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The Role of Synthetic Calcium Carbonate and Bone Marrow Combination in Healing Process of Critical Size of Redial Bone in Rabbits

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Abstract

THIS study aimed to assess the regenerative potential of synthetic calcium carbonate ($CaCO_3$) combined with bone marrow (BM) for treating extensive bone defects in a rabbit model. Critical-sized radial defects were surgically created and treated either with $CaCO_3$ alone or with $CaCO_3$ enriched with autologous BM harvested from the iliac crest. Postoperative monitoring included clinical observations, radiological assessments, and histological analyses to compare healing outcomes between the groups. The results demonstrated that adding BM enhanced the repair process, indicated by faster and more uniform new bone formation. Radiographic scoring showed greater bone regeneration in the $CaCO_3$ + BM group compared to $CaCO_3$ alone, although this difference was not statistically significant ($p > 0.05$). Histological sections confirmed improved osteogenesis, characterized by abundant osteoblast activity, increased vascular infiltration, and more advanced integration of the biomaterials with host tissue. In contrast, defects treated with $CaCO_3$ alone showed slower and incomplete repair, with some areas exhibiting persistent fibrous tissue. These findings support the notion that BM supplies essential osteoprogenitor cells and growth factors that work synergistically with the osteoconductive properties of $CaCO_3$, thereby accelerating bone regeneration. Overall, combining $CaCO_3$ with BM presents a promising and effective strategy that could enhance clinical outcomes in both veterinary and human bone reconstructive surgery.

Keywords: Bone graft, Bone marrow, Calcium carbonate, Rabbit, Radial defect.

Introduction

Several cells, growth hormones, and extracellular matrix constituents work in concert during the intricate and dynamic process of bone repair. Clinical scenarios, particularly those involving significant bone deficits, such as tumor resection or severe injury, often compromise bone regeneration. In these cases, a more advanced approach to regeneration is necessary to enhance bone repair and augment its mechanical qualities [1]. Bone grafts can accelerate the healing process, while normal bone healing follows a defined sequence that begins with hematoma, inflammation, soft callus formation, complex callus development, and remodeling. These materials must provide biocompatibility and deliver bioactive signals to facilitate osteogenesis, angiogenesis, and tissue regeneration [2].

Calcium carbonate, in the forms of calcite or aragonite, is recognized for its biocompatibility, which enhances interactions with osteoblasts and facilitates its integration with bone [3, 4]. The high porosity, surface area-to-volume ratio, non-toxicity, and biocompatibility with bodily fluids, along with the ability for surface modification, suggest that micro- to nano-sized $CaCO_3$ particles have considerable potential for use in medicine, catalysis, environmental science, food processing, and material reinforcement [5].

Calcitic calcium carbonate ($CaCO_3$) has emerged as a viable option due to its biocompatibility, biodegradability, and osteoconductive properties [6].

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Numerous *in vitro* studies have demonstrated that calcium carbonate-based scaffolds promote osteoblast adhesion and proliferation while maintaining a favorable inflammatory profile [6, 7]. Additionally, incorporating calcium carbonate into composite materials such as collagen, polymers, and cements has shown improvements in osteoconductivity and cellular support [6, 8, 9]. Various polymorphic forms, including aragonite and vaterite, have been explored for their enhanced solubility and increased bioactivity, while calcite offers a balance between structural stability and controlled biodegradation [10, 6].

Bone marrow plays a crucial role in bone healing by providing a rich source of mesenchymal stem cells (MSCs), hematopoietic cells, and bioactive growth factors that work together to support bone regeneration. BM-derived MSCs can differentiate into osteoblasts, which directly contribute to the formation of new bone [11]. Recent evidence highlights that the dynamic interaction among BM-MSCs, immune cells, and signaling molecules within the bone marrow niche is essential for effective bone repair [11, 12]. This immune–stromal crosstalk regulates inflammation, encourages angiogenesis, and coordinates osteogenesis [12]. Furthermore, integrating bone marrow cells with biomaterial scaffolds and advanced structures enhances cell localization, osteogenic differentiation, and vascularization, presenting a promising therapeutic strategy for bone repair [11, 12].

