

Effect of Hydrogen Peroxide on Autologous Cranioplasty in Rabbits

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ABSTRACT

Background: Craniotomy is used to treat open depressed skull fractures, often leading to a skull defect requiring cranioplasty. An alternative technique involving immediate reinsertion of fractured bone after cleaning and disinfecting was tested in rabbits.

Objectives: To evaluate autologous cranioplasty in treating open depressed skull fractures in rabbits, using 3% hydrogen peroxide to sterilize contaminated bone fragments before reimplantation.

Materials and methods: Four groups of local rabbits (3.1–4.5 kg, 3–5 months old), each with 4 animals, were examined after surgery. Group A was assessed at 1 week postoperative, Group B at 3 weeks, Group C at 6 weeks, and Group D served as a control with normal bone tissue for histological reference. Rabbits were euthanized under deep anesthesia, wounds were re-explored, and bone fusion was examined by microscope.

Results: All treated groups differed from the control. Group A showed inflammation without regeneration. Group B demonstrated moderate tissue response and regeneration. Group C showed significant remodeling and new bone formation. Group D had matured, stable bone with no inflammation or healing activity.

Conclusion: Hydrogen peroxide-treated bone fragments enhanced healing and graft integration after cranioplasty in rabbits. Group C exhibited the highest healing response, and the method reduced the need for foreign implants or repeated surgeries.

Keywords: Cranioplasty; Skull fracture; Hydrogen peroxide; Rabbit model; Bone healing.

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INTRODUCTION

To enhance the overall quality of life for patients and decrease the chance of complications related to skull defects or deformities, cranioplasty is a vital procedure that helps to improve physical integrity and mental stability [1, 2]. The use of cranioplasty is not only for human beings, but it can also be used successfully on an animal with similar functional and aesthetic concerns [3, 4]. Various surgical interventions were performed depending on the requirements of a particular patient, the severity of the cranial defect, and the preference of the surgeon. Such methods are titanium mesh reconstruction, autologous bone grafting, alloplastic implants, tridimensional printed implants, dermal layers, and hydroxyapatite cement [5–7]. The etiology of the defect, such as size and location,

general clinical condition of the patient, level of skills of the dealing surgeon, experience with the procedure, and presence of the required resources, are the critical considerations to choose the best method for treatment [8].

A combined approach is essential for achieving of successful outcomes in a few cases. Neurosurgeons, plastic surgeons, radiologists, and biomedical engineers often collaborate to achieve accuracy and success in surgeries of cranioplasty [9, 10]. However, these surgeries have disadvantages, especially regarding cranial site postoperative infections [11]. Infection is a critical risk owing to the proximity of the surgical region to the brain, as well as to potential contamination during surgery [12, 13]. Meningitis and abscess formation are life-threatening secondary complications from these infections, leading to extra surgeries as well as longer hospital stays [14]. Proper infection control plays a critical role in supporting bone healing and reducing the risk of negative outcomes [15].

This pilot study examined whether open depressed skull fractures in rabbits could be managed using the animal's own

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bone fragments for reconstruction. The bone pieces were treated with 3% hydrogen peroxide to reduce the risk of infection while still allowing the skull to heal properly. Although various methods of sterilizing skull fragments exist, studies on the efficacy and safety of hydrogen peroxide in autologous cranioplasty for open fractures are still insufficient, posing a challenge to achieving optimal surgical outcomes.

MATERIALS AND METHODS

Animals

A total of 16 healthy local rabbits weighing between 3.1 and 4.5 kg and approximately 3 to 5 months of age were included in this study, which were collected from the animal house of the Experimental Research Unit, College of Veterinary Medicine, University of Mosul, Mosul, Iraq. Animals were kept in specially designed cages (one rabbit per cage) in the laboratory under suitable environmental conditions for seven days before the experiment. The rabbits were fed a typical diet and water ad libitum. The study duration was approximately 2 months, from April 2024 to June 2024.

Groups and experimental design

The rabbits were randomly separated into four equal groups, with four rabbits in each. Subsequently, all rabbits were weighed before treatment to calculate their doses.

Group A was obtained after 1 week post operation, group B after 3 weeks post operation, group C after 6 weeks post operation, and group D was the control group obtained from normal cranial bone.

