Original Article

Histopathological Evaluation of Eggshell and DBM Combination on the Repair of Critical Size Experimental Calvarial Bone Defects in Rats

Masihipour, S[1], Sabiza, S[2*], Rezaie, A[3], Ghaderi Ghahfarokhi, M[4]

1. DVM Graduate, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
2. Assistant Professor, Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
3. Associate Professor, Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
4. Assistant Professor, Department of Food Hygiene, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

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Corresponding Author's E-Mail:
s.sabiza@scu.ac.ir

ABSTRACT

Fracture repair is a constant clinical challenge, and finding a method to promote and improve restoration is a primary goal for researchers. This is examined from various perspectives, such as fewer complications, increased speed, and cost-effectiveness. The present study aimed to investigate the effectiveness of eggshell powder, compared to the commercial form of demineralized bone matrix (DBM), in critical-size defects in rat calvarial bone. In this study, 40 adult male Wistar rats were selected and randomly divided into four groups of 10. The first group was the control group (C), the second was the eggshell powder group (E), the third was the DBM group (D), and the fourth was the one simultaneously receiving eggshell powder and DBM (DE). In these groups, a 5 mm diameter defect was created in the calvaria using a trephine. All animals received the appropriate treatment for their group. Each group was then divided into two subgroups of five. On days 30 and 60 post-surgery, these subgroups were euthanized, followed by sampling and histopathology examinations. After evaluating the repair percentage using Quick Photo software, the DE group had the highest repair percentage on days 30 and 60. Groups E and D had similar recovery percentages, with group D having a slightly higher one. There was a significant difference between all three groups and the control group. In conclusion, eggshell powder may potentially serve as a suitable substitute for some transplants.

Keywords: Bone healing, Eggshell, Rat

1. Introduction

Bone is a specialized type of connective tissue that, like other connective tissues, consists of cells and an extracellular matrix, including dense and spongy bone (1). The cellular components of bone comprise bone progenitor cells, osteoblasts, osteoclasts, osteocytes, and hematopoietic elements of the bone marrow (2). The healing of fractured bones occurs in three overlapping stages: 1) inflammatory, 2) repair, and 3) regeneration. Disorders in any of these stages can cause delayed union, malunion, or even nonunion (2). Bone tissue has a great capacity for self-regeneration; however, complicated injuries, such as fractures, or surgically induced lesions after tumor resection or osteomyelitis are common, which disrupts the normal bone healing procedure. Bone regeneration has involved the use of scaffolds, growth factors, and stem cells. Mesenchymal stem cells are multipotent adult stem cells that can differentiate into various connective tissue phenotypes, such as osteoblasts, chondrocytes, and adipocytes (3).

The use of allografts, autografts, as well as other substitutes and materials similar to bone grafts, enhances the quality of repair and accelerates the healing process (4). Hydroxyapatite is a natural compound that contains bone microminerals and stimulates cell divisions when used in bone defects (5). Hydroxyapatite powders are produced from two types of raw materials, falling into two categories: synthetic and natural (6). Natural hydroxyapatite can be extracted from materials such as sea shells and egg shells (7).

Compared to other sources, eggshell is a superior source of calcium, containing calcium oxide, calcium carbonate, calcium hydroxide, calcium phosphate, or hydroxyapatite (8). Natural hydroxyapatite is expected to offer better metabolic activity and more dynamic responses than its synthetic counterpart (9). Demineralized bone matrix (DBM) is another alternative that plays a crucial role in orthopedic surgeries (10). DBM is an important treatment option for appendicular, axial, and craniofacial skeletons (11).

The purpose of this study was to investigate the effect of eggshell powder on the repair of experimental bone defects in rats, either alone or in combination with DBM powder.

2. Materials and Methods

In this study, 40 adult white male Wistar rats with an average weight of 250 to 300 gr were randomly selected and used. The animals were randomly divided into four groups of 10. To facilitate the process, each group was further divided into two subgroups of five. Samples were collected from these subgroups on days 30 and 60 after surgery. The four main groups included the control group (C), the one receiving eggshell powder (E), the one receiving DBM powder (D), and the one receiving the combined powder of eggshell and DBM (DE).

The eggshells were thoroughly washed with tap water, disinfected with alcohol, and left intact for 24 h at room temperature to remove moisture. Afterward, the thin inner membrane of the eggshells was carefully removed with forceps. The eggshells were rinsed and washed again with distilled water, then placed in an oven at a temperature of 45°C for 24 h to dry. Subsequently, they were finely ground using a home grinder, and sieves with mesh sizes of 18 and 40 were employed to separate small and large particles, with particles larger than this value being discarded. The required number of samples was packed into 20 packages of 2 g and sterilized using gamma rays with a dose of 9 kGy (12).