This study investigated the natural process of bone healing in rabbits using synthetic Calcium Carbonate (Sigma Aldrich) and its association with autologous bone marrow. To evaluate their effectiveness as bone graft materials, radiographic and histological assessments were conducted. the examined parameters included callus formation, fracture line assessment, bone remodeling, union, spongiosa, cortex, and bone marrow, followed by statistical analysis. The results may provide a comprehensive analysis of the efficacy of Calcium Carbonate and Calcium Carbonate combined with bone marrow as bone graft materials, and compare these findings with advancements in orthopedic surgery and regenerative medicine.

Material and Methods

Thirty clinically healthy male New Zealand rabbits, weighing 2.4 ± 0.17 kg and aged four months, were subjected to experimental surgery. Prior to surgery, these animals underwent a 15-day acclimatization period in a controlled environment with regulated light and temperature. During this period, they were progressively accustomed to being handled. We provided the rabbits with commercial rabbit pellets and unrestricted access to water while keeping them in solitary confinement. The Ethical Committee of the Institute of Veterinary and Agricultural Sciences of the University of Batna 1, Algeria, sanctioned all drilling and handling protocols.

Experimental surgery

Two groups of rabbits were randomly selected for this study. Rabbits of the first group received a calcium carbonate (CC) powder implant, and were considered as positive control, while the animals of the second were treated with a combination of CC powder and autologous bone marrow (BM) as bone grafts. All groups experienced a 10 mm segmental defect on the mid-shaft of the radius. Bone healing process was assessed through radiographic and histological evaluations on postoperative days 30, 60, and 90; the rabbits were euthanized in each group.

Anesthesia and Animal Preparation

We fasted the animals for two hours before putting them under standard conditions for a surgical procedure. They were initially sedated via intramuscular injection of xylazine (Xyla®, 1 mg/kg). The right forelimb and hip area were shaved and disinfected with an iodized polyvidone solution (Dermadine®). We initiated general anesthesia by administering ketamine (Imalgene®, 40 mg/kg) intramuscularly.

Surgical procedure

Animals were placed in right lateral recumbency, and surgical drapes were employed. We performed a 3–4 cm incision on the medial face of the right forelimb, approximately midway between the elbow and carpal joints. We used fine, dull scissors to sever the radial muscles delicately.

Surgical operation was performed, an osteotomy on the mid-shaft of the radius using a suitable electric surgical saw. The identical procedure was repeated at 10 mm from the original osteotomy. Once the bone fragment detached from its muscular attachment, we excised it to create a bone defect. Members of the initial group were administered 500 mg of synthetic calcium carbonate (CC), while the subsequent group received both CC and bone marrow (BM). In the groups receiving bone marrow, 500 mg of synthetic CC was first implanted into the radial bone defect. The muscle layer was then sutured to secure the material in place. Bone marrow was subsequently aspirated from the iliac crest using an aseptic technique. After shaving and disinfecting the area, an 18G needle was inserted into the iliac crest with slight rotational pressure. A 20cc syringe was then attached, and negative pressure was applied while gently manipulating the needle until the desired volume was obtained. A total of 0.3 ml of freshly aspirated bone marrow was then injected directly into the implanted defect through the muscle using a fine sterile needle to ensure in situ cellular enrichment [13]. The muscles were stitched together with a continuous 4/0 polyglactin 910 suture, and the skin

was closed with separate 4/0 polyamide stitches. The rabbits receive daily care after surgery to monitor their overall health and check for signs of infection or swelling at the surgical site (Fig. 1).

Radiographic Evaluation

Following the surgery, we euthanized five (5) animals from each group at intervals of 30, 60, and 90 days. The right arm was sectioned off at the shoulder joint, and two X-ray images (side view and front view) were taken using a STATIX radiography system (Italray, Italy) equipped with a 35T081 X50 AH tube, a digital X-ray machine at 55 kV and 3.2 mAs. The radiographs were assessed for indications of new bone development, the degree and size of callus, gap bridges, and signs of bone remodeling. Radiographic observations were evaluated using a modified Lane and Sandhu scoring system [14] (Table 1). The average score for each parameter was computed, and the total of the scores served as the cumulative radiographic score for each group. The group with the highest scores was considered to possess exceptional healing capabilities.

