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A Comparative Study on Bone Defect Regeneration in a Rabbit Model using Cuttlebone as a Bone Graft Substitute

Mohamed Tahar Benlamari*, Ishak Ayoub Keraghel, Adel Aissi and Omar Bennoune

Laboratory of Health, Animal Production and Environment, Department of Veterinary Sciences, Institute of Veterinary and Agricultural Sciences, University of Batna 1, Batna, Algeria.

Abstract

BONE, grafting is widely used for treating substantial bone defects and losses, enhancing the healing process, and promoting successful bone union. Natural calcium carbonate powder has been used for a long time as a bone substitute material and as a scaffold for bone morphogenetic protein (BMP). The present study aims to evaluate bone defect regeneration, with 2 mm diameter and 10 mm length defects created in the right radial bone in 45 rabbits, comparing normal bone healing to cuttlebone (CB1) with a cylindrical shape (2 mm in diameter and 10 mm in length), and cuttlebone powder (CB2). Clinical, radiological, and histological evaluations were made 30, 60, and 90 days after surgery. Results of the radiological scores were comparable between the control group and CB1, followed by CB2, with no significant difference ($p > 0.05$). Histological scores were high in CB1, followed by the control group and CB2 without significant differences ($p > 0.05$). The results indicate that cylindrical cuttlebone graft closely mimics natural healing and may improve cortical regeneration. Conversely, cuttlebone in powdered form, demonstrated some limitations in maintaining structural integrity, callus formation, and remodelling.

Keywords: Bone graft, Cuttlebone, Powder, Rabbit, Radial defect.

Introduction

Bone repair is a complex and dynamic process that involves the coordinated engagement of multiple cells, growth hormones, and extracellular matrix components. Bone grafts are commonly used in bone loss and fracture repair. Bone grafts can expedite the healing process despite normal bone healing adhering to a specific sequence started by hematoma, inflammation, soft callus development, complex callus formation, and remodeling. These materials must provide biocompatibility and bioactive signals to promote osteogenesis, angiogenesis, and tissue regeneration [1].

The utilization of xenogeneic bone substitutes is not novel, yet the majority of its applications were predicated on clinical experience and effectiveness [2]. Nowadays, bone xenografts have emerged as a viable option to autografts for bone grafting, owing to their availability, cost-effectiveness, and diminished morbidity at donor locations [3,4,5].

Cuttlebone, the interior shell of *Sepia officinalis* (commonly known as cuttlefish), comprises many trace elements, such as sodium, magnesium, potassium, and strontium, which significantly enhance bone development. Moreover, it is a cheap, accessible raw material that is 84% $CaCO_3$ [6]. Its distinctive appearance and composition make it suitable for application as a powder in bone grafting procedures and as a scaffold. The porous structure of cuttlebone and its ability to promote cell adhesion and proliferation validate its application as a bone graft material [7]. Moreover, adequate vascularization, facilitated by the cuttlebone's porous nature, is crucial for successful bone regeneration [8].

This study examines the natural process of bone healing in rabbits without graft material and its interaction with a xenograft utilizing defatted cuttlebone from *Sepia officinalis* in cylindrical and powdered forms. To assess the effectiveness of cuttlebone as a bone graft material, radiographic and histological evaluations were conducted, utilizing

*Corresponding authors: Mohamed T. Benlamari, E-mail: mohamed.benlamari@univ-batna.dz Tel.: 9647504592159
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parameters including callus formation, fracture line assessment, bone remodeling, union, spongiosa, cortex, and bone marrow, followed by statistical analysis. The results may offer a thorough analysis of the efficacy of defatted cuttlebone xenografts, both in cylindrical and powdered forms, as bone transplant materials, and compare them with conventional bone healing, advancing orthopedic surgery, and regenerative medicine.

