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Original Article

Histologic and histomorphometric evaluation of the effect of lactoferrin combined with anorganic bovine bone on healing of experimentally induced bony defects on rabbit calvaria

Mojgan Paknejad[1,2], Amir Reza Rokn[2,3], Ali Akbar Sabur Yaraghi[4], Flora Elhami[1], Mohammad Javad Kharazifard[5], Neda Moslemi[6,7]

[1]Dental Research Center, [2]Department of Periodontics, School of Dentistry, [3]Implant Research Center, [4]Department of Nutrition and Biochemistry, School of Public Health, [5]Biostatistician, Dental Research Center, [6]Laser Research Center in Dentistry, [7]Department of Periodontics, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

ABSTRACT

Background: Recent studies have shown that lactoferrin promotes the proliferation and differentiation of osteoblasts and inhibits osteoclast-mediated bone resorption. Anorganic bovine bone (ABB) graft has been extensively used as an osteoconductive material in the bone reconstructive surgeries. The purpose of this study was to examine whether the combination of lactoferrin with Bio-Oss would improve ossification in experimentally induced bone defects in rabbit calvaria.

Materials and Methods: In this randomized, prospective animal study, a total of 32 bone defects with the diameter of 6 mm were created on the calvaria of 8 male New Zealand rabbits (4 defects in each animal). One defect was filled with ABB + Lactoferrin + Vehicle (BLV), the second one with ABB + Lactoferrin (BL), the third defect with ABB + V (BV), and the fourth defect was filled with ABB (B) alone. After 4 weeks, histologic sections were prepared and evaluated histologically and histomorphometrically. The type, percentage and vitality of newly formed bone, inflammation, percentage of residual material, and foreign body reaction were assessed for each specimen. Data were analyzed using Friedman tests.

Results: All groups were similar in terms of inflammation and vitality, type, percentage of new bone formation, and residual material. The percentage of new bone formation in BLV, BL, BV, and B groups were 14.73 ± 3.14%, 15.02 ± 1.51%, 15.95 ± 2.24% and 13.44 ± 2.89% ($P = 0.1$) and the amount of residual biomaterial were 11.85 ± 1.50%, 13.73 ± 1.80%, 13.02 ± 1.86%, and 15.41 ± 2.05%, respectively ($P = 0.392$).

Conclusion: Based on results of this study, the combination of lactoferrin and ABB did not show any significant improvement in bone regeneration compared with ABB alone in surgically induced bony defects in rabbit calvaria.

Key Words: Animal experimentation, bone regeneration, bovine bone, lactoferrin, osteogenesis

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Address for correspondence:
Prof. Neda Moslemi,
Ghods Street, Enghelabave,
Post code: 14147, Tehran,
Iran.
E-mail: neda_moslemi@yahoo.com

INTRODUCTION

Milk is a rich biological fluid with many growth factors that have a critical role in skeletal growth and development of neonate.[1] Lactoferrin is a fraction of whey protein that is extracted from milk. This agent not only has been found in milk, but also has been extracted from exocrine secretions of mammals and secondary granules of neutrophils. This iron-binding glycoprotein belongs to the transferrin family and has shown multifunctional properties that among them antimicrobial effects,[2] modulation of inflammatory and immune response,[3] and promotion of bone growth are prominent. *In vitro* studies showed that lactoferrin potently stimulates proliferation and differentiation of osteoblasts and inhibits apoptosis of osteoblasts and formation of osteoclasts.[4,5] It was demonstrated that lactoferrin promotes the ability of osteoblasts

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to synthesize and mineralize bone matrix, as well.[1] A recent study showed that bovine lactoferrin induces synthesis of the angiogenic factors (vascular endothelial growth factor and fibroblast growth factor-2) in osteoblasts.[6] An *in vivo* study evaluated the effect of lactoferrin on bone mass. This study showed that subcutaneous injection of lactoferrin in mice resulted in dramatic increase in local bone formation.[4] Anorganic bovine bone (ABB) is an osteoconductive material that is used extensively for bone augmentation (Bio-Oss; Geistlich Biomaterials). Due to lack of osteoinductive properties of this bone graft, several agents have been combined to enhance its regenerative properties.[7] Bone morphogenic protein and platelet rich plasma are frequently used as osteoinductive agents;[8,9] however, some of their drawbacks like high cost and patient morbidity have limited their common uses in practice.[10]

To the best of our knowledge, no study has evaluated the effect of combination of lactoferrin and ABB in bone healing. The aim of this study was to evaluate the influence of addition of lactoferrin to ABB in comparison with ABB alone in regeneration of experimentally induced bony defect in rabbit calvaria.