Histological Evaluation

The bone was fixed with 10% phosphate-buffered formalin for three days and then decalcified with 10% EDTA for one month. We passed the samples to an ethanol series for dehydration, embedded them in paraffin, and prepared sections measuring six μm in thickness. The sections were stained using hematoxylin and eosin (H&E). The histological observations were assessed individually using an optical microscope and graded based on the modified Lane and Sandhu grading system [15], as presented in (Table 2). The mean scores of the several histopathological parameters were calculated, and the total histopathological score was derived by summing the mean scores. The group exhibiting the most outstanding cumulative histopathological score was considered the optimal for bone consolidation.

Statistical Analysis

An independent Student's t-test was performed using IBM SPSS Statistics for Windows, version 26, to compare the radiographic and histological scores between the two experimental groups (n = 5 per group). A p-value of less than 0.05 was considered statistically significant [16].

Results

General Observations

All animals survived along the period of operation, and there were no anesthesia-related deaths during the procedures. After recovering from anesthesia, all animals promptly resumed their regular feeding and activity. Wound healing occurred with the first intention in all groups, with no signs of swelling or visible infection observed throughout the study.

Radiographic Examination

The mean total radiographic scores and corresponding standard deviations (±SD) are summarized in Table (3). After 30 days, the CC + BM group exhibited significant new bone growth and a marked periosteal reaction compared to the CC group alone, although this difference was not statistically significant ($p > 0.05$). Additionally, the CC + BM group demonstrated a slightly higher radiographic density score than the CC group (Fig. 2).

At 60 days, the score for reducing defect size in the CC group remained relatively high, while the CC+BM group exhibited no defects. Furthermore, the CC+BM group showed improved radiography scores compared to the CC group ($p > 0.05$). Early signs of remodeling were noted in the CC+BM group; however, no such signs were observed in the CC group (Fig. 2).

At 90 days, no fracture lines were observed in either the CC+BM or CC groups, indicating significant progress in healing. The radiographic density in the CC+BM group was greater than that in the CC group ($p > 0.05$). While remodeling characteristics were noted in the CC+BM group, initial signs of remodeling were identified in the CC group (Fig. 2).

Histological Examination

The histological findings aligned with the radiographic observations. Table (4) presents the mean values (±SD) of the comprehensive histological scores, which include union, spongiosa, cortex, and bone marrow at the defect site reduction scores.

At 30 days, the comparison between groups revealed no significant differences ($P > 0.05$). The CC+BM group exhibited a favorable score at the site of bone deficiency. In the CC group, which contained an inflammatory granuloma, the connection between the body's tissue and the new bone tissue was primarily fibrous, with some cartilage present. In contrast, the CC+BM group demonstrated endochondral ossification with minimal inflammatory granuloma. Additionally, the average score for spongy bone formation was higher in the CC+BM group. Moreover, during this period, no group displayed any signs of compact bone development (Fig. 3).

At 60 days, the comparison between groups revealed no significant differences ($P > 0.05$). Both groups demonstrated an average osteochondral union. The CC group had bone marrow occupying more than half of the area and exhibited minimal inflammatory granuloma, whereas the CC+BM group experienced full colonization by red bone marrow. Furthermore, the CC+BM group showed a high degree of organization and activity in the newly

formed spongy bone within the bone deficit, while the CC group only displayed early activity. The CC group had already initiated the production of compact bone, while the CC+BM group showed compact bone reorganized in the majority (Fig. 3).

After 90 days, no significant changes were observed ($P > 0.05$). Bone union occurred in both groups. In the CC group, the area was filled with red bone marrow, whereas in the CC+BM group, fatty bone marrow was present in the spaces of the new spongy tissue. Both groups exhibited enhanced trabecular bone structure and activity. The CC+BM group showed comprehensive remodeling and development of compact bone tissue, while only an initial phase of this process was seen in the CC group (Fig. 3).

