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Original Article

Periosteum-induced ossification effect in skull defect through interleukin-8 and NF-κB pathway: An experimental study with *Oryctolagus cuniculus* rabbits

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Received : 15 January 2022
Accepted : 07 March 2022
Published : 15 April 2022

DOI
10.25259/SNI_62_2022

Quick Response Code:

ABSTRACT

Background: The purpose of this study was to analyze the response of inflammatory cytokines interleukin-8 (IL-8) and NF-κB to the closure of skull defect with periosteum as a scaffolding material in bone healing used after surgery.

Methods: Thirty *Oryctolagus cuniculus* rabbits underwent a craniotomy to create a 20 mm diameter round defect in the parietal bones. The parietal bones were returned to its place and stabilized by an internal plate fixation. The defects were either left empty or implanted with periosteum. At 6 weeks, the specimens were euthanized and examined.

Results: Histological examination showed a more well-developed formation of woven bone in the periosteum group. Immunohistochemical examinations showed that the use of periosteum in the closure of skull defects reduced the NF-κB and IL-8 response which affected the ossification process.

Conclusion: The experiment showed that the use of periosteum was linked with IL-8 and NF-κB downregulation toward ossification effects at any point throughout the trial. Periosteum usage might be beneficial as a scaffolding material in bone healing for autograft cranioplasty in animal model and could be applied to clinical practice.

Keywords: Cranioplasty, Interleukin-8, NF-κB, Periosteum flap

INTRODUCTION

Decompressive craniectomy is a surgical procedure that involves the removal of the skull bone to protect the patient from increasing intracranial pressure.[8] If the circumstances permit, the bone will be reattached; this is referred to as cranioplasty. However, this treatment has certain risks for the patient. One of these potential problems is bone resorption. This kind of bone resorption may occur in individuals who have been fitted with bone and whose bone has been compromised in

terms of osteogenesis and vascularization as a result of the method involved.[9]

Numerous cranioplasty methods have been developed to address the problems of bone resorption. Duhamel and John Hunter performed one of them utilizing the periosteum membrane, which has osteogenic characteristics. The periosteal membrane is made up of two layers: an exterior (fibrous and smooth) layer and an interior layer (cambium). The outer layer is densely packed with collagen and the reticular layer is densely packed with osteoblasts and osteoprogenitor cells, while the inner layer is densely packed with osteoblasts and osteoprogenitor cells.[2] The mechanism of how pericranium-assisted autograph cranioplasty technique accelerates bone growth has not yet been clearly defined.

MATERIALS AND METHODS

Study design

The research protocol was authorized by the authorities of Universitas Airlangga, Department of Veterinary (Experimental Animal Studies, study number 2.KE.087.10.2020). The experimental study used 10 adults *Oryctolagus cuniculus* rabbits that were at least 5 months old and weighed between 4 and 5 kg. Animals were randomly divided into two groups (Group: with periosteum and without periosteum), each containing five rabbits. After 6 weeks, all animals were sacrificed and immunohistochemistry examination was performed.

Medication of animals

All operations were performed under general anesthesia, 65 mg kg^{-1} ketamine and 4 mg kg^{-1} xylazine, administered intramuscularly into the hind leg. Each animal received an intramuscular injection of 100,000 IU prophylactic benzylpenicillin. Following surgery, the animals received analgesics for 3 days, once daily, muscularly using phenylbutazone.

Craniotomy protocol

The rabbits' head was shaved between the eyes and the ears for surgical site preparation. The skin was sterilized with a solution of 10% iodine. After administering a local anesthetic (1 mL lidocaine and epinephrine solution); subcutaneously, a midline incision was created, and the skin and periosteum were reflected to reveal the skull. Round bone defects (diameter 20 mm) were bored into the inner cortex using a bone trephine with dural preservation. Following bone plate removal, the bone is returned and fixed using a long plate [Figure 1]. Periosteum sheets of each animal were applied over the bone defect in one group. Miniplates (Osteonic, Seoul, Korea) and surgical stitching (Silk 4.0, OneMed, Surabaya, Indonesia) for the fixation of bone and pericranial membranes are used throughout. The miniplate and thread utilized in this procedure are those used in neurosurgery. The skin was sutured properly. The animals were humanely euthanized with pentobarbital after 6 weeks of observation.

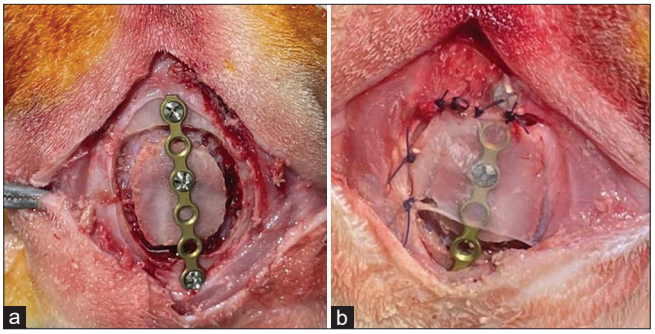

Figure 1: Autograft cranioplasty with long plate fixation following craniotomy procedure. (a) Defect placement without periosteum and (b) defect placement with periosteum.

