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Hussein B.J.✉, Ghani B.A.
University of Baghdad, Baghdad, Iraq

Effect of Local Application of Collagen and Beta-Tricalcium Phosphate on Bone Healing

Conflict of interest: nothing to declare.
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Contacts: medicalresearch68@yahoo.com

Abstract

Introduction. The healing of bone tissue, whether a fracture or fusion, is influenced by a number of biochemical, biomechanical, cellular, hormonal and other pathogenetic mechanisms associated with the regulation of phosphorus-calcium metabolism. β-Tricalcium phosphate (β-TCP) is a bioceramic material that represents a good balance among absorption, degradation and new bone formation and can preserve structural stability by releasing a large quantity of calcium (Ca^{2+}) and sulfate (SO_4^{2-}) ions, which are indispensable inorganic salts for new bone formation. Collagen (COL) is the major component in numerous tissues and can be extracted as a natural biomaterial for various medical and biological purposes.
Purpose. To evaluate the individual and combined effects of β-TCP and COL sponges on bone healing.
Materials and methods. A total of 20 adult male albino rats with average weights of 250–350 g (3–4 months) were used in this study. Four intrabony holes were induced in both femurs of each rat and filled with β-TCP, COL, and their combination in the experimental groups. The fourth group of holes, which were left to heal spontaneously, was used as the control. Sacrification of animals was performed after 2- and 4-week healing periods to prepare specimens for histological and histomorphometric analyses.
Results. Histological findings showed that the combined application of β-TCP and COL resulted in more bone formation and mineralization compared with the other groups. Highly significant differences regarding bone cells and bone marrow area were recorded among all groups for during both healing periods.
Conclusion. The combined application of β-TCP and COL was more effective in enhancing bone formation and maturation compared with the controls.
Keywords: beta-tricalcium phosphate, collagen, bone healing, mineralization, osteogenic cells, intrabony holes

Хусейн Б.Дж.✉, Гхани Б.А.
Университет Багдада, Багдад, Ирак

Влияние местного применения коллагена и бета-трикальцийфосфата на заживление повреждений костной ткани

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Этическое заявление. Данная работа одобрена Стоматологическим колледжем Университета Багдада. Эксперимент проводился в соответствии с этическими принципами обращения с лабораторными животными Стоматологического колледжа Университета Багдада (№ 429721).
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Контакты: medicalresearch68@yahoo.com

Резюме

Введение. На заживляемость костной ткани, будь то перелом или сращение, влияет целый ряд биохимических, биомеханических, клеточных, гормональных и других патогенетических механизмов, связанных с регуляцией фосфорно-кальциевого обмена. β-трикальцийфосфат (β-TCP) – это биокерамический материал, обеспечивающий оптимальный баланс между абсорбцией, разрушением и образованием новой костной ткани, который способен сохранять структурную стабильность за счет высвобождения большого количества ионов кальция (Ca^{2+}) и сульфата (SO_4^{2-}), представляющих собой неорганические соли, необходимые для формирования новой костной ткани. Коллаген (COL) является основным компонентом многих тканей и может быть использован в качестве природного биоматериала для различных медицинских и биологических целей.
Цель. Оценка влияния β-TCP и коллагеновых губок на заживление кости при отдельном и комбинированном применении.
Материалы и методы. В исследовании использовали 20 взрослых крыс-самцов – альбиносов со средним весом 250–350 г (3–4 месяца). В обеих бедренных костях каждой крысы было проделано по четыре внутрикостных отверстия, которые заполнили β-TCP, COL и их комбинацией (экспериментальные группы). В четвертой группе отверстия были оставлены для спонтанного заживления (контрольная группа). По истечении 2- и 4-недельных периодов восстановления животных умерщвляли и отбирали образцы для гистологического и гистоморфометрического анализа.
Результаты. Выполненное гистологическое исследование позволило установить, что комбинированное применение β-TCP и коллагена способствует более активному формированию и минерализации костной ткани по сравнению с результатами, полученными в других группах. Выраженные достоверно значимые различия в количестве костных клеток и площади костного мозга были зафиксированы во всех группах в течение обоих периодов восстановления.