Material and Methods

Forty-five clinically healthy, five-month-old New Zealand male rabbits, with an average weight of 2.7 kg, were included in experimental surgery. Before the procedures, these animals were kept for acclimatization for 21 days in an environment where light and temperature were controlled. They were gradually acclimated to being handled throughout this time. The rabbits received commercial rabbit pellets and unlimited water while housed in solitary confinement. The Ethical Committee of the Institute of Veterinary and Agricultural Sciences, University of Batna 1, Batna, Algeria, approved all drilling and handling procedures.

Cuttlebone Graft Preparation

Two cuttlebone (CB) graft forms were made: CB1, a cylindrical cuttlebone defatted and freeze-dried, and CB2, a powder form of defatted and freeze-dried cuttlebone. Both originate from the same cuttlefish (genus: Sepia, species: officinalis).

CB1 was sectioned into a cylindrical form with a diameter of 2 mm and a length of 10 mm. The cuttlebones were immersed in a 1:1 chloroform: methanol solution for six days, placed in a freezer for twenty-four hours, and freeze-dried for 72 hours at -80 degrees Celsius. Ethylene oxide gas was backfilled into the freeze-dried cuttlebones for twenty-four hours [9].

CB2 was made from freeze-dried CB1 using a mortar and pestle. The bone powder was then filtered through a 60-mesh screen to yield a fine powder to fill up bone loss. Ethylene oxide gas was then added to a secure specimen collection bottle, to which the powder was added and left for twenty-four hours [9].

Experimental surgery

The rabbits used in this study were randomly grouped into three appraisal groups. It is important to note that absolutely only normal saline were applied to the Control Group. The first treatment group received CB1, and the Second Treatment Group received CB2. For all the groups, a 10 mm segmental defect was made on the mid-shaft of the radius. The control group was allowed to heal by itself, while the first treatment group had defects filled with CB1 cylinders, and the second treatment group had theirs filled with CB2 powder.

Animals were assessed radiographically and histologically on the 30^{th}, 60^{th}, and 90th postoperative days; the rabbits were sacrificed in each group.

Anesthesia and Animal Preparation

After a 12-hour fasting, the animals were placed under standard conditions for a surgical procedure. They were initially treated with an intramuscular injection of xylazine (Xyla®, 1 mg/kg) to provide sedation. The right forelimb and hip region were shaved, and an antiseptic solution was prepared using an iodized polyvidone solution (Dermadine®). General anesthesia was induced with an intramuscular injection of ketamine (Imalgene®, 40 mg/kg).

Surgical procedure

Animals were positioned in right lateral recumbency, and surgical drapes were utilized. A 3–4 cm incision was made on the medial side of the right forelimb, about midway between the elbow and carpal joints. Fine, blunt scissors were used to split the radius muscles gently (Fig. 1).

An osteotomy was performed on the mid-shaft of the radius using an appropriate electric medical saw. The same process was repeated 10 mm away from the initial osteotomy. After the bone fragment separated from its muscle attachment, it was removed to form a bone defect. The control group were not treated and left to heal normally. The first treatment group members received CB1, while the second received CB2. Following that, a running 4/0 polyglactin 910 suture was used to suture the muscles, and a basic interrupted 4/0 polyamide suture was used to close the skin. The postoperative care of the rabbits is carried out daily to assess the general condition of the rabbits and to observe the operated site for swelling or infection.

Radiographic Evaluation

Five (5) animals in each group were sacrificed at 30, 60, and 90 days postoperatively. The right limb was transected at the glenohumeral joint, and two orthogonal radiographs (mid-lateral and anteroposterior views) were taken using a digital radiography system (XR 6000, GE Healthcare®) using 55 kV and 50 mAs. The radiographs were evaluated for evidence of new bone formation, callus extent and size, gap bridges, x-ray density, and signs of remodeling. Observations were interpreted using an X-ray scoring system modified by Lane and Sandhu [10] Table (1). The mean score for each parameter was calculated, and the sum of the scores was used as the cumulative radiographic score for each group. The group with the highest scores was considered to have excellent healing.