Discussion

Modern bone substitutes have introduced innovative strategies to enhance bone regeneration by addressing the limitations of traditional bone grafts, such as limited availability, high costs, the risk of zoonotic disease transmission, and potential immune reactions [17, 18]. Autologous bone grafting, especially when combined with effective treatments for nonunion, has been extensively studied and generally yields positive outcomes [19]. However, the use of autologous grafts has recently declined due to several drawbacks, including prolonged surgical procedures, postoperative pain, and complications related to bleeding [20, 21].

Calcium carbonate ($CaCO_3$) is a ternary ionic compound made up of calcium, carbon, and oxygen [22]. It is one of the most abundant minerals on Earth and is commonly found in geological formations worldwide [23]. Calcium carbonate plays a crucial role in biomineralization research and contributes to the formation of various natural structures, including marine sediments, mollusk shells, snail shells, coal balls, pearls, and eggshells [23, 24, 25]. $CaCO_3$ exists in several crystalline forms, notably calcite, aragonite, and vaterite [26]. This study aimed to assess the bone healing potential of synthetic calcium carbonate (CC) sourced from Sigma-Aldrich, both alone and in combination with bone marrow (CC+BM), using radiographic and histological evaluations in a rabbit radial bone defect model.

The CC+BM received the highest overall scores after 30 days. The radiological and histological data showed no significant differences across groups, which can be explained histologically by the minimal inflammatory granuloma observed in the CC+BM group compared to the CC group. Bone marrow, particularly through mesenchymal stem cells (MSCs), aids bone healing by promoting new bone formation and modulating inflammation [12, 27]. This process helps prevent or reduce granuloma formation, a common barrier in implant and graft healing. As a result, there was enhanced regeneration with fewer fibrotic or chronic inflammatory complications [28, 29].

At 60 days, the CC+BM group exhibited the highest radiological scores in terms of fracture line appearance, bone remodeling, and callus formation. Histological data indicated that bone regeneration occurs more rapidly in the CC+BM group, with no significant difference ($P > 0.05$). This observation can be attributed to the absence of inflammatory granuloma in the CC+BM group, while a minimal inflammatory granuloma persists in the CC group [28, 29]. The resorption rate of calcite is slower than that of hydroxyapatite (HA) and significantly slower than β-tricalcium phosphate (β-TCP) or amorphous calcium carbonate ($CaCO_3$) forms such as vaterite [30, 31]. Macrophages and osteoclasts play a role in the phagocytosis and degradation of $CaCO_3$ particles. These cells create an acidic microenvironment that promotes dissolution, which may explain the advantages of using BM in bone healing [32, 33].

At 90 days, CC+BM presented the highest radiological and histological score with no significative difference ($P > 0.05$). The histological results revealed that the lamellar bone repair score was much higher in CC+BM. Bone marrow remodeling is a dynamic process involving the coordinated activity of osteoclasts and mesenchymal stem cells (MSCs). Osteoclasts resorb bone by creating an acidic microenvironment that dissolves mineralized matrix, while MSCs differentiate into osteoblasts to form new bone [33]. This remodeling maintains both skeletal integrity and the hematopoietic environment. Recent studies highlight how inflammatory signals and cellular interactions within the marrow niche regulate this balance, with MSCs also contributing to immune modulation and vascular support during bone regeneration [34].

Conclusion

Combining calcium carbonate with bone marrow significantly enhanced bone regeneration compared to using calcium carbonate alone. While calcium carbonate ($CaCO_3$) provides a stable and osteoconductive scaffold, bone marrow supplies mesenchymal stem cells, growth factors, and immune-regulating elements, which expedited healing, improved vascularization, and decreased inflammation and granuloma formation. This collaboration resulted in faster and more effective bone repair.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study adheres to the ethics guidelines set forth by the Faculty of Veterinary Medicine Department of Veterinary Sciences at the Institute of Veterinary and Agricultural Sciences, University of Batna 1, Algeria (ethics approval number: 133/DV/ISVSA/UB1/2024).

TABLE 1. Lane and Sandhu radiological scoring system [14].