Заключение. Комбинированное применение β-TCP и коллагена способствует более эффективному формированию и развитию костной ткани по сравнению с контрольной группой.
Ключевые слова: бета-трикальцийфосфат, коллаген, заживление костной ткани, минерализация, остеогенные клетки, внутрикостные отверстия

■ INTRODUCTION

Bone is a dynamic biological tissue mainly composed of three components: cells, fibers, and matrix. Collagen (COL), which provides tensile strength, is the main component of the bone matrix. Calcium phosphate, which provides compressive strength, is the main mineral component of bone [1]. In the treatment of bone defects, scaffolds play an important role and can provide a bridge for new bone tissue growth into the gap and a platform for cells and growth factors to exert their physiological roles [2].

β-Tricalcium phosphate (β-TCP) is a calcium salt of phosphoric acid with the chemical formula $Ca_3(PO_4)_2$. This compound is also known as tribasic calcium phosphate; calcium phosphate is one of the main combustion products of bones. Calcium phosphate is also commonly derived from inorganic sources such as mineral rock [3]. β-TCP is a promising biomaterial for bone repair due to its osteoconductivity, osteoinductivity, biodegradation, and delivery of large amounts of calcium and phosphate ions into the defect environment. β-TCP is favored by maxillofacial and orthopedic surgeons in the restoration of bone tissue loss [4].

β-TCP is reabsorbed in a progressive manner, which leaves an area for bone neoformation. Multiple studies have been conducted on β-TCP and bone interaction, and histological examination showed initial bone neoformation in intergranular areas and porous surfaces, which guided new bone formation. Unlike hydroxyapatite, β-TCP is continuously and totally reabsorbed, which results in the discharge of calcium and phosphate ions that actively participate in new osseous tissue formation [5].

The critical geometry of β-TCP, in addition to its macro- and microporous structures, can result in the successful trapping of ions, osteogenic cells, and osteogenic proteins, which provides a suitable microenvironment for osteoinduction. In particular, the provision of calcium and phosphate ions by the dissolution of soluble β-TCP can contribute to a favorable environment for bone formation [6].

β-TCP is employed in the medical and dental fields and numerous dental operations, including pulp capping, apexification in endodontics, restoration of cleft palates and orbital rim deformities in maxillofacial surgery, and repair of osseous lesions in periodontics [7].

COL is a biological macromolecule and a key structural protein, the principal component of connective tissues in animals, and the most widely distributed functional extracellular matrix protein [8]. COL has been used for decades as scaffolding material in bone tissue engineering approaches where autografts are not feasible. Polymeric COL can be easily processed in a number of ways to manufacture biomaterials in the form of sponges, particles, or hydrogels for different applications [9]. When used as a scaffolding material, COL not only acts as a physical support for cells to attach to and grow on but also influences cell behavior; type I COL-based biomaterials are suitable and biocompatible in promoting successful bone healing when implanted into bone defects [10].

COL-based biomaterials (i) are biocompatible, (ii) have a tunable degradation rate, (iii) elicit a mild inflammatory response, (iv) are invaded by the host cells, and (v) can become vascularized. These features have made COL progressively popular for the enhanced healing processes [11]. In addition, COL stimulates angiogenesis in vitro and in vivo. Different strategies have been tested to increase the vascularization of COL-based biomaterials and cell proliferation and enhance osteogenic differentiation [12].

COL has been widely used in clinical settings for a number of years and exhibits good biodegradability, biocompatibility, and absorbability [13]; it is close to the natural organic structure of bone and exerts positive effects on cell fate. COL can also facilitate cell attachment and can be easily absorbed by organisms. Moreover, COL can inhibit the growth of bacterial pathogens [14].

A COL sponge is a biocompatible material that fills the extraction socket and acts as a scaffold to prevent the collapse of soft tissue after extraction. In conclusion, it can reduce the patient's postoperative complications at an early stage, enhance the initial healing of soft tissue, and minimize periodontal defects [15].

■ PURPOSE OF THE STUDY

To evaluate the individual and combined effects of β-TCP and COL sponges on bone healing.