On the day of the experiment, all animals were transferred to the surgery room and anesthetized through intraperitoneal administration of 2% xylazine (10 mg/kg) and 10% ketamine (100 mg/kg). After anesthesia, the calvaria was clipped from the muzzle to the back of the neck through a clipper. Following a preliminary scrub, a betadine scrub was performed on the skin of the site and its surroundings. The animals were then transferred to the operating table and placed in the sternal position. A 2 cm long incision was made in the middle of the skull by a surgical blade. The skin

and periosteum were removed from the skull, and a bone disk was created and removed in the calvaria using a trephine with an outer diameter of 7 mm. According to the assigned groupings, the bone defects were filled with the desired powder.

Next, the layers were closed in a single layer using a simple single pattern and non-absorbable nylon thread. On the 30th and 60th days after surgery, rats were euthanized by injecting a high dose of 2% xylazine and 10% ketamine. A piece of the skull containing the graft was separated and placed in 10% formalin. The samples were preserved in 10% formalin for seven days, and after calcium removal by 90% formic acid in a 125 ml mixture with 125 ml of distilled water, they were sectioned in the middle. After molding and preparing slides, hematoxylin-eosin and Goldner's trichrome staining were performed.

The SPSS software (version 24, IBM Corporation, NY, USA) was used for data analysis. One-way analysis of variance and the LSD post-test were employed to compare the data between the studied groups. The results were presented as mean±standard error, and $P \leq 0.05$ values were considered significant.

3. Results

3.1. Evaluation on the 30th Day after the Defect was Created

Microscopic examination of the samples prepared after 30 days in the control group revealed the formation of soft callus tissue, consisting of connective fibers with newly formed vessels and numerous inflammatory cells along the defect. These cases were observed in all four samples. Bleeding was also observed in some areas due to the looseness of the walls of the newly formed vessels (Figures 1 and 2).

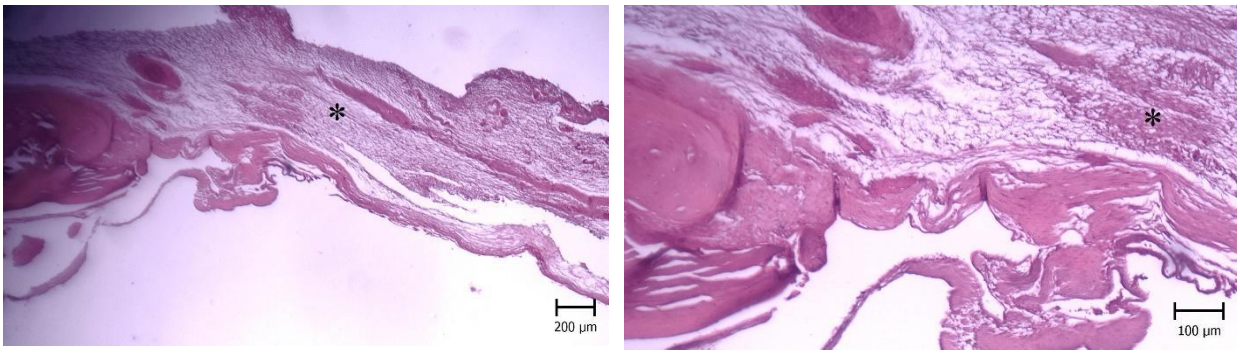

Figure 1. Defect in calvaria bone. control group. Wistar rat. 30 days after the defect occurs. Pay attention to the presence of soft callus tissue (black star) in the defect site (hematoxylin and eosin staining).

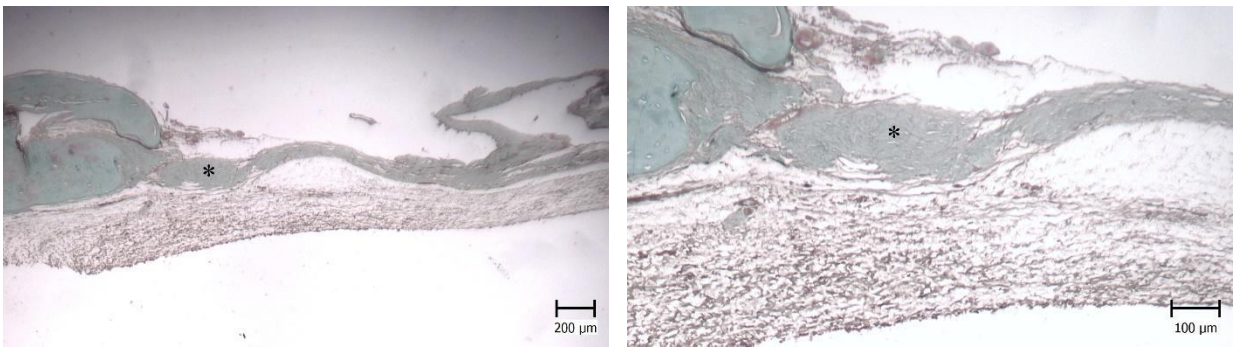

Figure 2. Defect in calvaria bone. control group. Wistar rat. 30 days after the defect occurs. Pay attention to the presence of soft callus tissue (black star) in the defect site (Goldner's Trichrom staining).

