased osteoclastogensis are mainly responsible for increased bone trabeculae thickness and decreased bone marrow area. This agrees with[19] who found that by promoting the differentiation of osteoblasts

Table 1: Group comparison difference by ANOVA test for osteocyte in each healing duration

Duration	Study group	OC		*F* value	*P* value
		Mean ±SD	Range		
2 W	Control	23.7±2.1	21-27	8.36	<0.001*HS
	HY	27.5±2.3	24-30		
	HA/β-TCP	26.6±3.2	22-31		
	HA/β-TCP + HY	29.7±1.6	27-32		
4 W	Control	34.1±2.9	30-39	18.6	<0.001* HS
	HY	39.2±2.3	36-43		
	HA/β-TCP	38.7±2.1	35-42		
	HA/β-TCP + HY	43.3±2.3	40-47		

HS: highly significant
*ANOVA test, significant ≤0.05

Table 2: Group comparison difference by ANOVA test for osteoblast in each healing duration

Duration	Study group	OB		*F* value	*P* value
		Mean ± SD	Range		
2 W	Control	32.2±2.6	29-37	31.01	<0.001* HS
	HY	42.5±2.4	39-46		
	HA/β-TCP	44.1±2.8	40-48		
	HA/β-TCP + HY	40.7±2.7	37-45		
4 W	Control	19.7±1.6	17-22	37.8	<0.001* HS
	HY	24.2±1.7	22-27		
	HA/β-TCP	22.5±2.4	19-26		
	HA/β-TCP + HY	31.2±2.9	26-35		

HS: highly significant
*ANOVA test, significant ≤0.05

Table 3: Group comparison difference by ANOVA test for osteoclast in each healing duration

Duration	Study group	OCL		*F* value	*P* value
		Mean ± SD	Range		
2 W	Control	1.5±0.2	1.2-1.9	1.94	0.145* NS
	HY	1.7±0.1	1.5-1.9		
	HA/β-TCP	1.8±0.2	1.5-2.2		
	HA/β-TCP + HY	1.7±0.1	1.5-2		
4 W	Control	0.75±0.2	0.3-1.2	2.81	0.058* NS
	HY	0.5±0.2	0.2-0.9		
	HA/β-TCP	0.61±0.25	0.2-0.9		
	HA/β-TCP + HY	0.41±0.2	0.1-0.7		

and osteogenic mesenchymal tissue, as well as the early apposition of osteoid tissue, HY, an osteoconductive substance, enhances and expedites osseointegration around titanium implants after 2 and 4 weeks.

When compared to the control group, 2 weeks after receiving HA/β-TCP therapy, a microscope inspection indicated new bone production as proved by the existence of numerous new bone trabeculae and an increase in osteoblast numbers. This finding proved with[20] who used HA/β-TCP as a bone graft material in healing of induced jaw bone defects. They found that the amount of new bone formation in HA/β-TCP groups was significant than control group. According to a study achieved by,[21] the implantation of mesenchymal stem cells adherent to HA/β-TCP in the alveolar socket sites of dogs showed signs of a constant rise in bone production there. Comparing the HA/β-TCP treated group to the control group, an increase in trabecular bone thickness and a decrease in bone marrow area were seen after 4 weeks. Also this finding proved with,[22] who discovered that 4 weeks after creating significant defects in the calvarias of rats, HA/β-TCP was able to induce new bone growth with thicker lamellar bone bridging in biphasic calcium phosphate group among the other synthetic biomaterials groups.