Surgical operation

A depressed skull fracture was made using a sterile small screwdriver, hammered into the skull after intramuscular injection of 1 ml of ketamine (35 mg/kg) and xylazine (5mg/Kg) for sedation. The scalp was shaved, disinfected, and then a longitudinal incision was made over the site of injury. A craniectomy of 5 mm diameter was created around the depressed fracture site, and the depressed bone segment was removed. Upon inspection, the dura mater was intact in some cases and injured in others; the injured dura was left without surgical repair. Bleeding was controlled using direct pressure or direct instillation of 3% hydrogen peroxide. The bone fragments were cleaned from any dirt or hair that could be attached to them and then disinfected by immersing the fragments in a surgical bowl filled with 3% hydrogen peroxide for about one minute, the bone fragments were reinstalled to fill the skull defect, and the wound was closed in a sterile condition using 3/0 cutting silk suture (Figure 1). Dipyrene solution was given intramuscularly at a dose of 125 mg per rabbit for 4 days after the operation for managing postoperative pain. Oxytetracycline spray 2% was applied to the wound twice daily, and 0.5 ml penicillin G 200000 IU (with dihydrostreptomycin 200 mg) injection for four days was administered with close monitoring of the wound site for any signs of infection or wound discharge. Stitches were removed at day 7 for all rabbits.

Histological preparation and evaluation

Fixation in buffered formalin was necessary for histological preparation of cranial bone to preserve cellular detail. The bone was then decalcified using 5% formic acid in a low vacuum oven at 56°C and the removal of calcium was verified by

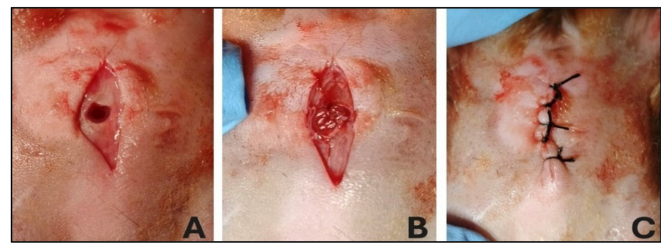


Figure 1. Experimental craniectomy model in an adult male local rabbit at the site of depressed skull fracture. B: Reinstallation of the bone fragment on the craniectomy site. C: Closure of the wound by cutting the silk suture.

ammonium oxalate testing [16]. Specimens were thoroughly cleaned, then double-embedded using the Peterfi technique and sectioned with a rotary microtome at a knife tilt of 45 to 50 degrees, usually at a thickness of 5 to 7 μ m [17]. Staining techniques often started with Masson's Trichrome for connective tissue contrast, followed by H&E.

A quantitative analysis of cranial bone was conducted by examining cells using a simple counting method under a light microscope (Novex B-Series Binocular Microscope, Novex, Amsterdam, Netherlands). This was done using a 48-megapixel digital camera (AmScope MU4800, AmScope, Irvine, California, USA) with a microscope, and a special fixer took a digital picture of the section. The number of cells from five fields for each rabbit was counted once from the center and again from the edge of the slide. The primary average taken from each rabbit (five fields) was added to the other averages of the same group for calculating the final average. The ImageJ 1.54 (NIH, USA) software was used to count the neutrophils, osteoblasts, osteoclasts, and osteocytes in the cranial bone sections. Cell / 400X field served as the cells' counting unit [18].

Statistical analysis

Data were obtained using microscopic cell counting from the rabbit's cranial bone. Normal distribution was verified using the Kruskal-Wallis's test, and the results were given as mean $\pm$ SEM ($M \pm SEM$). Differences between groups were analysed using one way ANOVA test. Statistical analysis was conducted using IBM SPSS Statistics version 27 (Statistical Package for the Social Sciences; IBM Corp., Armonk, New York, USA) with a significance level of P-value < 0.05 [19].

RESULTS

Microscopical findings

Haematoxylin and Eosin stain

Group A (Figure 2; A) shows late inflammatory phase features: areolar tissue with neutrophils and hematoma. Group B (Figure 2; B) demonstrates active healing with areolar tissue deposition, osteoblast proliferation, and active osteoclasts. Group C (Figure 3; A) exhibits normal architecture with a defined defect and callus zone separated from native bone by a reactive osteoblastic layer. Group D (Figure 3; B) shows normal skull histology with intact fibrous symphysis.

Masson's trichrome stain

In the first week, cellular granulation tissue dominates with disorganized woven bone indicates an inflammatory phase and

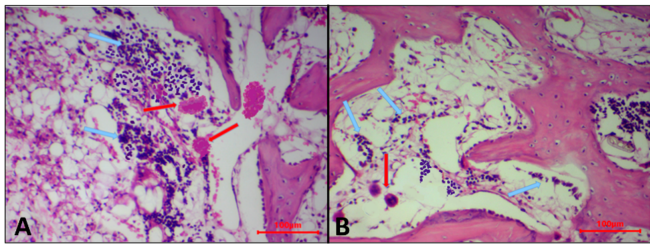


Figure 2. Depressed skull bone fracture in a rabbit. A: Group A at 1 week postoperatively showed deposition of areolar tissue (Blue arrow), hematoma (Red arrow) [H&E, 100X]. B: Group B at 3 weeks postoperatively showed osteoblast (Blue arrow), Osteoclast (Red arrow) [H&E, 100X].