Histopathological examination

For 4 h, the specimens were preserved in cold 4% formaldehyde. The tissues were frozen in optimum cutting temperature compound (Sakura Finetek, Torrance, CA). Sections were cut to a thickness of 5 mm, attached on charged slides, and allowed to air dry for 5 min before staining. Staining with hematoxylin and eosin was carried out according to the procedure. The samples were analyzed using a light microscope (Fluorescence Microscope Olympus IX71).

Immunohistochemistry examination

Immunohistochemical responses were performed on rabbit skull tissue obtained 6 weeks after periosteum implantation. A monoclonal mouse anti-human NF-κB antibody was used to determine the expression of the NF-κB marker (ready-to-use antibody; 2J10D7; Novus Biologicals, CO, USA). Interleukin-8 expression was quantified using antibodies, anti-interleukin-8 (IL-8) antibody (monoclonal mouse, ready to use; MBS2025703; MybioSource, San Diego, USA). At room temperature, all sections were treated with the primary antibodies for 1 h. In addition, antigen determinants of all proteins were exposed by boiling sections for 15 min at 98°C in 10 mM citrate buffer (pH 6.5). Light microscope was used to examine the materials using a Fluorescence Microscope Olympus IX71 (Olympus, Tokyo, Japan).

RESULTS

Thirty animals were utilized in the research. All animals recovered normally and were terminated as scheduled. The two groups, rabbit skull following placement of periosteum

and without periosteum, were compared from all skull fragments. The final study comprised 30 animals, and [Figure 2] illustrates the skull after 6 weeks.

In those with periosteum, the woven bone formation could be observed on the defect walls without bridging the defects. The defect with the periosteum showed woven bone formation to the level of the original cortex [Figure 3]. In one of the sections without periosteum, a small area space reaction was observed in the soft tissue covering the defect. Otherwise, the space displayed is disjointed from the fault.

NF-κB expression/6 weeks

Statistical analysis showed a significant difference between each group at week 6 posttreatment ($P < 0.05$). Bone defects without periosteum showed the highest number of NF-B compared to cranioplasty with pericranium grafts [Figures 4 and 5].

IL-8 expression/6 weeks

Statistical analysis showed no significant difference ($P > 0.05$) between the use of periosteum and without periosteum at week 6 posttreatment. Bone defects without periosteum showed the highest IL-8 expression compared to the use of periosteum [Figures 6 and 7].

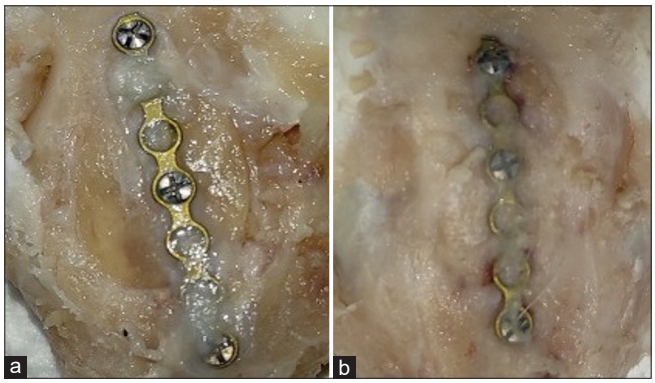

Figure 2: The defect of the rabbit skull following 6 weeks of observation. (a) Defect placement without periosteum and (b) defect placement with periosteum.

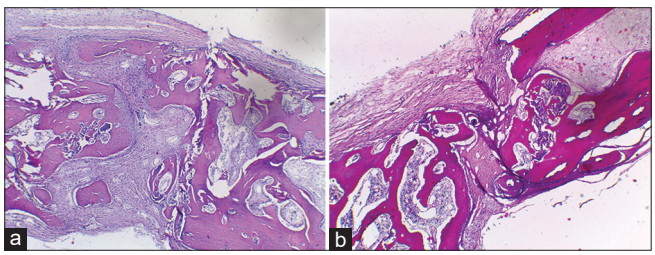

Figure 3: Hematoxylin and eosin stained sections of skull from animal after 6 weeks. (a) ×20 of skull defect without periosteum and (b) ×20 of skull defect after placement with periosteum.