Category	Description	Score
Callus	No callus	0
	Callus occupying 25% of the defect	1
	Callus occupying 50% of the defect	2
	Callus occupying 75% of the defect	3
	Callus occupying 100% of the defect	4
Fracture line	Clear fracture line	0
	Relatively clear fracture line	1
	Partial fracture line	2
	Almost vanished	3
	Completely vanished	4
Bone remodeling	No bone remodeling	0
	Remodeling of the intramedullary channel	2
	Complete cortical remodeling	4

TABLE 2. Modified Lane and Sandhu scoring system [15].

2	Description	Score
Union	No sign of union	0
	Fibrous union	1
	Osteochondral union	2
	Bone union	3
	Complete reorganization	4
Spongiosa	No sign of cellular activity	0
	Early bone formation	1
	Active new bone formation	2
	Reorganized spongiosa formation	3
	Complete reorganized spongiosa	4
Cortex	Absence of cortex	0
	Early detection	1
	Initiation of formation	2
	Reorganization in majority	3
	Complete organization	4
Bone marrow	Not available	0
	Detection of fibrinous material	1
	Defect occupying more than half	2
	Fully occupying the red bone marrow	3
	Adult type fatty marrow	4

TABLE 3. Radiographic scores parameters for healing assessment (mean ± SD).

Days	Index	Callus	Fracture line	Bone remodeling
30 days	CC	3.0 ± 1.26	2.67 ± 1.03	1.33 ± 1.03
	CC+BM	3.33 ± 1.03	3.0 ± 0.89	2.0 ± 1.79
60 days	CC	3.17 ± 0.98	3.0 ± 0.89	2.33 ± 1.51
	CC+BM	3.5 ± 0.84	3.33 ± 0.82	2.67 ± 1.63
90 days	CC	3.83 ± 0.41	3.33 ± 0.82	2.67 ± 1.63
	CC+BM	3.83 ± 0.41	3.67 ± 0.52	3.33 ± 1.03

*p<0.05 between groups. SD: Standard deviation. CC: Calcium Carbonate. BM: Bone Marrow.

TABLE 4. Histological mean ± SD scores of different parameters in groups (CC and CC+BM), and time periods (30, 60, and 90 days).

Day	Group	Union	Spongiosa	Cortex	Bone marrow
30	CC	2.0 ± 0.71	1.8 ± 0.84	1.8 ± 0.84	1.8 ± 0.84
	CC + BM	2.2 ± 0.84	2.0 ± 0.71	2.0 ± 0.71	2.2 ± 0.84
60	CC	2.2 ± 0.84	2.2 ± 0.84	2.2 ± 0.84	2.4 ± 1.14
	CC + BM	3.2 ± 0.84	3.2 ± 0.84	3.0 ± 0.71	2.8 ± 0.84
90	CC	3.0 ± 1.0	3.2 ± 0.84	3.2 ± 0.84	3.0 ± 0.71
	CC + BM	3.6 ± 0.55	3.6 ± 0.55	3.6 ± 0.55	3.6 ± 0.55

*p<0.05 between groups. SD: Standard deviation. CC: Calcium Carbonate. BM: Bone Marrow.

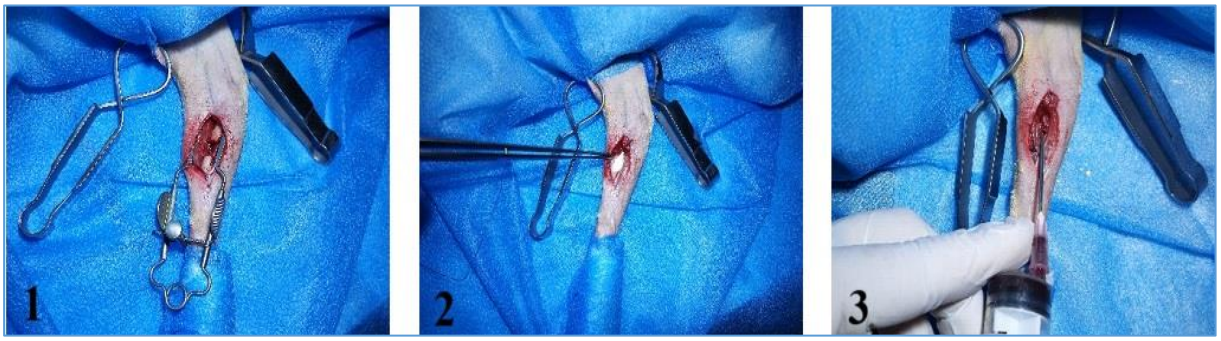

Fig. 1. Osseous defect in the mid-shaft of the right radius.