Histological Evaluation

The bone was fixed using 10% phosphate-buffered formalin for three days and then decalcified using 10% formic acid for at least seven days. The samples were then subjected to an ethanol series for dehydration, embedded in paraffin, and sections with a 6 mm thickness were prepared. The sections were stained with hematoxylin and eosin (H&E). The histological observations were individually evaluated with an optical microscope (Leica) and scored according to the modified Lane and Sandhu scoring system [11], as in Table **(**2). Therefore, the mean scores of the various histopathological parameters were computed, and the total histopathological score was obtained by adding the mean scores. The group with the highest total histopathological score was thus considered the group with the best bone consolidation

Statistical Analysis

A two-way analysis of variance (ANOVA) was performed using the 26th version of IBM SPSS Statistics for Windows to assess the radiographic and histological scores among the three experimental groups. A p-value below 0.05 was deemed statistically significant.

Results

General Observations

All animals survived until sample collection, and no deaths due to anesthesia occurred during the experimental procedure. All animals resumed normal food intake and activities after waking from anesthesia. The wounds healed by the first intention in all groups, and no swelling or visible signs of infection were observed.

Radiographic Examination

Table (3) shows the averages (±SD) of the total radiographic scores, remodeling signals, radiographic density, and defect site reduction scores.

At 30 days, the control group had considerable new bone growth and a pronounced periosteal reaction. Conversely, the CB1 and CB2 groups exhibited merely a slight periosteal response. Compared to CB1 and CB2, the control group's defect site reduction score was not significantly greater ($p > 0.05$). Furthermore, radiographic density was increased across the study groups; however, the control group had a slightly higher radiographic density score than CB1 and CB2 ($p > 0.05$) (Fig. 2).

At 60 days, the defect site reduction score in the CB2 group remained constant, while the control and CB1 groups demonstrated a considerable reduction in defect size. CB1 and the control group exhibit superior radiographic scores compared to CB2 ($p > 0.05$). Initial indications of remodeling were observed in the control and CB1 groups; however, none were detected in the CB2 group (Fig. 2).

At 90 days, no fracture lines were observed in the control and CB1 groups, signifying advanced healing. Nevertheless, the deficiency persisted in the CB2 group. Radiographic density in the CB1 group was more significant than in the control and CB2 groups ($p > 0.05$). Remodeling characteristics were noted in the control and CB1 groups; however, none were identified in the CB2 group (Fig. 2).

Histological Examination

The histology findings were consistent and closely aligned with the radiography observations. Table (4) presents the averages (±SD) of the entire histological scores, including union, spongiosa, cortex, and bone marrow at the defect site reduction scores.

At 30 days, a statistically significant difference in bone union and bone marrow was noted between the groups ($P<0.01$). A favorable score was observed at the location of bone deficiency in the control group. The integration between the host tissue and the newly formed bone tissue was fibrous to osteochondral in the CB1 and CB2 groups. In the CB1 and CB2 groups, early attachment of the new bone was noted, whereas the control group had histologically more active spongy bone tissue at the deficiency level ($P<0.05$). The control group exhibited a superior average score for spongy bone development. Moreover, no compact bone growth was detected in any groups throughout this period (Fig. 3).

At 60 days, the comparison between groups showed no significant differences ($P>0.05$). An average osteochondral union was seen in the control and CB1 groups, while the CB2 group remained fibrous to osteochondral. In the CB2 group, bone marrow was present in only over half of the deficiency in animals, whereas complete colonization by red bone marrow occurred in the control and CB1 groups. Furthermore, the newly developed spongy bone in the bone deficit exhibited only initial activity in the CB2 group, whereas in the control and CB1 groups demonstrated a high level of organisation and activity. The group CB2 exhibited an early manifestation of compact bone, whereas both the control and CB1 groups had already commenced the formation of compact bone (Fig. 3).