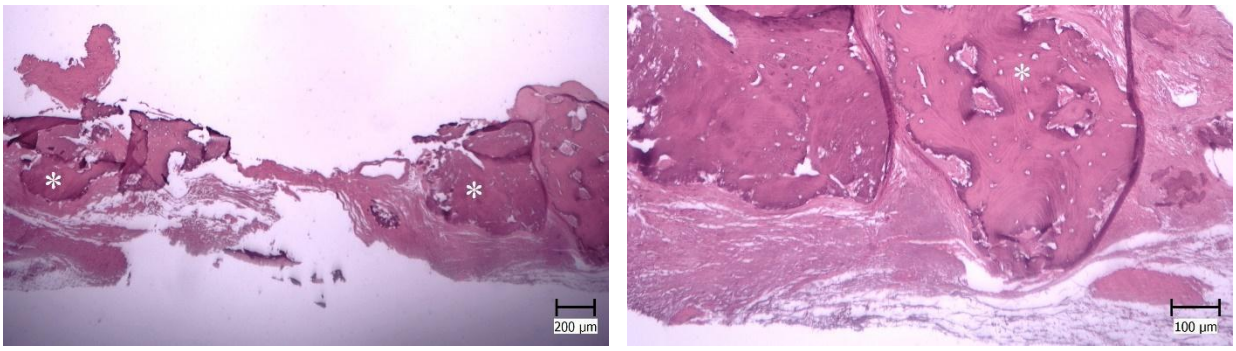

Figure 3. Defect in calvaria bone. Group D. Wistar rat. 30 days after the defect occurs. Pay attention to newly formed bone masses in the defect site (white star)(hematoxylin and eosin staining).

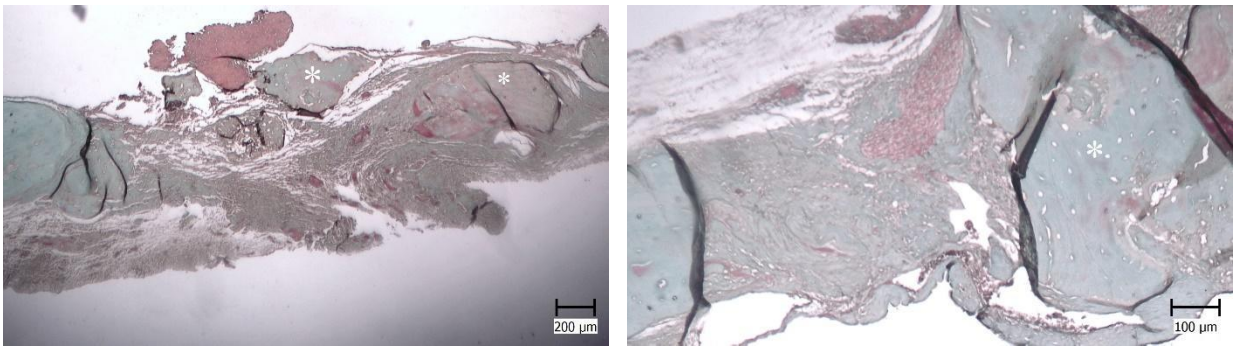

Figure 4. Defect in calvaria bone. Group D. Wistar rat. 30 days after the defect occurs. Pay attention to newly formed bone masses in the defect site (white star) (Goldner's Trichrom staining).

In three samples from group D, multiple masses of newly formed cancellous bone were identified at the defect site. The formed trabeculae had no specific order and were oriented in different directions, with osteocytes visible inside the lacunae. In one sample, only a small mass of newly formed bone was visible. Additionally, osteoblasts were observed around them. Among the cancellous bone mass, dilated and large vessels containing numerous erythrocytes and leukocytes were observed (Figures 3 and 4). In all rats of the third group, group E, numerous foci of bone tissue formation were observed among the callus connective tissue. In the center of most of these foci, spaces containing basophilic substances were seen. These newly formed lumps were of the spongy bone type (Figures 5 and 6).

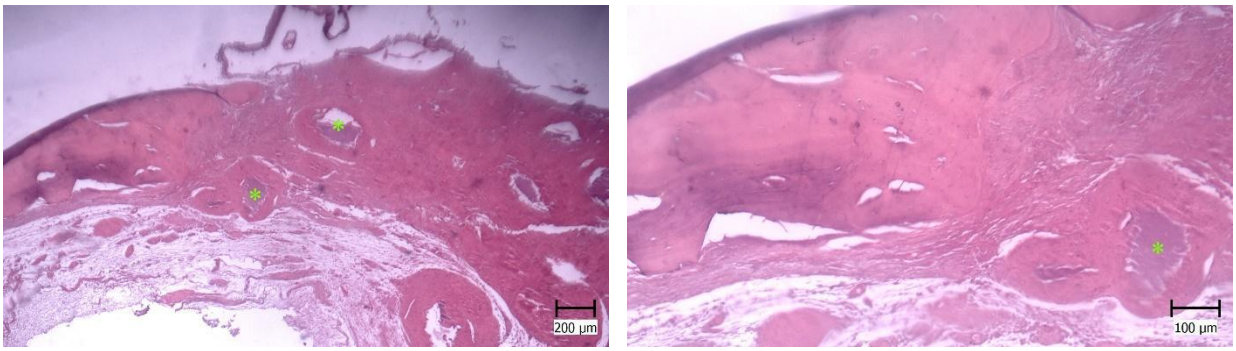

Figure 5. Defect in calvaria bone. Group E. Wistar rat. 30 days after the defect occurs. Pay attention to newly formed bone masses in the defect site (white star) and numerous spaces in the defect site with the presence of basophilic substances in it (green star) (hematoxylin and eosin staining).