■ MATERIALS AND METHODS

Study design and setting

Twenty adult male albino rats weighing approximately 250–350 g and aged 3–4 months were used in this study. The rats received intramuscular injection of 50 mg ketamine hydrochloride (1 mL/kg body weight) plus 2% xylazin (0.2 ml/kg body weight). Surgery was performed under sterile conditions by making an incision on the skin and underlying fascia. Then, reflection was performed to expose the rat femurs. Induction of four intrabony holes approximately 3 mm in depth and 2 mm in width was conducted with intermittent drilling and continuous cooling with normal saline using a micro engine that was set at a rotary speed of 2500 rpm. The operation sites were washed with normal saline to remove debris. Group A (20 holes), as the control group, was left untreated for spontaneous healing. The intrabony holes in Groups B (20 holes), C (20 holes), and D (20 holes) were filled with COL, β-TCP material, and combined COL and β-TCP materials, respectively.

Animals

All animals were scarified after 2 and 4 weeks of healing, and bone specimens were immediately fixed in 10% freshly prepared formalin and left for 2 days for fixation. Bone decalcification was performed using a formic acid sodium citrate solution, which was freshly prepared from two solutions (125 cc formic acid (90%) and 125 cc distilled water; 50 mg sodium citrate and 250 cc distilled water) [16] and the specimens were processed into paraffin blocks. Tissue sections of 5 µm thickness were prepared followed by hematoxylin and eosin staining and histological examination using a light microscope (OpticaB-350, Italy).

Histomorphometric analysis of the following bone microarchitectures was performed. Bone cell counting involved the following:

1. Osteoblast (OB) cell number (OB/mm^2).
2. Osteocyte cell number (OC/mm^2).
3. Osteoclast cell number (OCL/mm^2).

Trabecular and bone marrow areas were also measured:

1. Trabecular area (mm^2).
2. Bone marrow area (mm^2).
3. Trabecular number (mm^2).

Statistics

Measurement of trabecular and bone marrow area was performed using a program software (ImageJ.exe). The recorded data were analyzed statistically by analysis of variance and least significant difference test.

■ RESULTS

Histological findings

After 2 weeks of healing, the microphotography of bone sections from Group A (control group) showed new trabeculae rimmed by OBs, osteocytes embedded in bone, and osteoclasts (Figure 1). Group B (COL group) exhibited deposition of new bone trabeculae enclosing the marrow tissue rimmed by OBs (Figure 2). Group C (β-TCP group) revealed osteocytes embedded in newly formed bone and osteoclasts (Figure 3). Group D (COL + β-TCP group) presented new bone trabeculae rimmed by OBs and osteocytes embedded in the bone (Figure 4).

Four-week duration of bone healing

Histological examination of bone sections from Group A (control group) showed dense bone and Haversian canals surrounded by osteocytes (Figure 5). Group B (COL group) exhibited newly formed bone that was more densely rimmed by OBs and osteocytes in (Figure 6). The microphotograph view of Group C (β-TCP group) after 4 weeks of healing

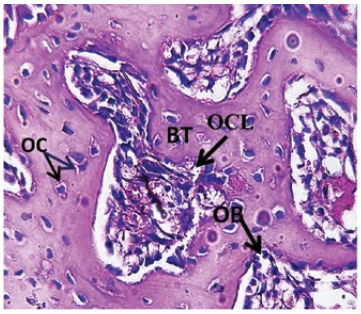

Fig. 1. After 2 weeks of healing, the control group showed new bone trabeculae (BT), osteocytes (OCs) osteoblasts (OB), and osteoclasts (OCL). H&EX 40

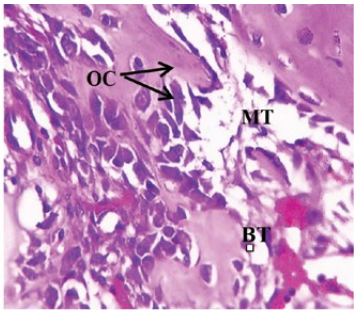

Fig. 2. After 2 weeks of healing the collagen group showed thin bone trabeculae (BT), marrow tissue (MT), and osteocytes (OC). H&EX40