At 90 days, no significant differences are seen ($P>0.05$). The union was consistently osteochondral (sometimes fibrous) in the CB2 group, whereas a bony union was noted in the control and CB1 groups. The bone marrow development noted in the CB2 group was somewhat underdeveloped and sparse compared to the mature, adipose bone marrow in the cavities of the newly produced spongy tissue of the control and CB1 groups. The CB2 group initially developed spongy tissue, whereas the control and CB1 groups exhibited better organization and activity. Significant remodeling and formation of

compact bone tissue were observed in the control and CB1 groups; however, in the CB2 group, only an initial phase was detected (Fig. 3).

The results show that CB1, which has a cylindrical cuttlebone graft, heals very similarly to the control group and may even help the cortical regeneration process more. On the other hand, the powdered form of CB2 was not good at keeping the structure intact, making a callus, or remodelling.

Discussion

Modern bone substitutes have implemented novel strategies for bone regeneration by overcoming the drawbacks of bone grafts, including scarcity, elevated costs, transmission of zoonotic diseases, and immune responses, among others [12,13,14]. Autologous bone grafts combined with effective nonunion treatment have been extensively studied and have favorable outcomes [15,16]. This approach has recently been limited due to disadvantages such as prolonged surgical time, pain, and hemorrhaging [12,17,18]. Cuttlebone exhibits a high concentration of calcium carbonate ($CaCO_3$) and a porous structure, making it an ideal scaffold for bone tissue engineering [19,20]. Calcium carbonate is used extensively as a biomaterial in bone regeneration due to its biocompatibility, bioactivity, and osteoconductive characteristics. It functions as a scaffold for creating new bone tissue and is essential for supplying the calcium ions required for bone mineralization [21]. This study investigated the bone healing benefits of two forms of Cuttlebone (CB1 and CB2) utilizing X-ray and histological analysis in a rabbit radial bone defect model.

The control group received the highest overall scores after 30 days. The radiological data showed no significant differences across all groups. However, the histological findings demonstrated that bone repair progressed more swiftly in the control group, exhibiting a statistically significant difference ($P<0.05$). Groups CB1 and CB2 were surrounded by a dense, fibrous tissue that was full of inflammatory cells, such as neutrophils and macrophages, which are known to break down bone [9, 22, 23].

At 60 days, the control group exhibits the highest radiological scores in fracture line and bone remodeling while sharing identical scores with CB1 in callus formation. Histological data revealed that bone regeneration progressed more swiftly in the control group, with no significant difference ($P > 0.05$), and it had equivalent scores to CB1 in the spongiosa and bone marrow. This change can be attributed to osteoclasts' gradual destruction and resorption, particularly in week 4 [22]. The porous structure additionally enhances angiogenesis [24]. CB2 has a low score because the small particles are resorbed more rapidly due to their increased surface area, which expedites graft replacement with new bone. However, fast resorption may compromise mechanical stability in the early recuperation phases [25]. At 90 days, the control group and CB1 exhibit equal scores. Nonetheless, CB2 persistently receives a low rating. The histological results revealed that the lamellar bone repair score was much higher in CB1, exhibiting equivalent scores to the Control group in union, spongiosa, and bone marrow, with no statistically significant difference ($P>0.05$). In 10 mm defect sizes, the grade of bone growth was relatively high, which can explain bone healing in the control group [26]; nonetheless, the standard deviation is considerable (1.41). The high score of CB1 can be clarified by the increased acceleration of bone repair after the inflammatory response and the gradual resorption and replacement by fresh bone due to the graft resorption and porosity of cuttlebone with high vascularization stimulating lamellar bone formation [8]. Histological sections demonstrate graft material and mature lamellar bone, with osteoclasts resorbing the graft. The CB2 is gradually replaced and assimilated by new bone tissue [25]. Histological analysis demonstrates the formation of braided bone encasing the particles, which later remodels into mature lamellar bone.

Based on our recent findings, further research is needed to search for more effective biomaterials that can enhance bone regeneration beyond those used in this study. Furthermore, more research is needed to find ways to keep bone grafts from getting encapsulated, to find the best biomaterials for bone induction, and to get the most out of CB2 by adding binders or structural stabilizers to make its osteoconductive and osteoinductive properties even stronger.

