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# Recycling calcium hydroxide from waste quail eggshell to reconstruct mandibular gap in dogs: A histopathological and immunohistochemical changes

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**Abstract**

The current investigation studied a deliberately induced mandibular bone gap in dogs, which included immunohistochemistry, histological images, and assessment of processed egg quail. In this experiment, twenty-four adult dogs were split evenly between control group and a quail eggshell group (QESCH). The intended publication details the use of the hydrothermal approach to produce circular mandibular bone gaps and their reconstruction using calcium hydroxide powder recycled from quail. We analyzed the scores at 7, 15, and 30 days and evaluated the clinical, histological, and immunohistochemical outcomes. When comparing the QESCH group to the control group, the histological results showed that new bone production was present, along with an inflammatory reaction, a rise in osteoblasts and osteoclasts, and the emergence of Haversian canals. The immunohistochemistry results showed that at 30 days, the QESCH group had a slight positive expression of Alkaline phosphatase ALP, in contrast to the control group, which had an extremely weak expression. Finally, the control group's mandibular bone defect did not spontaneously mend to the QESCH group control group, and histopathological and immunohistochemical alterations were noticeably better in the QESCH group. Since quail egg shells are biocompatible and do not induce inflammatory or immunological reactions, they can be used as bone substitute biomaterials, aiding the healing process.

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## Introduction

Normal bones have the ability to mend and regenerate, but when there are significant size flaws, which can occur due to many factors such as becoming older, having tumors removed, being in a vehicle accident, or having a fracture that does not heal properly, the healing process cannot be achieved on its own (1). A mandibular defect is characterized by the disorder of the lower jaw. According to research, it is the second most prevalent type of facial bone fracture in mammals (2). Most dog mandibular defects are of the body type, meaning they extend from the canine teeth to the angle of the mandible (3). Because the jaw is anatomically complex, mandibular abnormalities typically require soft tissue healing rather than reconstruction (4). Postoperative infections owing to danger from oral flora impair its restoration, accounting for approximately 59% of all face abnormalities (5). Although trauma and injury to the trigeminal and mental nerves are the most common complications associated with the surgical approach to mandibular defect reconstruction, the majority of these injuries may be treated, and the animals regain sensibility without any problems (6). Biomaterial bone substitutes should not cause cancer, have light fibrosis, and not trigger an inflammatory or immunological rejection response (7). One of the essential sources of biomaterials is avian

eggshells. Due to its high calcium and relatively low protein content, quail eggshell is a popular biomaterial bone substitute (8). Additionally, compared to other calcium sources such as limestone, quail eggshell powder has a higher solubility, suggesting it is more bioavailable than these sources. The quail shell has recently emerged as a sustainable resource for calcium (9). Eggshells are considered a possible natural source for medicinal purposes due to the abundance of inorganic components, the most common of which are calcium carbonate ($CaCO_3$), magnesium carbonate (MgCO3), and calcium phosphate ($Ca_3(PO_4)_2$) (10). Eggshells are a great source of calcium and have few harmful components, which is only one of their numerous advantages. Their abundance of the amorphous crystal calcite, composed of calcium carbonate, is yet another perk (11).

No research has yet been conducted on quail eggshells restore damaged or missing bone.

## Materials and methods

### Ethical approve

Following the protocols and procedures set out by the University of Mosul, Animal Care and Committee numbered UM.Vet.2022.050, all studies and animal care were conducted following the highest standards of ethical behavior.

### Experiment design

The method outlined in (12) was used to make the calcium hydroxide powder. The quail eggs, which were not cracked, were gathered from a farmer in Tikrit City, Iraq. To ensure the quail eggshell was dirt-free, it was washed with deionized water and then cooked for approximately 30 minutes. An electrical mortar grinder (Retsch, RM200, China) was used to reduce the quail shells to a fine powder, which was subsequently calcined in a muffled furnace (Prothrom, Turkey) at 1200 °C for 2 hours. Calcium carbonate ($CaCO_3$) is transformed from the quail eggshell into calcium oxide (CaO) during this stage. Then, the (CaO) powder was mixed with distilled water in a beaker. In addition to producing a white powder (Figure 1), this reaction also produced an extremely high liberated temperature ($Ca(OH)_2$), which is calcium hydroxide.