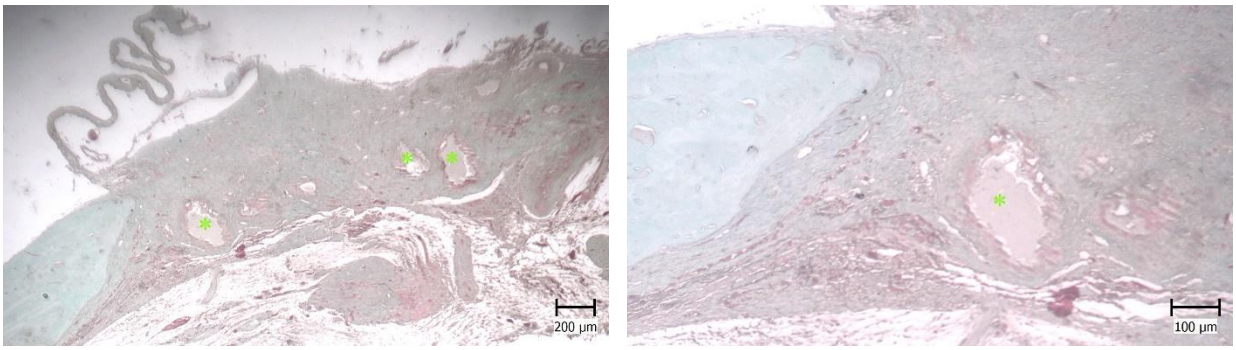

Figure 6. Defect in calvaria bone. Group E. Wistar rat. 30 days after the defect occurs. Pay attention to newly formed bone masses in the defect site (white star) and numerous spaces in the defect site (green star) (Goldner's Trichrom staining).

In the rats of the fourth group, the DE group, large foci of spongy bone tissue were observed. In two samples, the masses were larger and occupied a wide area. Additionally, in the center of some of these foci, numerous spaces containing basophilic substances were seen (Figures 7 and 8).

3.2. Evaluation on the 60th Day after the Defect was Created

Microscopic examination of the samples prepared after 60 days in the control group showed the presence of soft and hard callus tissue, as well as small foci of newly formed bone in all samples. This ossification

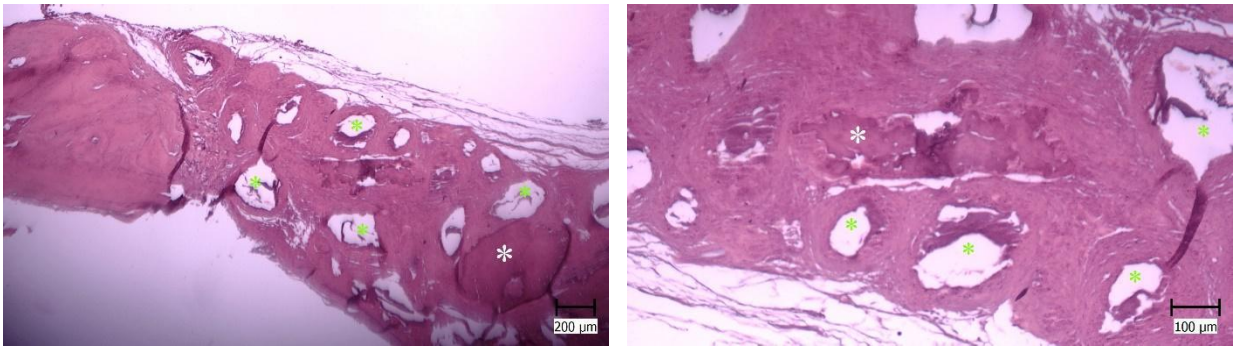

Figure 7. Defect in calvaria bone. DE group. Wistar rat. 30 days after the defect occurs. Pay attention to newly formed bone masses in the defect site (white star) and numerous spaces in the defect site with the presence of basophilic substances in it (green star) (hematoxylin and eosin staining).