Conclusion

The findings show that a cylindrical cuttlebone graft may enhance cortical regeneration and closely resemble spontaneous healing. In contrast, cuttlebone powder showed some limits in remodeling, callus formation, and structural integrity. Cuttlebone powder is suitable for filling minor, irregular imperfections when mechanical stability is not critical.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study follows the ethics guidelines of the Faculty of Veterinary Medicine Department of Veterinary Sciences, Institute of Veterinary and Agricultural Sciences, University of Batna 1, Batna, Algeria (ethics approval number; 77/DV/ISVSA/UB1/2024).

TABLE 1. Lane and Sandhu radiological scoring system [10].

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

Maximum total score: 12

TABLE 2. Modified Lane and Sandhu scoring system [11]

Category	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).

	Index	Callus	Fracture line	Bone remodeling
	Control group	3 ± 1	2.6 ± 0.89	1.2 ± 1.1
30 days	CB1	2.6 ± 1.34	2.2 ± 1.3	0.8 ± 1.1
	CB2	2.2 ± 1.1	2 ± 0.71	0.4 ± 0.89
	Control group	3.6 ± 0.89	3.4 ± 0.84	2.8 ± 1.79
60 days	CB1	3.6 ± 0.89	3.2 ± 0.84	2.4 ± 1.67
	CB2	2.6 ± 1.34	2.6 ± 1.34	0.8 ± 1.1
	Control group	3.6 ± 0.89	3.6 ± 0.89	2.8 ± 1.79
90 days	CB1	3.6 ± 0.89	3.6 ± 0.89	2.8 ± 1.79
	CB2	2.8 ± 0.84	2.8 ± 0.84	1.2 ± 1.79

*p<0.05 between groups. SD: Standard deviation. CB1: Cuttlebone cylindrical shape. CB2: Cuttlebone powder.

TABLE 4. Histological mean ± SD scores of different parameters in groups ("Control group", "CB1", and "CB2"), and times (30, 60, and 90 days).

Days	Index	Union	Spongiosa	Cortex	Bone Marrow
	Control group	1.60 ± 0.55**	2.40 ± 0.55*	1.60 ± 0.55*	1.80 ± 0.45**
30 days	CB1	1.40 ± 0.55	1.80 ± 0.84	1.20 ± 0.45	1.40 ± 0.55
	CB2	1.40 ± 0.55	1.60 ± 0.55	1.20 ± 0.45	1.40 ± 0.55
	Control group	2.80 ± 0.45	2.80 ± 0.45	2.40 ± 0.89	2.80 ± 0.84
60 days	CB1	2.60 ± 0.55	2.80 ± 0.45	2.20 ± 0.84	2.80 ± 0.45
	CB2	2.60 ± 0.55	2.60 ± 0.55	1.60 ± 0.55	2.40 ± 0.55
	Control group	3.40 ± 0.89	3.40 ± 0.89	3.00 ± 1.41	3.20 ± 0.84
90 days	CB1	3.40 ± 0.89	3.40 ± 0.89	3.20 ± 0.84	3.20 ± 0.84
	CB2	2.80 ± 0.84	2.80 ± 0.84	2.40 ± 0.89	2.80 ± 0.84

*p<0.05, **p<0.01 between groups. SD=Standard deviation, CB1=Cuttlebone cylindrical shape, CB2=Cuttlebone powder

Fig. 1. Osseous defect in the mid-shaft of the right radius. CG: The control group proceeded independently to recuperate; CB1: deficiency is filled with a cylindrical cuttlebone, CB2: cuttlebone powder fills the deficiency.