The anesthetic regimen for all animals udergoing surgery includes injecting 15mg/kg of xylazine and 10mg/kg of ketamine HCL (from Alfasan in Holland) intramuscularly (13). The procedure began with a five-centimeter-long skin incision along the premolar/molar area. The mandibular bone was then exposed with blunt dissection of the deep fascia between the masseter and digastric muscle. Using a slow-speed electric bone drill and a cylindrical diamond hole saw, a circular mandibular bone was created into each of the experimental animals' jaws through a gap defect in their mandibles' caudal borders. To do this, a Juster (j3901, China) was designed to cut through full-thickness bone tissues without perforating the underlying buccal mucosa. The bone was then continuously irrigated with saline solution to avoid heat damage (Figure 2). The fault gap was 14 mm in diameter and about 0.5mm deep (Figure 3).


Figure 1: A, the quail eggshell prior to manufacturing. B, the calcium hydroxide powder made from scrambled quail eggs.

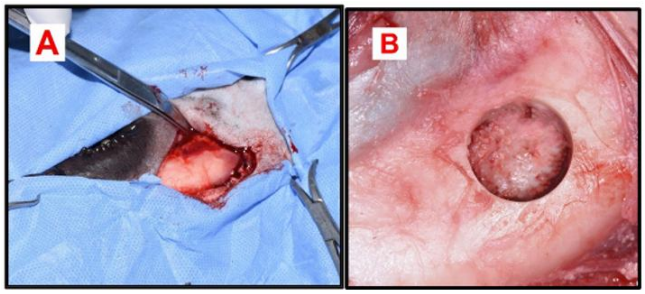


Figure 2: A. The inferior aspect of the mandibular arch. The experimental procedure involved utilizing a bone drill square to create a circular mandibular gap defect while constantly irrigating with saline solution (B).


Figure 3: A mandibular bone artificially created as a gap defect, B, measuring fourteen millimeters in diameter and fifty-five millimeters in depth**.**

**Histopathological evaluation**

Aseptically, bone samples were taken from each group seven, fifteen, and thirty-days following surgery. In order to maintain the integrity of the tissue, the samples were preserved for 48 hours in a fixing solution containing 10% neutral buffer formalin (NBF). Afterward, the bone tissue underwent decalcification, which involved using an ethylenediamine tetraacetate acid (EDTA) solution for 12 weeks, with weekly refreshments, to remove minerals and calcium salts. At this point, 10 grams of EDTA were dissolved in 100 milliliters of distilled water to create a 10% solution. To finish the calcium removal process, the bone sample was treated with the final solution, which had to be refilled daily (14). Decalcification was considered complete when the tissue became easily punctured or sliced with a needle. The other procedures in processing the decalcified tissue included dehydration, cleaning, infiltration, and embedding. Eventually, the tissue was contained in a hard paraffin block. The samples were subsequently sectioned using a conventional rotary microtome from Leica Microsystems, Germany, to a thickness of 5 µm. The next step was to use the conventional stains of eosin and hematoxylin to color them. Lastly, a light microscope (AX80T, Olympus, Japan) was used to examine the slide. In addition, a semi-qualitative analysis was conducted using a histopathological healing score based on the modified (14) to assess the healing process and new bone production at the site of bone gap deficiencies.