MATERIALS AND METHODS

Eight healthy 6-month old New Zealand white male rabbits with average weight of 3.5 ± 0.3 kg were included in this randomized blind experimental study. The animal study protocol was approved by Ethical Committee for animal experiments of Tehran University of Medical Sciences. Each rabbit was anesthetized with an intramuscular injection of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone Merieux, Toulouse, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany). The animals were accommodated in separate cages and had free access to food and water from one month before surgical intervention.

Non-iron saturated bovine lactoferrin (Sigma Chemical Company; St. Louis, USA) was diluted in distilled water (1:10) to obtain the concentration of 500 μg/ml.[11,12] Seventy-microliter lactoferrin was used in each defect.

Tragacanth was used as a vehicle for lactoferrin. Tragacanth has been proposed as a carrier for lactoferrin.[13] This natural gum is obtained from *Astragalus gummifer Labillardiere* and other species of *Astragalus* grown in western Asia. Tragacanth gum is used as a suspending and emulsifying agent in a variety of pharmaceutical formulations. Its wide use is due to its stability in a wide range of temperature and pH, and its effectiveness as an emulsifying agent with extremely long shelf life.[14] As tragacanth is a natural polymer, it is nontoxic and biocompatible, making it a suitable medium for cell growth.[15,16]

Surgical procedure

The heads of animals were shaved, and the cutaneous surface was disinfected with povidone iodine solution prior to the operation. The calvaria bone was exposed through a skin incision of 10 cm in caudo-cranial direction. A periosteal elevator was used to separate the periosteum from the bone surface. Four similar circular bicortical defects (6-mm diameter) were made in the parietal bone using a trephine on a slow-speed electric handpiece with physiologic saline irrigation [Figure 1a]. For the first rabbit, randomly, one defect was filled with ABB + Lactoferrin + Vehicle (BLV), the second one with ABB + Lactoferrin (BL), the third defect with ABB + V (BV), and the fourth defect was filled with ABB (B) alone. For next ones the positions were changed rotationally (clockwise) [Figure 1b]. Closure of periosteum and subcutaneous tissues was done with 4/0 bioabsorbable polyglactin 910 suture (Vicryl, Ethicon Inc., Somerville, NJ), while the skin was relocated with Nylon 3/0 suture (Apositos Sanitarios Aragoneses, Huesca, Spain). After surgery, each animal received subcutaneous injection of Ketoprofen (0.1 ml daily for 3 days) and enrofloxacin (Baytril, Bayer Corp, Shawnee, KS, USA) (0.7 ml daily for 7 days). The animals were sacrificed with an overdose of sodium pentobarbital IV (Dolethal; Vetoquinol) after four weeks.

Histology and histomorphometry

All samples were prepared for hematoxilin and eosin (H and E) staining. In each animal, the entire calvarium was removed using a reciprocating saw.

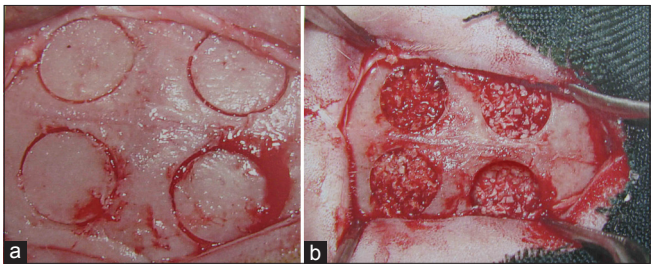

Figure 1: (a) Four bicortical defects (6-mm diameter) were made in the parietal bone using a trephine. (b) Defects were randomly filled with ABB+Lactoferrin+Vehicle, ABB+Lactoferrin, ABB+Vehicle, and ABB alone. (ABB: Anorganic bovine bone)

Samples were fixed in 10% buffered formalin for 2 weeks and then decalcified by immersion in nitric acid solution. Finally, samples were dipped in lithium carbonate solution for neutralization.

After bisecting each defect, specimens were dehydrated by graded alcohol and embedded in paraffin. Then, they were sectioned at 5-μm sections, stained with H and E, and examined under a light microscope (BX 51, Olympus Co., Tokyo, Japan). On average, three most central sections were obtained from each specimen for histologic and histomorphometric analyses. A masked, expert pathologist examined the following criteria in the histologic sections:

Inflammation was assessed based on inflammatory cell (IC) counts under a high power field (×40) as follows: grade 0 (no IC), grade 1 (1-100 IC), grade 2 (100-250 IC), grade 3 (more than 250 IC).[17]

Foreign body reaction was manifested as the presence of foreign body giant cells in a granulomatous response. The digital photographs were analyzed by Adobe Photoshop CS2 (Adobe Systems, San Jose, CA). In this analysis, the pixels within the marked area were counted using the software. The percentage of newly formed bone was calculated as follows: The number of pixels in the newly formed bone/total number of pixels in the defect × 100. Furthermore, the percentage of remaining material was calculated as the number of pixels in the area of remaining material/total number of pixels in the defect × 100.