DISCUSSION

Bone regeneration is a multistep process that begins with the mitotic expansion of progenitor cells at the sites of bone formation and continues with differentiation of these progenitor cells into functional osteoblasts. Our research sought to investigate if periosteum might be utilized as a scaffold for bone grafts, assisting in the multistep process of bone regeneration. In addition, we evaluated the osteogenic capacity of periosteum through the use of

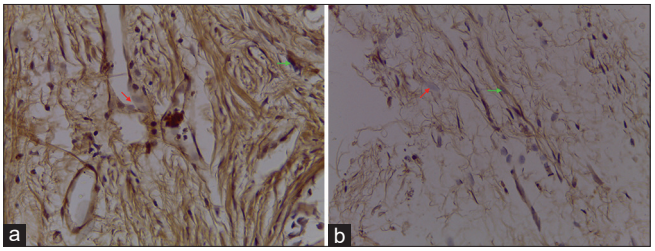

Figure 4: Staining of NF-κB antibody at week 6 provides immunoreactivity to macrophage cells indicated by green arrows. Macrophage cells that do not exhibit immunoreactivity are indicated by red arrows. (a) With periosteum and (b) without periosteum.

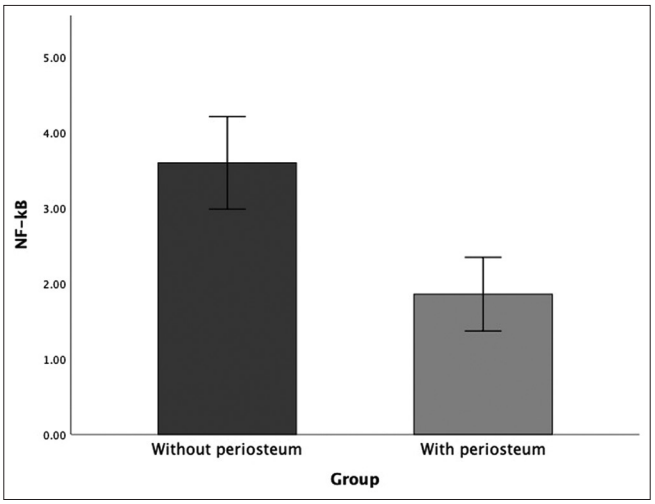

Figure 5: The mean difference of NF-κB expression at 6 weeks of observation.

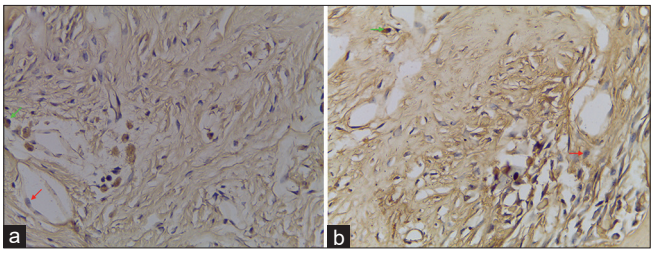

Figure 6: Staining of IL-8 antibody at week 6 provides immunoreactivity to macrophage cells indicated by green arrows. Macrophage cells that do not exhibit immunoreactivity are indicated by red arrows. (a) With periosteum and (b) without periosteum.

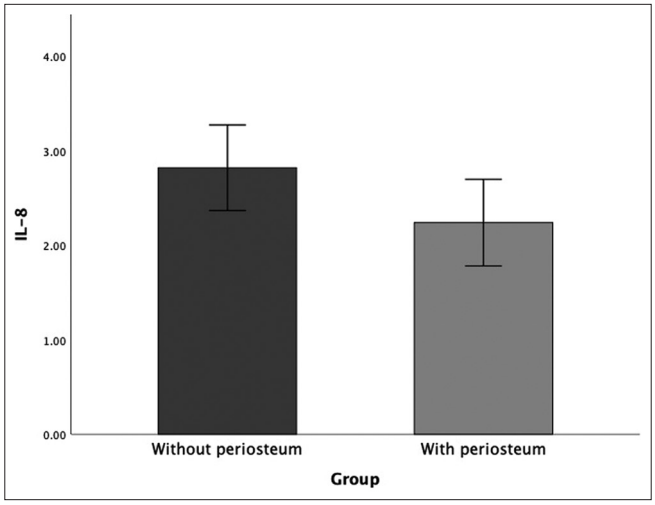

Figure 7: The difference in the mean value of interleukin-8 expression at 6 weeks of observation.

different osteoblastic phenotypic markers. Any bone graft material or scaffolding must possess the characteristics required to promote the growth of bone-forming cells in an optimum microenvironment. It was shown in the histologic examination that a more well-formed woven bone formation was observed in the periosteum group, indicating a faster bone healing compared to no periosteum flap.