**Immunohistochemistry (IHC) evaluation**

Using a modified avidin-biotin immunoperoxidase approach, immunohistochemistry was performed 30 days following surgery (15). Before being employed in the immunohistochemistry approach, the tissue sections were deparaffinized, rehydrated, and deactivated using the avidin-biotin immunoperoxidase process. For 7 minutes at room temperature, a mixture of 3% hydrogen peroxide and methanol inhibited endogenous peroxidase. Following a thorough washing with a pH 7.3 PBS solution that contained 0.01% thiomersal and 50% glycerol, the sample was then incubated at room temperature with 10% normal goat serum for 30–40 minutes. The slides were then exposed to primary antibodies—specifically, Alkaline Phosphatase (ALP) Polyclonal antibodies—diluted 1:100 (Elabscience, USA) and incubated at 4 Cº for 24 hours. After that, the slides were washed twice with PBS for three minutes. Subsequently, they were incubated with poly-HRP goat anti-rabbit IgG, a secondary antibody, diluted 1:400 (Wuhan Fine Biotech, China), which was used for 30 minutes at room temperature. Lastly, the slides were rinsed with PBS. Then, the DAB staining procedure was carried out. Subsequently, after each slide was dehydrated, a cover slide was applied, and the nuclei were counterstained with hematoxylin for 30 seconds at room temperature. Following this, the slides were washed with cold water. A digital video camera (Leica ICC50, Leica, USA) and a light microscope (Leica, USA) were used to photograph the slides in a controlled environment. Four groups were formed from the ALK expression data according to the degree of staining. The expert pathologist produced the interpretation using the modified procedure outlined by (16). Positive expressions were categorized as either weak, mild, moderate, or high.

**Results**

There were no signs of infection, hematoma development, wound dehiscence, or swelling in either group of surgical animals, and they seemed to be in good health overall. By the first goal, all wounds had healed within ten to fifteen days following the operation. Without cracks at the side of mandibular gap problems, every animal in the experiment showed normal behavior, including an appetite, mastication, salivation, and barking. In the control group, the histopathological findings at 7 days showed blood clots and hemorrhage encircling a large area of fibrous tissue that separated from the normal mandible bone edges (Figure 4). In contrast, the QESCH group showed newly formed connective tissue surrounding the remnants of $Ca(OH)_2$ materials, along with a new woven bone formation that included numerous osteoblast cells (Figure 5).

Histological analysis performed fifteen days following surgery in the control group revealed a bleeding site, densely granulated tissue, mature connective tissue, newly woven bone, and freshly produced blood vessels (Figure 6). In the QESCH group, new woven bone development and highly mature connective tissue partially blocked the site of a hole in the mandibular bone histological section taken 15 days after surgery (Figure 7).

During the 30 days post-operatively, the control group showed a significant amount of mature, vascular connective tissue filling up the bone hole, as well as a small area of freshly produced woven bone surrounding the mandible's edge (Figure 8). In contrast, during the same period in the QESCH, the findings showed that mature connective tissue with many blood vessels and fully formed new woven bone formed at the site of the obstruction (Figure 9).

Within the IHC sections Compared to the experimental group, the control group's cartilaginous and ossification zones demonstrated only mild expression of ALP. The connective tissue zone, on the other hand, showed no enzyme activity (negative expression) and was central to the damaged area (Figure 10). In the QESCH group, the ALP activity appeared moderate in the cartilaginous zone, central to the damaged area, and moderately expressed in the ossification zone. The ALP expression activity was lowest in the ossification zone and highest in the cartilaginous zone, according to the distribution of ALP activity (Figure 11).

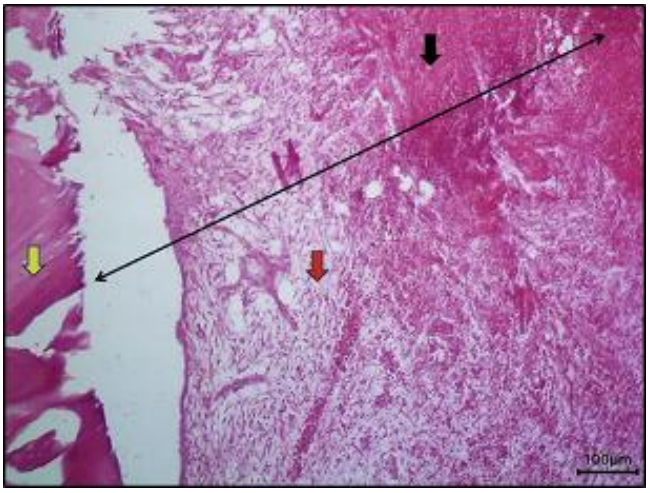


Figure 4: The control group's mandible bone at 7 days shows a hole (↔) with noticeable bleeding or a blood clot (black arrow) in the histological section. The area around the hole is surrounded by granulation tissue (red arrow), and the margin of the bone is marked with a yellow arrow. It is a 100X H&E stain. Scale bar=100µm.