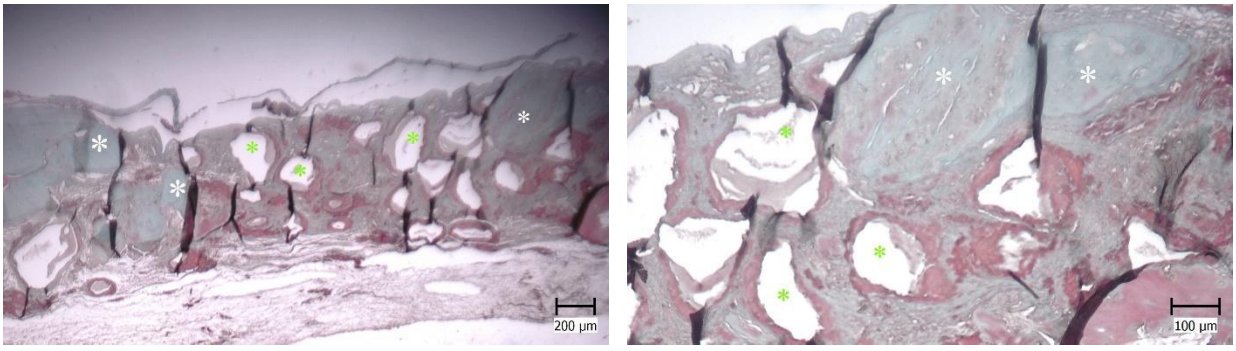

Figure 8. Defect in calvaria bone. DE group. Wistar rat. 30 days after the defect occurs. Pay attention to spongy bone tissue (white star) and multiple spaces in the defect site (green star) (Goldner's Trichrom staining).

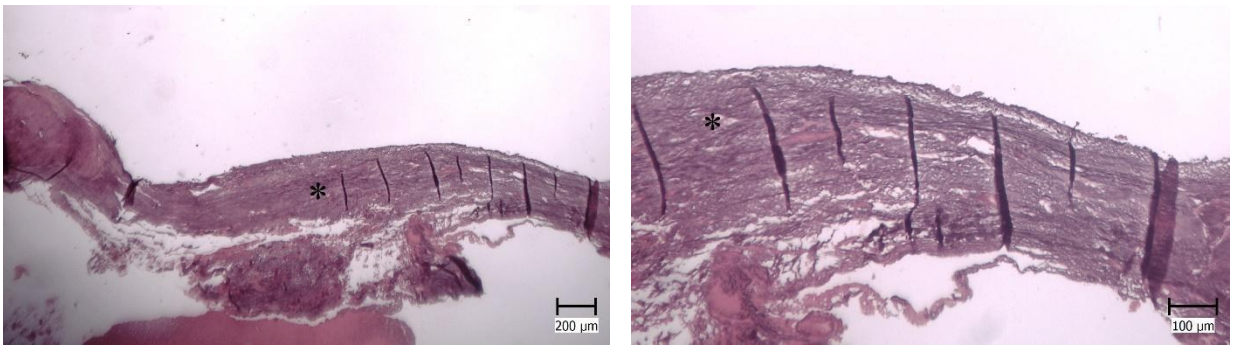

Figure 9. Defect in calvaria bone. control group. Wistar rat. 60 days after the defect occurs. Soft callus tissue (black star) (hematoxylin and eosin staining).

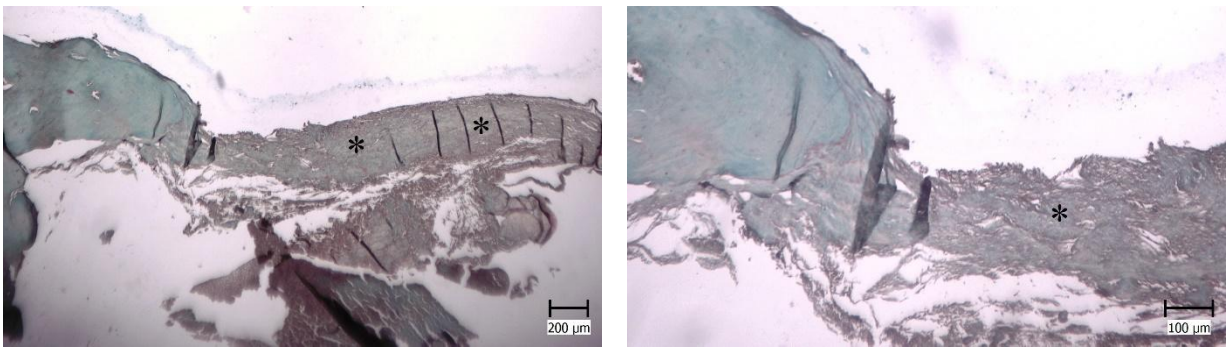

Figure 10. Defect in calvaria bone. control group. Wistar rat. 60 days after the defect occurs. Soft callus tissue (black star) (Goldner's Trichrom staining).

had started from the sides of the defect, and the new bone mass was forming toward the center (Figures 9 and 10). In group D, numerous masses of spongy bone were observed, along with the formation of lamellar bone masses. Regular blades of lamellar bone were observed along the defect. Additionally, in the lamellar bone, the vessels among the lamellae were atrophied, and a small number of osteocytes were seen in the lacunae. These mentioned cases were completely seen in three samples, while in one sample, less lamellar bone was formed, and the defect site was filled with spongy bone (Figures 11 and 12).