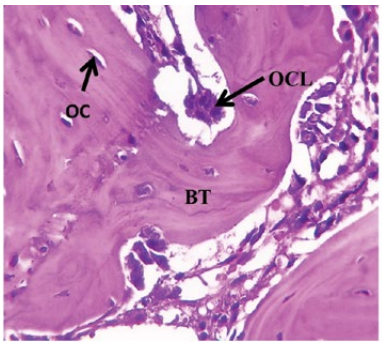

Fig. 3. After 2 weeks of healing, the β-TCP group showed bone trabeculae (BT), osteocytes (OC), and osteoclasts (OCL). ×40

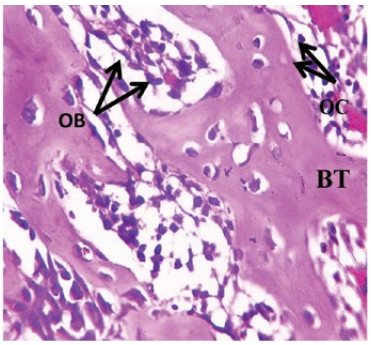

Fig. 4. After 2 weeks of healing, the β-TCP + collagen group showed new bone trabeculae (BT), osteoblasts (OB), and osteocytes (OC). ×40

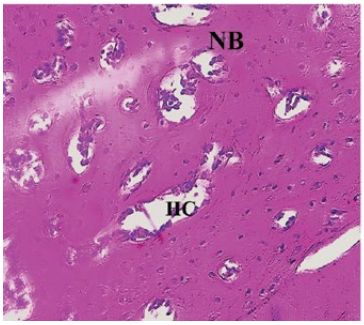

Fig. 5. The control group showed new bone (NB) and Haversian canal (HC) after 4 weeks of healing. H&E ×20

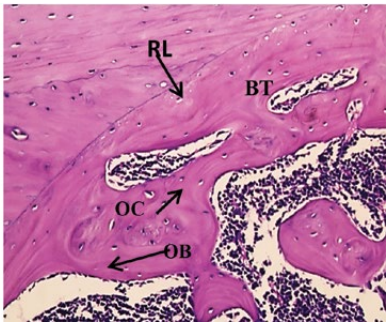

Fig. 6. After 4 weeks of healing, the collagen group revealed dense bone trabeculae (BT), osteoblast (OB), osteocytes (OC), and reversal line (RL). H&E ×20

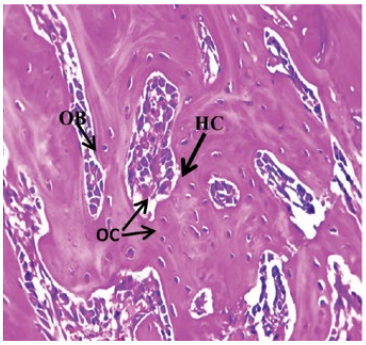

Fig. 7. After 4 weeks of healing, the TCP group exhibited thick bone trabeculae (BT) with regularly arranged osteocytes (OC) and osteoblasts (OB). H&E ×20

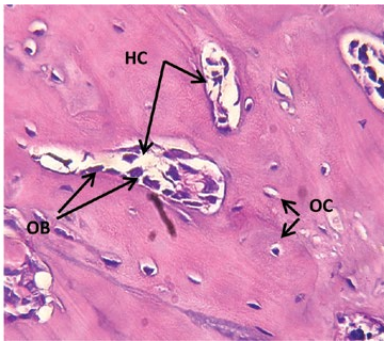

Fig. 8. After 4 weeks of healing, the combined treatment group showed Haversian canal (HC), osteocytes (OC), and osteoblast (OB) cells. H&E ×40

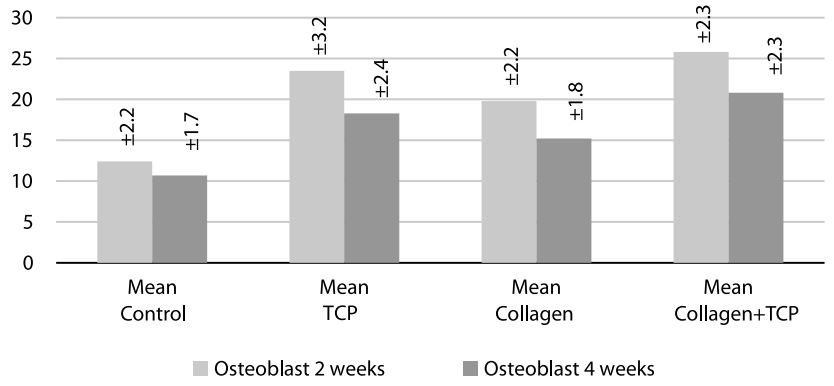

Fig. 9. Bar chart representation of the mean and standard deviation of bone cells after 2- and 4-week healing intervals for OBs in the control, COL, β-TCP, and COL + β-TCP groups