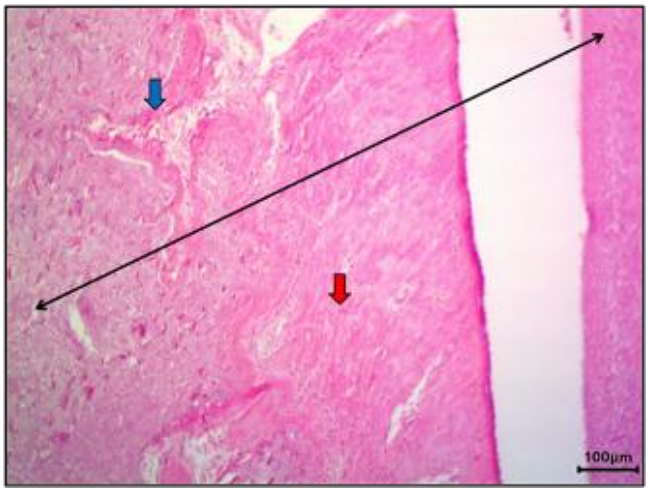


Figure 5: This histological section of the mandibular bone from the QESCH group (7 days) shows the location of the hole (↔) encircled by mature connective tissue (red arrow) and new woven bone development with osteoblasts (blue arrow).100X H&E stain. Scale bar=100µm.

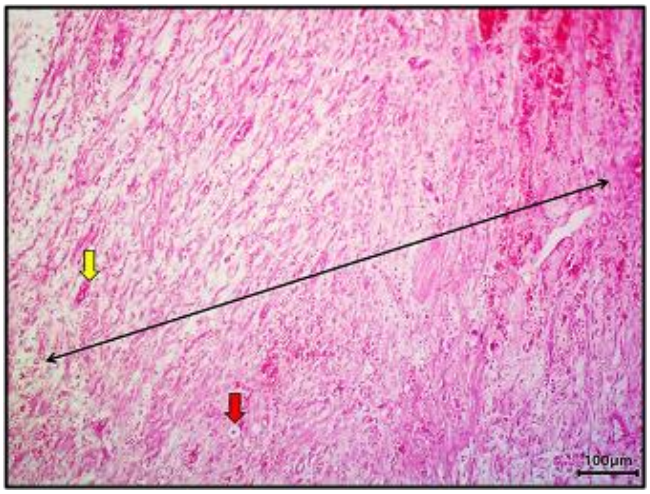


Figure 6: This is a histological slice of the control group's mandible bone taken fifteen days after the procedure. It shows the location of the hole (↔) blocked by a hemorrhagic clot (black arrow), densely packed connective tissue (red arrow), and, finally, existing or newly formed blood vessels (yellow arrow). The stain is 100X H&E. Scale bar=100µm.

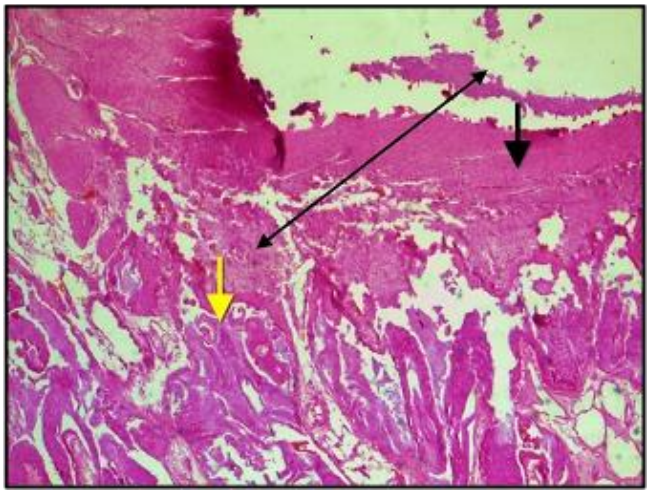

Figure 7: A 15-day histological slice of the QESCH group's mandibular bone reveals the hole's location (↔) with dense connective tissue (black arrow) with newly formed woven bone (yellow arrow). 100X H&E. Scale bar=100µm.