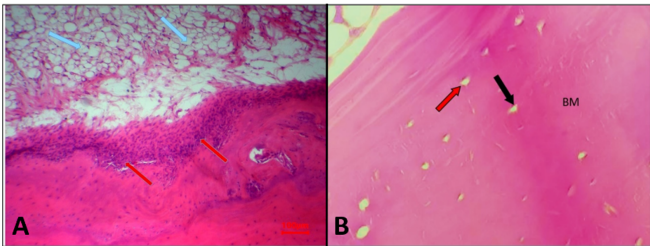


Figure 3. Depressed skull bone fracture in a rabbit. A: Group C at 6 weeks postoperatively showed callus healing area (Red arrow), adipose tissue (Blue arrow) [H&E, 100X]. B: Group D obtained from normal cranial bone showed normal histologic features, lacuna (Red colour), Osteocyte (Black colour), BM: Bone matrix. [H&E, 400X].

early osteogenesis (Figure 4; A). In the third week, the hard callus stage with cohesive woven bone mass filling the gap, showing maximal osteogenic activity without lamellar structure. In the sixth week, mature bone marrow with adipocytes and organized woven bone indicates the remodeling phase (Figure 4; B), marking restoration of the bone environment and replacement of woven bone with lamellar bone. Finally, in the control group, organized, dense lamellar bone with embedded osteocytes, typical of mature cortical bone (Figure 4; C).

Histological analysis

Histological analysis revealed significant differences between groups (Table 1). Inflammation was most pronounced in group A, which recorded the highest neutrophil count, while group C showed virtually no neutrophilic activity, but it had active bone formation, as evidenced by the highest osteoblast and osteoclast counts among all experimental groups. Osteocyte numbers progressively increased from group A through group C, approaching values comparable to the mature bone of group D.

We observed promising results; the bone showed clear progression toward normal architecture, no animals had adverse reactions to hydrogen peroxide, and the histological evidence consistently demonstrated good bone integration.

DISCUSSION

Cranioplasty is a well-known neurosurgical operation used on humans as well as animals to treat cranial defects. Bone

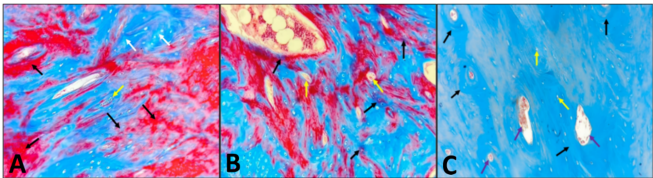


Figure 4. A: The early stage of bone healing of the skull bone in a rabbit of Group A at 1 week postoperatively showed granulation tissue (Black arrow), immature woven bone (White arrow), and osteoblasts (Yellow arrow), [Masson’s trichrome, 100X]. B: The advanced hard callus and early remodelling phase of the skull bone in a rabbit of group C at 6 weeks postoperatively showed remodelling bone matrix (Black arrow), bone marrow cavity (Yellow arrow), [Masson’s trichrome, 100X]. C: The normal, adult architecture of the skull bone in a rabbit in group D obtained from normal cranial bone showed lamellar bone matrix (Black arrow), osteocytes in lacunae (Yellow arrow), and haversian/vascular canals (Purple arrow), [Masson’s trichrome, 100X].

Table 1. Cell counts per group presented as mean ± standard error of the mean (M ± SEM). Different superscript letters (A,B,C) indicate statistically significant differences between groups (P-value < 0.05)*.

Groups	Polymorphonuclear cells number (M±SEM)	F score	P-value
A	187.75±12.59 ^C	194.95	0.001
B	66.00±0.40 ^B		
C	2.00±0.40 ^A		
D	0.00±0.00 ^A		
Groups	Osteoblast cells number (M±SEM)	F score	P-value
A	24.25±0.85 ^A	497.56	0.001
B	33.50±0.64 ^A		
C	240.75±9.95 ^B		
D	0.00±0.00 ^C		
Groups	Osteoclast cells number (M±SEM)	F score	P-value
A	1.25±0.25 ^A	20.00	0.001
B	3.50±0.64 ^B		
C	5.50±0.64 ^C		
D	0.75±0.25 ^A		
Groups	Osteocyte cells number (M±SEM)	F score	P-value
A	6.50±0.64 ^A	62.68	0.001
B	23.50±0.64 ^A		
C	74.50±8.87 ^B		
D	102.00±6.75 ^C		

* Abbreviations: P-value: Probability value; statistical significance was set at P-value < 0.05, F: F-statistic; the ratio of variance between groups to variance within groups, derived from one-way Analysis of Variance (ANOVA).