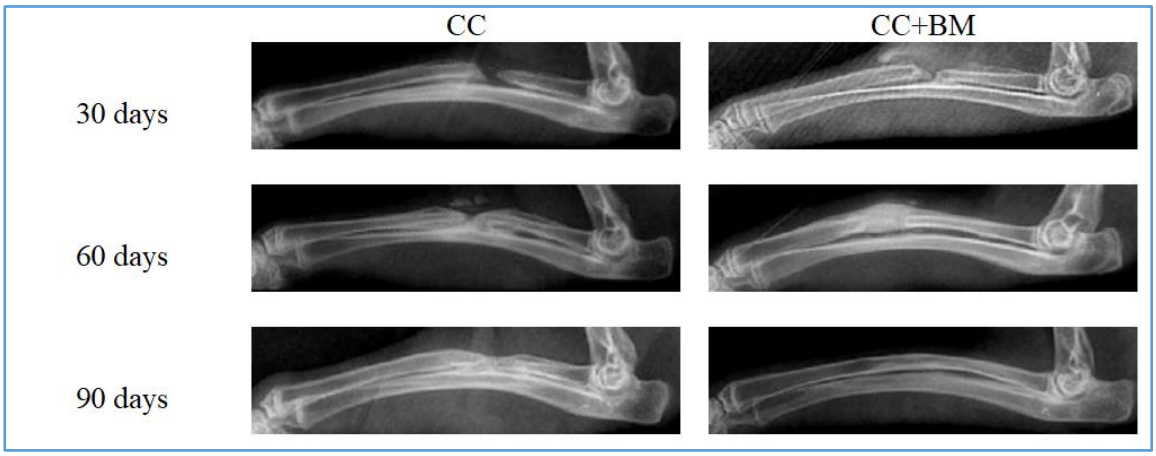

Fig. 2. Mediolateral radiographs after surgery of rabbits of "Calcium Carbonate (CC)" and "CC + Bone Marrow (BM)" groups. mm of osseous defect in the mid-shaft of the right radius; 2: deficiency is filled with Calcium Carbonate, 3: deficiency is filled with Calcium Carbonate and injection of autologous Bone Marrow.