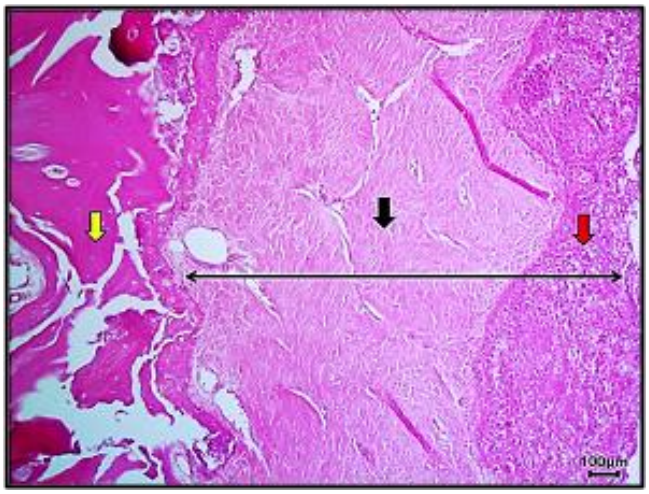


Figure 8: After 30 days, the new woven bone begins to form (red arrow), the edge of the mandible bone (yellow arrow), and the position of the hole (↔) blocked by completely grown connective tissue (black arrow) can be seen in this histological investigation of the control group's mandible bone. 100X H&E stain was used. Scale bar=100µm.

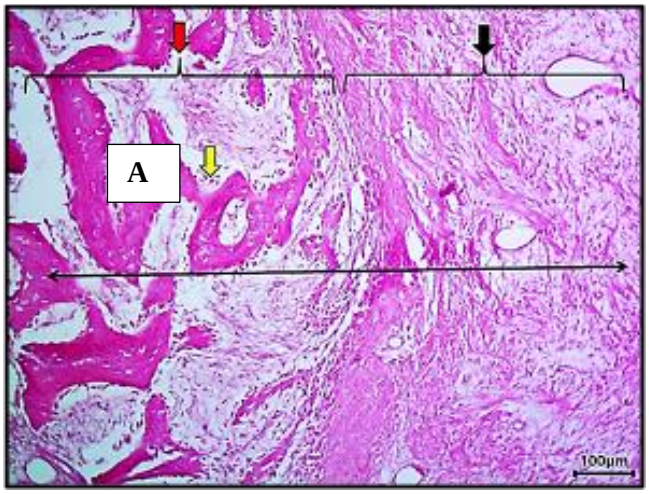


Figure 9: Histological analysis of the mandibular bone from the QESCH group at 30 days shows a variety of changes, including the formation of freshly woven bone (red arrow), several osteoblasts (yellow arrow), and a gap filled with mature connective tissue and numerous blood vessels (black arrow). It is a 100X H&E stain. Scale bar=100µm.

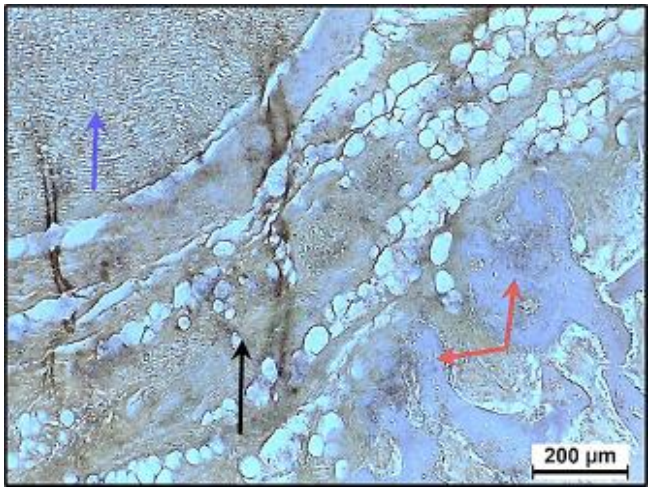


Figure 10: The control group's mandible bone defect area at 30 days PS showed (golden-brown) staining when we immunohistochemically stained for ALP activity under the light microscope. This indicated that the enzyme was weakly expressed in the cartilaginous zone (black arrow) and the ossification zone (red arrow. However, no enzyme activity in the connective tissue zone (negative expression led to staining with hematoxylin, scale bar=200µm.