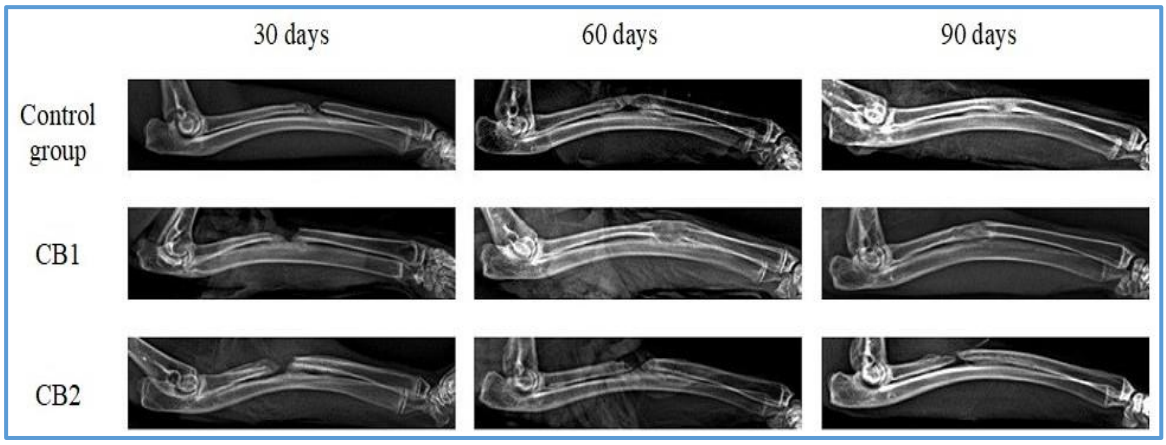

Fig. 2. Mediolateral radiographs after surgery of rabbits of the control, cuttlebone cylindrical shape (CB1), and cuttlebone powder (CB2) groups.