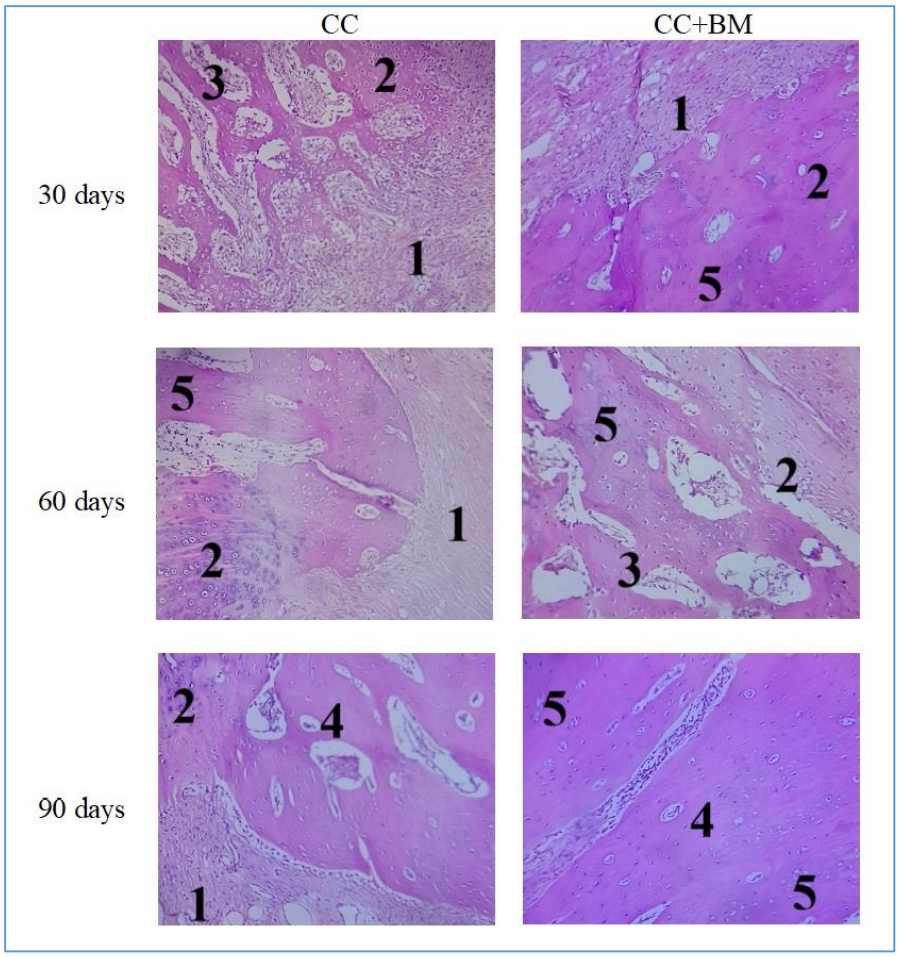

Fig. 3. Histological analysis of defect sites at different days post-surgery (H&E).
CC: Calcium Carbonate, BM: Bone Marrow, 1: fibrous tissue, 2: cartilage formation, 3: woven bone, 4: Haversian system formation, 1: new bone.

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دراسة دور مزيج كربونات الكالسيوم الصناعية ونخاع العظم في عملية التئام العيوب العظمية الواسعة باستخدام نموذج الأرنب

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الملخص

هدفت هذه الدراسة إلى تقييم القدرة التجديدية لكربونات الكالسيوم الاصطناعية ($CaCO_3$) الممزوجة بنخاع العظم (BM) لعلاج عيوب عظمية واسعة النطاق في نموذج أرنب. تم إنشاء عيوب شعاعية بالغة الأهمية جراحيًا وعولجت إما باستخدام $CaCO_3$ وحده أو باستخدام $CaCO_3$ المدعم بنخاع عظم ذاتي مأخوذ من العرف الحرقفي. تضمنت المراقبة بعد الجراحة ملاحظات سريرية وتقييمات إشعاعية وتحليلات نسيجية لمقارنة نتائج الشفاء بين المجموعتين. أظهرت النتائج أن إضافة نخاع العظم عززت عملية الإصلاح، مما يدل على تكوين عظام جديدة أسرع وأكثر تناسقًا. أظهر التقييم الشعاعي تجددًا عظميًا أكبر في مجموعة $CaCO_3$ + نخاع العظم مقارنةً بمجموعة $CaCO_3$ وحده، على الرغم من أن هذا الاختلاف لم يكن ذا دلالة إحصائية ($p > 0.05$). أكدت المقاطع النسيجية تحسنًا في تكوين العظم، الذي اتسم بنشاط كبير للخلايا العظمية، وزيادة في ارتشاح الأوعية الدموية، وتكاملًا أكثر تقدمًا للمواد الحيوية مع أنسجة العائل. في المقابل، أظهرت العيوب المعالجة بكربونات الكالسيوم ($CaCO_3$) وحدها إصلاحًا أبطأ وغير مكتمل، مع وجود أنسجة ليفية ثابتة في بعض المناطق. تدعم هذه النتائج فكرة أن نخاع العظم يُوفر خلايا سلفية عظمية أساسية وعوامل نمو تعمل بتآزر مع خصائص كربونات الكالسيوم في توصيل العظام، مما يُسرّع عملية تجديد العظام. بشكل عام، يُمثل الجمع بين كربونات الكالسيوم ($CaCO_3$) ونخاع العظم استراتيجية واعدة وفعالة يُمكن أن تُعزز النتائج السريرية في كل من جراحة إعادة بناء العظام البيطرية والبشرية.

الكلمات الدالة: طُعم عظمي، نخاع عظم، كربونات الكالسيوم، أرنب، عيب شعاعي.