fragments could be used on the same patient or animal if they are treated with a disinfectant material like 3% hydro-

gen peroxide. Although hydrogen peroxide is toxic to various cells due to its potent oxidative nature [20], it is considered a powerful stimulator of bone formation based at dilute concentrations and rapid exposure [21]. The application of this material in this study agrees with established clinical protocols for managing contaminated bone tissue. Supporting evidence was provided by a recent prospective investigation conducted by Elnoamany et al. [22], which involved washing depressed bone fragments with diluted hydrogen peroxide for 15 minutes in a cohort of 59 patients suffering from traumatic brain injury. The previous technique produced good aesthetic outcomes and kept patients satisfied, with complications occurring in just 8.5% of cases. Similarly, Zhang et al. [23] achieved positive results when they used preserved skull bone treated with povidone-iodine, maintaining low infection levels. The protocol adopted in the current study for the deployment of hydrogen peroxide shows similar results to those established approaches, and this method disinfects the bone tissue effectively while preserving its ability to heal (the core objective of this research).

The histological results showed distinct phases of bone healing at the experimental time points, which matched recognizable phases of fracture healing. In group A in the initial week, abundant inflammation was evident with high neutrophil counts (187.75 ± 12.59), plenty of areolar connective tissue, and hematoma formation. The initial phase of inflammation is pivotal in initiating healing, as it is through this that debris is cleared from necrotic cells and preparative signals are delivered that call for mesenchymal stem cells to come to the site of injury, as seen in bone healing studies [24].

In group B, the inflammation was significantly reduced by the count of neutrophils (66 ± 0.4), and active bone formation was indicated by elevated osteoblast activity (33.5 ± 0.64) with the formation of woven bone callus. This finding referred to the reparative phase, in which osteoprogenitor cells differentiate to become osteoblasts that begin forming new bone matrix. Masson's trichrome staining showed vascular and cellular granulation tissue along with disorganized woven bone, supporting that the tissue healing was at the hard callus stage. These histological findings agreed with those reported by Shapiro and Wu [25] on rabbit calvarial models.

Most significant changes occurred in group C, in which elevated osteoblast activity was recorded (240.75 ± 9.95), accompanied by increased numbers of osteoclasts (5.5 ± 0.64), indicating bone remodelling was occurring. The presence of mature bone marrow with fat cells, a well-organized lamellar bone pattern, and an increased number of osteocytes (74.5 ± 8.87) indicated progression into the remodelled phase. These findings aligned with those of rabbit calvarial bone regeneration reported by recent studies [26, 27], who indicated robust bone formation within 6 to 12 weeks' postoperative periods, attesting to the use of rabbits in cranioplasty studies.

In this study, several limitations need clarification. Firstly, there is an absence of an untreated control group (normal saline instead of hydrogen peroxide), making it difficult to compare the effect of the treated and untreated groups. Secondly, there were no microbiological cultures or assessments of clinical signs of infection, which limits the information about antimicrobial effectiveness or safety. Third, all animals received broad-spectrum antibiotics as recommended by the ethics committee, making it impossible to determine hydrogen peroxide's independent role in preventing infection.

Fourth, the study depends on histological analysis without biomechanical testing or imaging techniques like micro-computerized tomography; therefore, we cannot confirm the actual strength or mineralization of the healed bone. Finally, the six-week follow-up in rabbits may not reflect what happens in human patients over longer periods.

CONCLUSION

This pilot study concluded that autologous cranioplasty, using treated bone fragments with hydrogen peroxide, is an effective method to repair depressed skull fractures in rabbits. Tissue analysis revealed that healing proceeded through the normal stages of inflammation, repair, and remodeling. The best results came after 6 weeks, when the activity of bone-forming cells increased, bones were still being reshaped, and normal bone structure was restored. These findings confirm the effectiveness of this treatment method and need further investigation in more controlled studies, as it could potentially eliminate the need for a secondary cranioplasty procedure following craniectomy for traumatic brain injury with depressed skull fractures.

ETHICAL DECLARATIONS

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Ethics Approval and Consent to Participate

The study was carried out in accordance with institutional guidelines and received ethical approval from the Medical Research Ethics Committee, University of Mosul, Mosul, Iraq, under certificate number UOM/COM/MREC/23-24/MAR6.

Consent for Publication

Not applicable.

Availability of Data and Material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing Interests

The authors declare that there is no conflict of interest.

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The research is self-funded, and no external funding has been received.

Use of Artificial Intelligence

Claude was used for grammar checking.

Authors' Contributions

All listed authors made significant, direct, and intellectual contributions to the work. They have read and approved the final version of the manuscript.

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