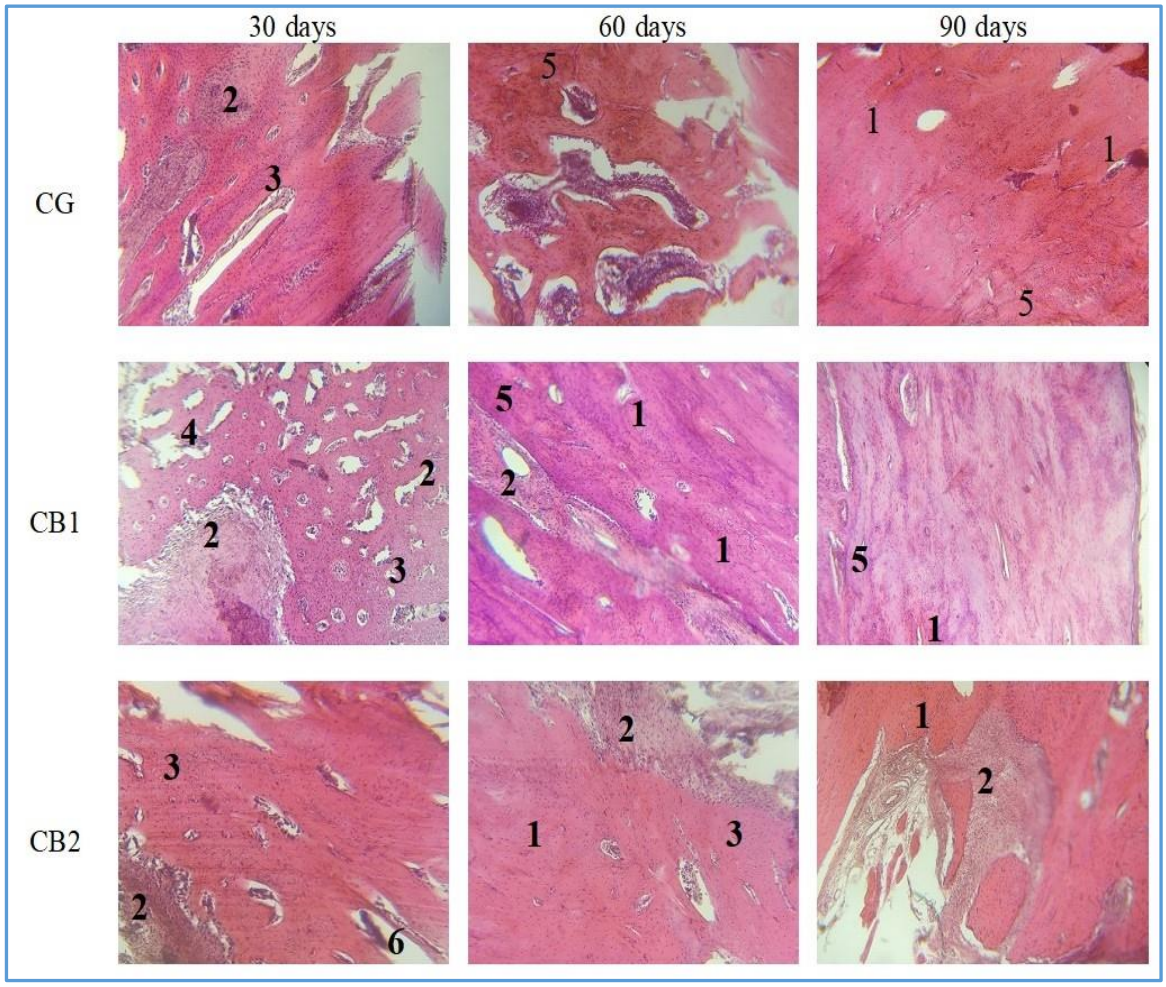

Fig. 3. Histological analysis of defect sites at different days post-surgery (H&E). CG: control group, CB1: cuttlebone cylindrical shape, CB2: cuttlebone powder, 1: new bone, 2: fibrous tissue, 3: cartilage formation, 4: woven bone, 5: Haversian system formation, 6: graft materials.

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دراسة مقارنة حول تجديد عيوب العظام في نموذج أرنب باستخدام عظم الحبار كبديل لطعوم العظام

محمد الطاهر بن لعماري*، إسحاق أيوب كراغل، عادل عايسي وعمار بنون

مخبر الصحة والإنتاج الحيواني والبيئة، قسم علوم البيطرة، معهد علوم البيطرة والعلوم الفلاحية، جامعة باتنة 1، باتنة، الجزائر.

الملخص

يُستخدم ترقيع العظام على نطاق واسع لعلاج عيوب العظام الكبيرة والخسائر، وتعزيز عملية الشفاء، وتعزيز التئام العظام بنجاح. وقد استُخدم مسحوق كربونات الكالسيوم الطبيعي لفترة طويلة كمواد بديلة للعظام وكدعامة لبروتين تكوين العظام. تهدف هذه الدراسة إلى تقييم تجديد عيوب العظام، مع عيوب يبلغ قطرها 2 مم وطولها 10 مم في عظم الشعاعي الأيمن لدى 45 أرنبًا، ومقارنة التئام العظام الطبيعي بعظم الحبار ذي الشكل الأسطواني (قطره 2 مم وطوله 10 مم)، ومسحوق عظم الحبار. أُجريت تقييمات سريرية وإشعاعية ونسيجية بعد 30 و60 و90 يومًا من الجراحة. كانت نتائج الدرجات الإشعاعية قابلة للمقارنة بين المجموعة التحكم وعظم الحبار، تليها مسحوق عظم الحبار، دون وجود فرق كبير. كانت الدرجات النسيجية مرتفعة في مجموعة عظم الحبار، تليها المجموعة التحكم ودون وجود فروق كبيرة. تشير النتائج إلى أن طعم عظم الحبار الأسطواني يحاكي الشفاء الطبيعي عن كثب وقد يحسن تجديد القشرة. على العكس من ذلك، أظهر مسحوق عظم الحبار بعض القصور في الحفاظ على سلامة هيكل العظم وإعادة تشكيله.

الكلمات الدالة: طُعم عظمي، عظم الحبار، مسحوق، أرنب، عيب شعاعي.