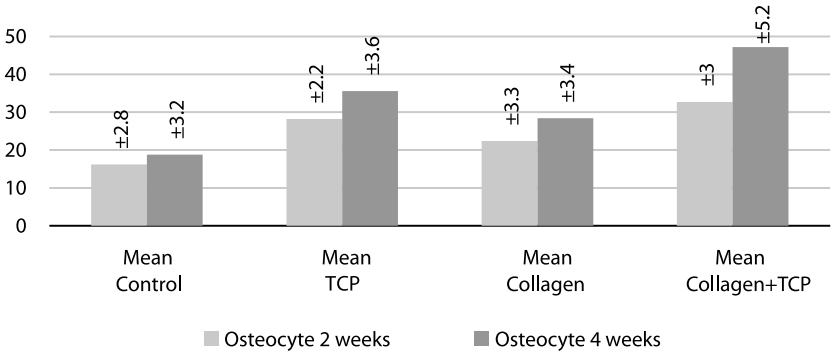

Fig. 10. Bar chart representation of the mean and standard deviation of bone cells after 2- and 4-week healing intervals for osteocytes in the control, COL, β-TCP, and COL + β-TCP groups

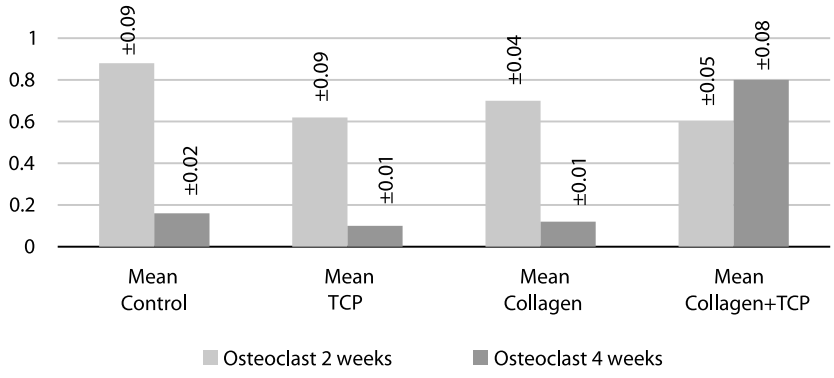

Fig. 11. Bar chart representation of the mean and standard deviation of bone cells after 2- and 4-week healing intervals for osteoclasts in the control, COL, β-TCP, and COL + β-TCP groups

revealed dense bone trabeculae with Haversian canals and osteocytes (Figure 7). After 4 weeks of healing, bone sections in Group D (COL + β-TCP group) presented dense bones with Haversian canals lined by OBs and surrounded by osteocytes (Figure 8).

Table 1
Comparison of bone cells in different healing durations in all studied groups

Variable	Duration	Control		COL		β-TCP		COL + β-TCP	
		T test	P value	T test	P value	T test	P value	T test	P value
OB	Between 2 and 4 weeks	1.734	0.110	4.987	0.010*	5.855	0.000**	5.414	0.000**
Osteocyte	Between 2 and 4 weeks	–3.129	0.042*	–6.326	0.000**	–7.897	0.000**	–15.788	0.000**
Osteoclast	Between 2 and 4 weeks	35.477	0.000**	30.911	0.000**	25.023	0.000**	–19.383	0.000**

Notes: * significant result; ** highly significant result.