In group E, several foci of spongy and lamellar bone tissue were observed among the callus connective tissue. Furthermore, in the center of some of these foci, spaces containing basophilic

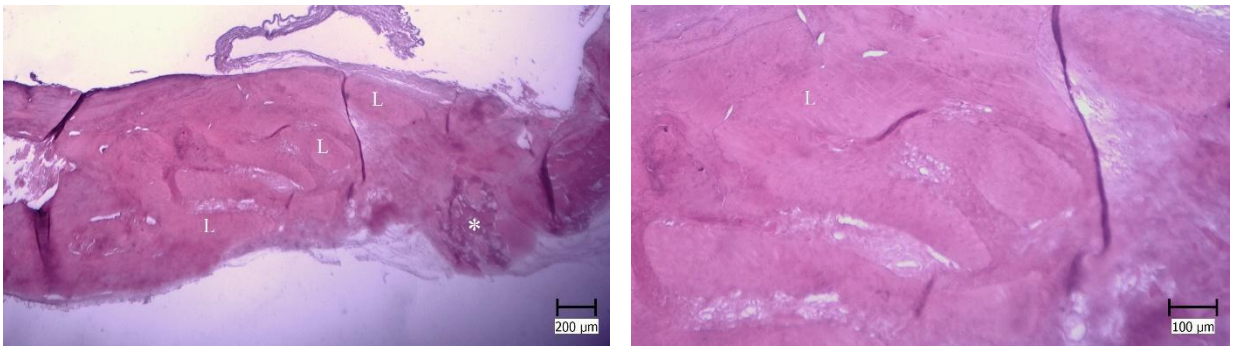

Figure 11. Defect in calvaria bone. Group D. Wistar rat. 60 days after the defect occurs. Lamellar bone tissue (L) can be seen in parallel blades (hematoxylin and eosin staining).

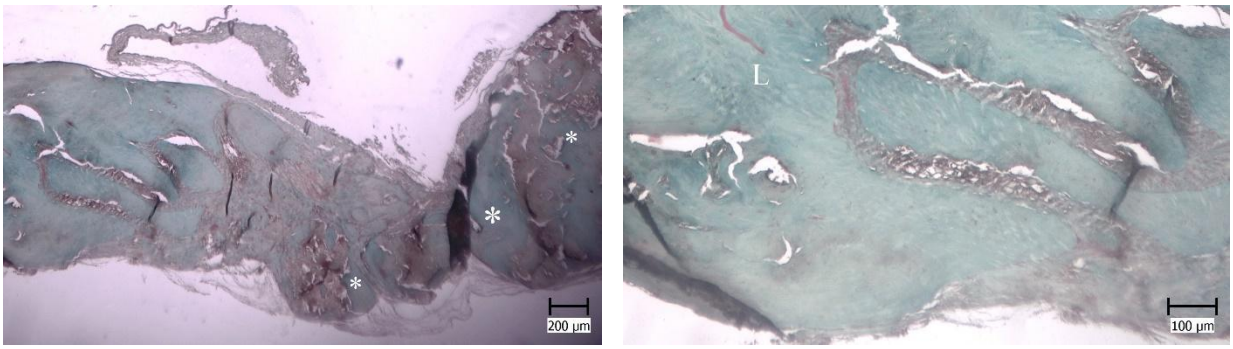

Figure 12. Defect in calvaria bone. Group D. Wistar rat. 60 days after the defect occurs. Spongy bone tissue (white star) and lamellar bone tissue (L) are seen in parallel blades (Goldner's Trichrom staining).

substances were seen. It should be noted that in one case, the masses were larger, and in the other three cases, the bone masses were smaller (Figures 13 and 14). In three samples from the DE group, the entire length of the defect contained lamellar bone masses and foci of spongy bone tissue with numerous spaces containing basophilic materials (Figures 15 and 16).

3.3. B-Percentage of Ossification of the Experimental Calvarial Bone Defect

According to the histopathological evaluation, the highest percentage of bone formation on the 30th day after the defect was assigned to group DE, while the lowest was assigned to group C. This difference was statistically significant (*P*=0.001) (Table 1 and Chart 1). On the 60th day after creating the defect, the highest

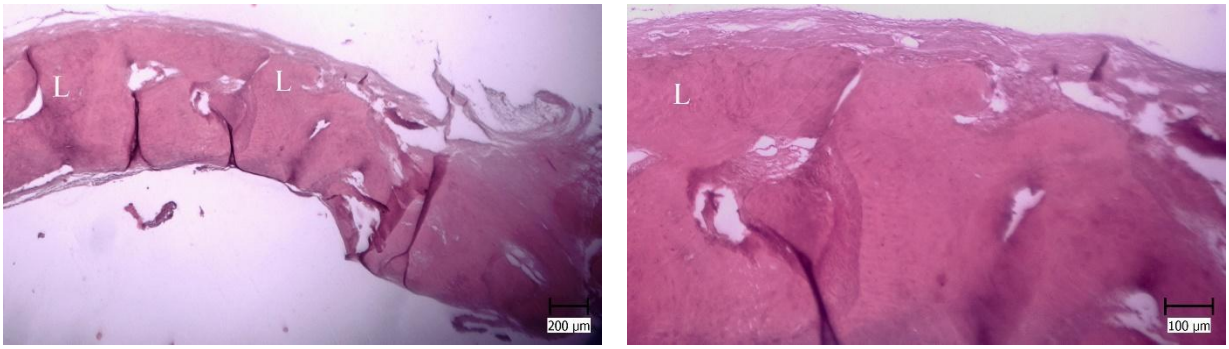

Figure 13. Defect in calvaria bone. Group E. Wistar rat. 60 days after the defect occurs. Lamellar bone tissue (L) can be seen in parallel blades (hematoxylin and eosin staining).