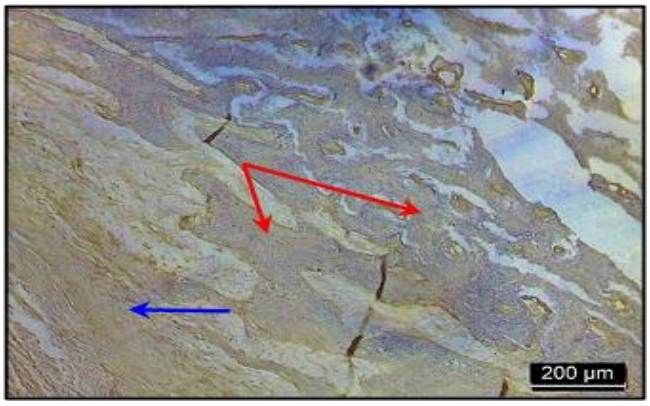


Figure 11: At 30 days post-operatively, immunohistochemical staining was used to identify the expression of ALP activity in the second group's mandible bone defect area under a light microscope. A moderate amount of expression was indicated in the cartilaginous zone and the ossification zone, as the stain appeared as a golden-brown tint using hematoxylin as a stain. Scale bar=200µm.

**Discussion**

Eggshells are considered a possible natural source for medicinal purposes due to the abundance of inorganic components, the most common of which are calcium carbonate ($CaCO_3$), magnesium carbonate ($MgCO_3$), and calcium phosphate ($Ca_3(PO_4)_2$) (10). Eggshells are a great source of calcium and have few harmful components, which is only one of their numerous advantages. Another perk is that they contain a lot of calcium carbonate, which is present in nature as the amorphous crystal calcite (17). When the drill machine, tool, force, angle, and speed are precisely controlled during the drilling procedure, the clinical results show that it can successfully induce canine mandibular bone

defects of a crucial size without causing harm to the bone tissues (17). Furthermore, there was no evidence of wound dehiscence after the wounds in both groups healed, which is particularly important when accessing the mandibular bone from the side is necessary for the surgery. According to a recent study, using a lateral technique was associated with fewer wound-related problems (18).

In neither group has histological analysis shown any obvious signs of bone necrosis or aberrant cellular activity (such as osteocyte distraction) around the lesion. So, the drilling force did not damage the bones or generate heat. Osteocytes, the cells in the bone matrix that sense mechanical strain, are also very sensitive to this kind of stress. Another sign that mechanically loaded force was not present in the bone during drilling is the presence of osteocytes within the lacune in the native bone edge (19). Histopathological examinations of the experimental animals' mandibular bone defect regions revealed varying degrees of new bone growth in both groups.

Compared to the control group, the QESCH groups had significantly more mature bone tissue and an increase in osteoblast, osteocyte, osteoclast, and bone trabeculae development at7,14 and 30 days post-surgery, according to the histopathological scores. The QESCH and control groups showed no statistically significant changes regarding bone-bridged creation. We concur with the prior research that found calcium hydroxide to activate osteoblasts at a later stage and that this process starts with a slow rise in tissue alkalinity to a pH of around 10.5, which triggers osteoblast differentiation and growth (20). In addition, some studies have shown that experimental dogs need approximately fifteen to thirty days following treatment for the calcium hydroxide to begin calcifying the pulp root canals (21). Due to the severe cytotoxic effects of the pure powder of $Ca(OH)_2$ and the strong alkaline environment, researchers are apprehensive about utilizing calcium hydroxide alone. Therefore, to increase the rate of osteogenic activity, some writers thought that calcium hydroxide mixed with other substances was the best option. To avoid the cytotoxic effect of calcium hydroxide in the neighboring tissue and to promote quick osteogenic qualities with sustained antibacterial activity (22).