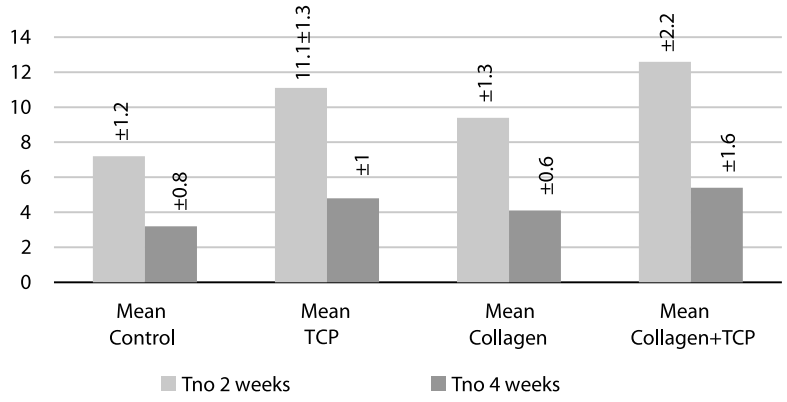

Fig. 12. Barchart representation of the mean and standard deviation of TN in bone cells after 2- and 4-week healing intervals

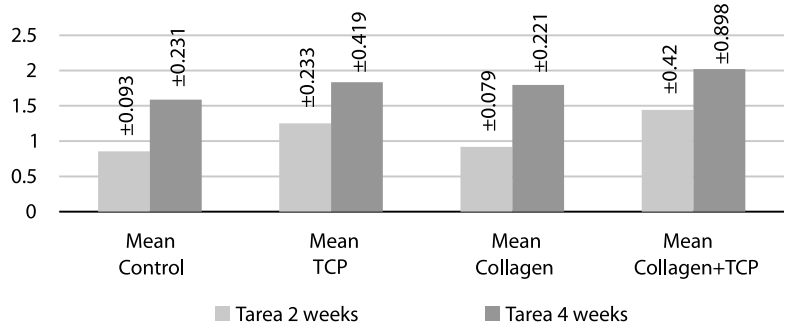

Fig. 13. Bar chart representation of the mean and standard deviation of bone cells in the T area after 2- and 4-week healing intervals

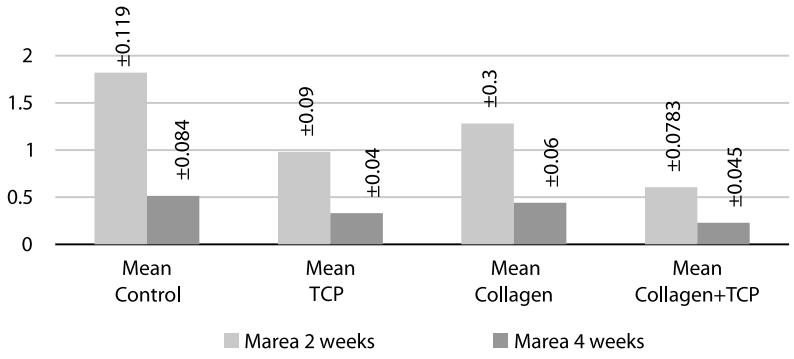

Fig. 14. Bar chart representation of the mean and standard deviation of BMA in bone cells after 2- and 4-week healing intervals

Table 2
Healing duration comparison for TN, TA, and BMA in all studied groups

Variable	Duration	Control		β-TCP		COL		COL + β-TCP	
		T test	P value	T test	P value	T test	P value	T test	P value
TN	Between 2 and 4 weeks	4.112	0.019*	6.275	0.000**	5.798	0.000**	7.109	0.000**
TA	Between 2 and 4 weeks	–18.363	0.000**	–31.119	0.000**	–38.313	0.000*	–27.707	0.000**
BMA	Between 2 and 4 weeks	30.118	0.000*	28.997	0.000**	40.947	0.000**	33.309	0.000**

Notes: * significant result; ** highly significant result.

Histomorphometric analysis of bone architecture parameters

Comparison of bone cells in all studied groups using paired T test showed a highly significant difference (P<0.01) between 2 and 4 weeks of healing periods (Figures 9, 10, and 11 and Table 1).

With the use of paired T test, comparison of TN, TA, and BMA in all studied groups showed highly significant results (P<0.01) between 2 and 4 weeks of healing, except for TN in the control group (Figures 12, 13, and 14 and Table 2).