The osteogenic capabilities of the osteoblasts were investigated for 6 weeks in our study. For assessing the osteogenic potential, Declercq, 2004, study and Aronow, 1990, study utilized time points ranging from 2 to 6 weeks. Investigators chose these different time points based on the component of osteoblasts they were studying. The 6-week endpoint appeared acceptable because we were evaluating the total response of the osteoblasts from all sources. Periosteum itself does not provide structural support, but it is ideal for covering faults and voids in the bone. It could be employed as a stand-alone substance to boost the osteogenic process. Periosteum can reduce the amount of artificial compound required to fill a defect, resulting in smaller donor sites and lower morbidity. The periosteum produced the least amount of IL-8 and NF-κB in our investigation. As a result of this discovery, we can deduce that the healing process will accelerate if bone defects are covered with periosteum. Our research demonstrated that when the graft is exposed to a sufficient supply of cytokines, nutrients, and hormones, osteoprogenitor cells become activated and stain positive for osteogenic potential indicators.

NF-κB (activated B-cell core factor) is a part of the transcription factor, which was first identified as a regulator of B-cell differentiation by its ability to bind to the B site of the kappa light chain gene in B cells. They were later shown to be involved in innate and adaptive immunity in response to pathogens and autoimmune stimuli and many aspects of normal cellular function.[3] The role of NF-κB in bone was first identified in the mid-1990s when two groups of researchers produced NF-κB1/2 double knockout (dKO) mice and found unexpectedly that they had tooth eruption failure and osteopetrosis in the absence of osteoclasts by producing both NF-κB1 and -2 single knockout mice which shown modest immune deficiency, which translate into severe B-cell and T-cell differentiation defects and are in that respect similar to RANKL and RANK KO mice, which are produced later after RANKL.[3]

NF-κB itself is one of the cytokines that have been known to play an essential role in the tissue healing process.[5] Various complex cascades related to tissue healing occur in the presence of activation of NF-κB. Activation of NF-κB causes a further activation process of more than 200 genes involved in inflammation, cell migration, cell proliferation, cell cycle, cell survival, and inhibition of apoptosis.[5] Specific to bone tissue, inhibition of NF-κB has been reported to be an effective means of inhibiting osteoclast activity and bone resorption.[1]

IL-8 has been reported by a previous study by Edderkaoui[4] to have an effect in bone healing conditions. In the case of human long bone fractures under 72 h, Hoff *et al.*[6] reported an increase in IL-8 levels in the hematoma area arising from fractures and the surrounding bone marrow, with higher levels in the hematoma area itself. These data suggest an increased inflammatory reaction in the lesion area, which was a fracture, which was similar to the case we studied. In our study, the same thing happened wherein the craniotomy case on the rabbit skull, IL-8 in that area was increased.[4,6] However, the decreased levels in the case of pericranium implantation could have several explanations, one of which is a reduction in the inflammatory reaction in the area affected by bone and/or pericranial grafts. Further studies may be needed to analyze further the inflammatory factors affected by the treatment and the healing of fractured lesions that occur in the bone tissue.[7]

This research has several limitations, two of which are the considerably low number of samples and short period of follow-up for the new bone formation. This experiment also does not evaluate the critical bone defect size, which may play an important role in the practice of autograft osteoplasty procedure.

CONCLUSION

This study demonstrates that the periosteum has the elements required for osteogenesis. Periosteum also exhibits lower IL-8 production and NF-κB laying down properties, according to the study. Because of its porous nature and the fact that it is autogenous, it causes no immunologic reaction, making it easy to assimilate into the defect. The periosteum is ideal for covering bone defects because it is readily available and has a layer-like consistency. Periosteum proved to be effective treatment evaluated in terms of promoting ossification and

inhibiting bone resorption inside bony defects produced in a rabbit calvarium model.

Acknowledgments

The authors would like to thank all who have contributed to the process and completion of this report, including the teaching staffs and fellow residents of the Department of Neurosurgery of Faculty of Medicine, Universitas Airlangga, Dr. Soetomo General Academic Hospital, Surabaya, Indonesia. We also want to thank the Faculty of Veterinary Medicine, the Department of Orthopaedics and Traumatology and Department of Public Health and Preventive Medicine, and the Department of Anatomical Pathology Faculty of Medicine Universitas Airlangga – Dr. Soetomo General Academic Hospital. We would also like to extend our gratitude toward the Department of Neurosurgery, Faculty of Medicine Universitas Hasanuddin – Dr. Wahidin Sudirohusodo Central General Hospital.

Declaration of patient consent

Institutional Review Board (IRB) permission obtained for the study.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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How to cite this article: Apriawan T, Widjiati W, Utomo DN, Al Fauzi A, Subagio EA, Utomo B, *et al*. Periosteum-induced ossification effect in skull defect through interleukin-8 and NF-κB pathway: An experimental study with *Oryctolagus cuniculus* rabbits. Surg Neurol Int 2022;13:140.