The manufactured calcium hydroxide particles, which showed up in big, irregular morphology, are associated with substantial reductions in bone tissue and bone bridge formation in the QESCH. Previous research corroborated our findings by clarifying that big particles reduce the area available for bone growth (23). At the 30-day mark, histological sections from the control group revealed cartilaginous callus formation and immature bone tissue filling the gap. In contrast, QESCH revealed varying degrees of mature bone formation, regeneration, and endochondral ossification (24).

The immunohistochemical assessments give an extra layer of proof regarding the early reaction of biomaterials after implantation. Progenitor or developing osteoblasts produce ALP, a marker enzyme (25). Additionally, on day 15 following the bone defect, several osteoblasts lining the trabecular bone had positive immunostaining for alkaline phosphatase. Additionally, alkaline phosphatase was expressed by certain cells lining the bone bridge (26,27). Immunohistochemistry findings revealed deficient levels of ALP expression associated with granulocyte presence in connective tissue 30 days following surgery in the control group.

## Conclusion

We concluded that the processed quail egg shells are biocompatible and do not induce inflammatory or immunological reactions, they can be used as bone substitute biomaterials, aiding the bone tissue healing process.

## Acknowledgments

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## Conflict of interest

The authors declare no conflict of interest. Funding The authors funded this study.

## Editorial board note

Muneer S. Al-Badrany and Dhafer M. Aziz the editors of the Iraqi Journal of Veterinary Sciences, did not participate in any stage of the decision-making process for this article.

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# إعادة تدوير هيدروكسيد الكالسيوم من قشر بيض السمان لإعادة بناء الفجوة الفكية في الكلاب: التغيرات النسيجية المرضية والمناعية الكيميائية

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[1]فرع الجراحة والتوليد، كلية الطب البيطري، جامعة تكريت، تكريت، [2]فرع الجراحة وعلم تناسل الحيوان، كلية الطب البيطري، جامعة الموصل، الموصل، العراق

**الخلاصة**

كانت الفجوة العظمية الفكية التي تم إنشاؤها في الكلاب موضوع تحليل هذه المخطوطة، والذي تضمن الفحوصات المناعية الكيميائية والصور النسيجية وتقييم بيض السمان المعالج. في هذه التجربة، تم تقسيم الحيوانات الى أربعة وعشرين كلبا بالغا بالتساوي بين مجموعتين: مجموعة السيطرة وقشر بيض السمان. توضح النشرة المقصودة استخدام الطريقة الحرارية المائية لإنتاج فجوات عظمية فكية دائرية وإعادة بنائها باستخدام مسحوق هيدروكسيد الكالسيوم المعاد تدويره من قشر بيض السمان. في اليوم ٧ و١٥ و٣٠، قمنا بتحليل النتائج وتقييم النتائج السريرية والنسيجية والمناعية الكيميائية. عند مقارنة مجموعة قشر بيض السمان بمجموعة السيطرة، أظهرت النتائج النسيجية وجود إنتاج عظمي جديد، جنبا إلى جنب مع رد فعل التهابي، وارتفاع في الخلايا العظمية والناقضة للعظم، وظهور قنوات هافرس. وأظهرت نتائج المناعة النسيجية أنه في اليوم الثلاثين، كان لدى مجموعة قشر بيض السمان تعبير إيجابي طفيف عن الفوسفتيز القلوي، على عكس مجموعة التحكم التي كان تعبيرها ضعيفا للغاية. أخيرا، لم يلتئم عيب عظم الفك السفلي لمجموعة التحكم تلقائيا كما هو الحال عند مقارنة مجموعة قشر بيض السمان بمجموعة السيطرة، كانت التغيرات النسيجية والمناعية أفضل بشكل ملحوظ في مجموعة قشر بيض السمان. نظرا لأن قشور بيض السمان متوافقة حيويا ولا تسبب تفاعلات التهابية أو مناعية، فيمكن استخدامها كمواد حيوية بديلة للعظام، مما يساعد في عملية الشفاء.