■ DISCUSSION

The bone healing process is indicated by the deposition of bone matrix and the creation of trabecular bone, which decreases in number but grows in thicknessthroughout the healing process. The amount of newly formed bone in osseous defects is one of the most critical elements in determining the degree of bone regeneratio [17]. These reported findings are in line with the results of this study, as illustrated by histological examination of bone sections, which showed newly formed trabeculae rimmed by OBs that decreased in number as they thickened over time. This finding was more evident in the experimental groups, specifically the combined treatment group.

Rico et al. (2021) [18] used COL powder in a rabbit alveolar cleft model and observed considerably improved bone repair. COL acted as a scaffold on which cells were retained and induced to differentiate, which increased osteogenesis. In line with this study, the COL group mostly showed increased OB differentiation and reduced osteoclastogenesis through the inhibition of osteoclast activity from 2 weeks to 4 weeks. This finding is supported by that of Dawood and AL-Ghaban (2021) [19], who demonstrated that bone defects treated with marina COL exhibited new bone formation in the form of numerous bone trabeculae, which suggests the role of COL in providing an osteoconductive environment for the migration, proliferation, and differentiation of bone-forming cells. The effect of β-TCP application on bone healing revealed an increased number of OBs, which agrees with the finding of Gilev et al. (2020) [20], who augmented the tibia of New Zealand rabbits with β-TCP alone and revealed β-TCP as an optimal material for inducing OB differentiation, mineralization of the extracellular matrix, and subsequent bone formation.

Moreover, the recorded mean values of TN and TA were higher in the β-TCP groups than in the control group, which is in agreement with the result of Lu et al. (2021) [21], who implanted β-TCP in femur defects in rabbits and observed that β-TCP affected bone repair by interacting with the surrounding tissues through the regulation of growth factors, cytokines, and ions. A possible mechanism of β-TCP-regulated osteogenesis is that β-TCP can directly or indirectly (via the release of ionic components, such as Ca^{2+} and PO_4^{3-}) facilitate OB differentiation, promote vascularization, regulate the release of growth factors, and alter blood clot formation, which promote bone healing at defect sites.

The combination group showed a high count of OB cells after 2 weeks of healing compared with the control group and more bone trabecular areas than in any of the other groups. This result agrees with that of Tebyanian et al. (2019) [22], who showed that the introduction of β-TCP powder into the COL matrix improved vascularization and the biological and mechanical properties of the COL scaffold and evaluated the effects of a complex COL/β-TCP scaffold on bone formation in rabbit calvarial bone defects to assess the potential use of COL/β-TCP as a bone substitute to repair bone defects.

In this study, the trabecular area thicknesses increased compared with that of the control group after 4 weeks, which is in agreement with the finding of Kim et al. (2017) [23], who concluded that the combined use of COL and β-TCP in the long bone fracture led to improved bone regeneration. Xiao (2015) [24] stated that the composition of the bone scaffold must be kept similar to that of natural bone to provide a desirable environment for cell attachment and proliferation. COL and β-TCP can mimic the basic composition of bone and have been used as bone graft substitutes. The results of this study are also consistent with those of Mohseni et al. (2018) [25], who showed the positive effect of nanocomposite β-TCP/COL scaffold in comparison with hydroxyapatite scaffold in the healing of femoral defects in rabbits; they also observed the improved quantity and velocity of bone formation and quantity of newly formed lamellae in the healing site of the nanocomposite TCP/COL group.

The results of the current study are also supported by those of Ho et al. (2016) [26], who combined β-TCP-COL into the tooth socket of beagle dogs and observed that the horizontal dimensions of alveolar bone were more evident in the β-TCP COL groups and accompanied by new bone formation at 4 weeks compared with those in the COL and control groups. This finding was obtained possibly because COL/β-TCP simultaneously

acted as osteoinductive carrier and osteoconductive bone grafting material to accelerate bone regeneration.

■ CONCLUSION

Bone defects treated with COL/β-TCP as a combination scaffold increased the rate of bone regeneration by enhancing OB proliferation and decreasing osteoclast activity and mineralization of the bone matrix was superior to that of the control group.

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