Table 4: Group comparison difference by ANOVA test for trabecular number in each healing duration

Duration	Study group	Trabecular No.		*F* value	*P* value
		Mean ± SD	Range		
2 W	Control	8.7 ± 1.4	7.5-9.9	2.1	0.12* NS
	HY	10.2 ± 2.1	8.4-12		
	HA/*β*-TCP	10 ± 2	8.3-11.6		
	HA/*β*-TCP + HY	11 ± 1.6	9.6-12.3		
4 W	Control	6 ± 1.3	4.9-7	1.4	0.25* NS
	HY	5 ± 2	3.3-6.6		
	HA/*β*-TCP	5.2 ± 1.2	4.1-6.3		
	HA/*β*-TCP + HY	4.5 ± 1.1	3.5-5.4		

NS: non-significant
*ANOVA test, significant ≤0.05

Table 5: Group comparison difference by ANOVA test for trabecular area in each healing duration

Duration	Study group	Trabecular area		*F* value	*P* value
		Mean ± SD	Range		
2 W	Control	0.15 ± 0.01	0.13-0.18	14.89	<0.001* HS
	HY	0.18 ± 0.01	0.16-0.2		
	HA/*β*-TCP	0.19 ± 0.02	0.16-0.22		
	HA/*β*-TCP + HY	0.21 ± 0.01	0.18-0.23		
4 W	Control	0.4 ± 0.1	0.2-0.6	11.91	<0.001* HS
	HY	0.6 ± 0.1	0.4-0.8		
	HA/*β*-TCP	0.65 ± 0.1	0.5-0.8		
	HA/*β*-TCP + HY	0.82 ± 0.1	0.6-1.1		

Table 6: Group comparison difference by ANOVA test for bone marrow area in each healing duration

Duration	Study group	Bone marrow area		*F* value	*P* value
		Mean ± SD	Range		
2 W	Control	0.81 ± 0.1	0.6-1.1	3.24	0.037* S
	HY	0.65 ± 0.1	0.4-0.9		
	HA/*β*-TCP	0.63 ± 0.1	0.4-0.8		
	HA/*β*-TCP + HY	0.58 ± 0.1	0.4-0.8		
4 W	Control	0.34 ± 0.02	0.3-0.38	29.2	<0.001* HS
	HY	0.29 ± 0.02	0.25-0.34		
	HA/*β*-TCP	0.28 ± 0.02	0.24-0.32		
	HA/*β*-TCP + HY	0.22 ± 0.02	0.19-0.26		

In combination group, mature bone trabeculae encircling the Haversian canal with regularly distributed osteocyte (OC) were noticed in the present study. After 2 weeks, there was a higher number of osteoblast cells than in the control group, and there was more trabecular bone in combination group than in any of the other groups. Those findings were in agreement with[23] who discovered that at 2 weeks, HY + HA/*β*-TCP samples had a greater ratio of new bone development than the control group. The incorporation of HY to bone grafts improved handling characteristics in clinical settings in addition to promoting osteoconduction. At the end of postoperative week 4, detected increased trabecular area thicknesses compared to the control group. The present results were also agreeing with[24] who found higher new bone formation within HA/*β*-TCP+HY critical-size calvarial defects of rats compared with the control defects after 4 weeks, indicating that HY enhanced bone formation.

Also this finding proved with[25] who evaluated the benefit of HA/*β*-TCP with HY for alveolar socket preservation by a histomorphometric investigation. He found that comparing samples of HA/*β*-TCP with and without HY revealed that the HY is more effective in promoting osteoconduction, suggesting that the latter may be a promising method for preserving alveolar sockets. Also This agree with[26] who suggested that a novel composite mix

of HY and HA/*β*-TCP can enhance the osteoconductive qualities of bone grafts and act as a supporting structure to increase the efficiency of bone grafts and promote the creation of new bone in the lateral femoral condyle defect of rabbits.

Conclusion

In conclusion, the combined use of HY and HA/*β*-TCP for bone regeneration with in a little amount of HA/*β*-TCP can act as a potential treatment alternative for accelerate bone healing than using these biomaterials alone. In addition, this novel material makes handling during surgery more effective. Overall, it shows a lot of potential for use in guiding bone regeneration and encouraging the growth of new bone for the treatment of bone defects.

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Conflicts of interest

There are no conflicts of interest.

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