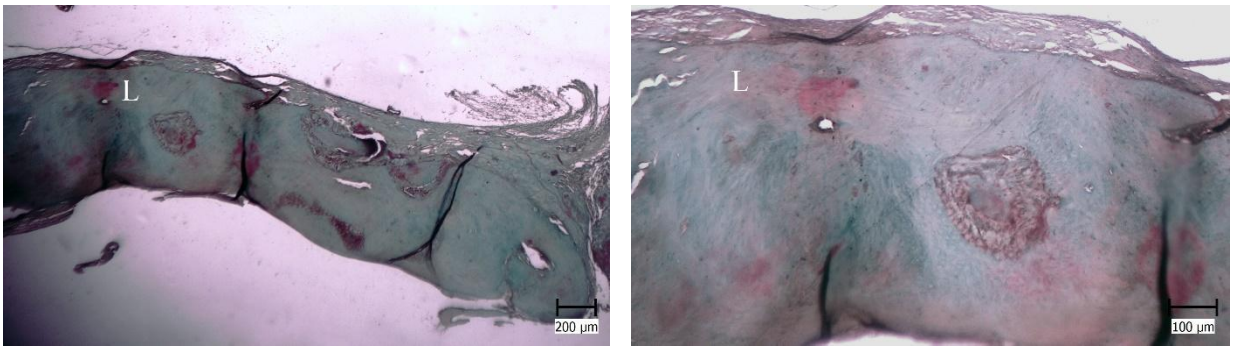

Figure 14. Defect in calvaria bone. Group E. Wistar rat. 60 days after the defect occurs. Lamellar bone tissue (L) (Goldner's Trichrom staining).

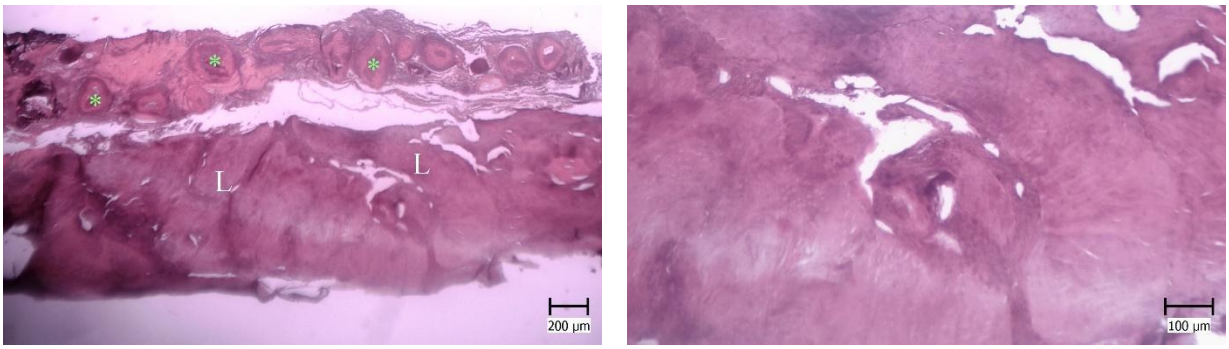

Figure 15. Defect in calvaria bone. DE group. Wistar rat. 60 days after the defect occurs. Lamellar bone tissue (L) and multiple spaces in the defect site (green star) (hematoxylin and eosin staining).

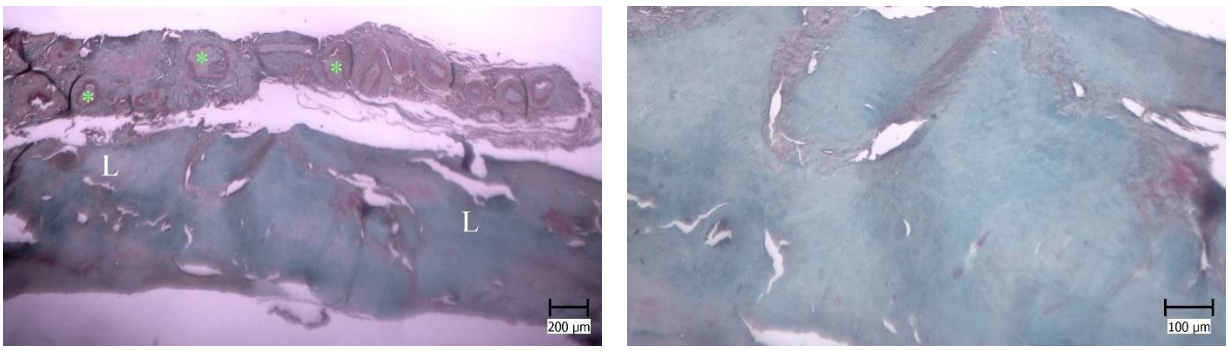

Figure 16. Defect in calvaria bone. DE group. Wistar rat. 60 days after the defect occurs. Lamellar bone tissue (L) and multiple spaces in the defect site (green star) (Goldner's Trichrom stain).

percentage of ossification was assigned to group DE and the lowest to group C. The difference between these two groups was statistically significant (*P*=0.001) (Table 1).

Table 1. Mean ± standard error of ossification percentage of experimental calvarial bone defect in rats

group / day	30th day evaluation	60th day evaluation
Control(C)	1.87±3.81 D, DE	3.31±16.22 E, D, DE
Eggshell powder(E)	3.32±14.19 D, DE	4.61±57.00 C, DE
DBM powder(D)	1.38±27.78 C	1.17±63.28 C
Combined powder of DBM and Eggshell (DE)	6.72±27.41 C, E	1.84±67.34 C, E

Inserted letters means there is a significant difference with the target group ($p<0.05$).

4. Discussion

The choice of an ideal bone graft depends on several factors, including tissue viability, defect size, size, shape, and volume of the graft, biomechanical properties, transport of the graft, cost, ethical issues, biological characteristics, and complications (13). Although autografts are considered the gold standard of transplantation, their use is impractical, especially in extensive bone injuries, due to limited resources. Additionally, taking a transplant from a sick patient is associated with the risk of contamination. The use of metal implants also has the risk of releasing harmful ions and their accumulation in different tissues, subsequently increasing the incidence of cancer. For this reason, researchers are seeking materials that reduce these problems, and in this direction, biodegradable biomaterials and tissue engineering have been developed (14,15).

Hydroxyapatite is a natural compound that includes bone microminerals, and when used in bone defects, it stimulates cell divisions (5). According to studies, the eggshell powder had a positive effect on the defects created in the tibia bone of 10 male dogs (12). Additionally, Alhussary et al. (1), in their study conducted in 2020, reported the effectiveness of eggshell powder on experimental defects in the mandible of 16 rabbits, which was done in line with the current research. Another study conducted by Kattimani et al. (5) investigated the effect of eggshell hydroxyapatite on cystic bone defects in the human maxilla. This group reported that at the end of eight weeks after surgery, all defects filled with eggshell hydroxyapatite were completely healed. With radiological evaluation, they concluded that bone density in the group receiving the eggshell was very close to the surrounding bone, and compared to the group that did not receive any substance, it significantly improved the healing process. This point was also observed in the current research. In the present study, the histopathology results were examined on the 30th and 60th days after surgery. In the resulting samples, the eggshell-receiving group had significantly better repair of the defect than the control group, and the recovery percentage of this group significantly increased, compared to that of the control group.

It is well-known that the size of particles used in bone grafts is an important factor in determining their absorption rate (16). In this study, micrometer-sized particles were used, with eggshell particles measuring 425 µm and DBM particles ranging from 150 to 1000 µm. Urist et al. (17) successfully employed human-derived DBM for repairing long bone and lumbar spine defects for the first time in 1965, reporting promising results. In the conducted study, the improvement observed in the group receiving DBM was significantly different, compared to the control group, indicating the positive effect of this substance on defect repair. However, it was less superior to the group receiving eggshells, with their repair percentages being very close to each other. This is likely due to the easier availability of hydroxyapatite from eggshells.

Both of these materials are used either as scaffolds for cell migration or as foci for ossification. In both groups, most of the restoration occurred in such a way that these materials acted as foci. By the 60th day, some of these foci were in contact with each other, overlapping, and significantly more numerous and larger than on the 30th day. Burwell (18) first suggested in 1964 that the bone induction potential of all implants could be maximized when combined with autografts. Later, the decalcified bone surface allogeneic graft was used as bone graft trays to preserve autogenous spongy marrow. The combination of autogenous bone and bone marrow with demineralized products increases the osteogenic potential of both bone marrow and demineralized matrix alone (11, 19).

In the current study treatment groups, the best result and most bone formation were observed in the group receiving both eggshell and DBM simultaneously. This group had a higher percentage of restoration than all other groups, indicating that although eggshell and DBM have positive effects on bone formation, their combined use enhances the potential of each substance individually. According to the results and investigations, eggshells had a positive effect on repairing critical defects in rats, with outcomes very close to a proven material such as DBM. It was also demonstrated that by combining these two substances, bone repair and healing can be further improved, yielding better quality restoration and an acceptable speed.

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Authors' Contribution

Investigator, data recording and manuscript

preparation: S. M.

Thesis, Surgery and manuscript preparation: S. S.

Histopathology assessments: A. R.

Egg shell preparation: M. GH.

Ethics

The project was approved by the local Committee of the Institutional Animal Care and Use of Shahid Chamran University of Ahvaz, Ahvaz, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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