Statistical analysis

The parameters were analyzed using Friedman test. Significance for the analyses were set at $P < 0.05$.

RESULTS

Healing was uneventful in all cases. A mild inflammation (grade I) was seen in one of specimens in BL group and moderate inflammation (grade II) was seen in one of samples in BV group. Other specimens showed no inflammatory reaction.

Foreign body reaction was not observed in any specimen.

Type of newly formed bone was a mixture of woven and lamellar bone in all specimens.

Mean (SD) of percentage of new bone and residual material is demonstrated in Table 1. The differences between groups were not statistically significant with regard to the amount of new bone formation ($P = 0.1$). In addition, four defects were not statistically different in terms of residual material ($P = 0.392$) [Figure 2a-d].

DISCUSSION

The results of this study showed that addition of lactoferrin did not have any positive influence on the amount of new bone formation in experimentally induced bone defects in rabbit calvaria.

Table 1: Mean (SD) of percentage of new bone and residual material in experimental defects

Treatment groups	Newly formed bone (%)	Remnant material (%)
B-L group	14.73±3.14	11.85±1.50
B-V group	15.02±1.51	13.73±1.80
B-L-V group	15.95±2.24	13.02±1.86
B group	13.44±2.89	15.41±2.05

B: Anorganic bovine bone, L: Lactoferrin, V: Vehicle

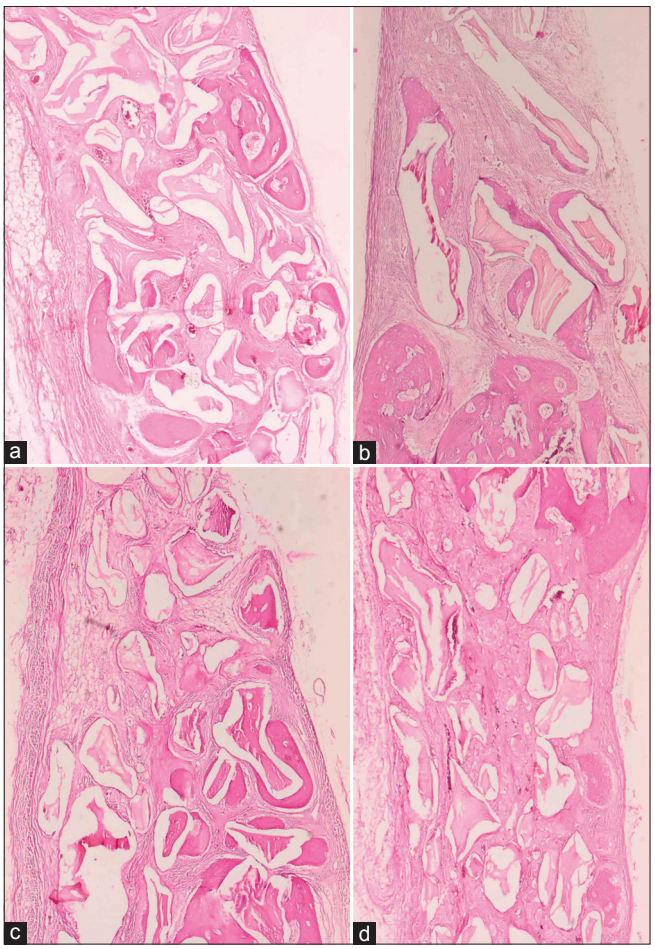

Figure 2: Hematoxylin and Eosin-stained section of defects. New bone formation and residual particles are observed in all defects (original magnification ×20). (a) ABB + Lactoferrin; (b) ABB + Vehicle; (c) ABB + Lactoferrin+ vehicle; (d) ABB alone. ABB: Anorganic bovine bone

Previous studies on rabbit calvaria stated that critical size defects in the range of 11-15 mm are suitable.[18-22] Analysis of the ability of different graft materials to create complete bone healing of a defect necessitates the use critical size defect, in order to prevent spontaneous bone regeneration. Since the present study was designed to investigate the early phases of regeneration, it was possible to use smaller defects. In addition, it is not possible to create four critical-size defects in rabbit calvaria, since it is too small. On the other hand, the results of a recent study showed that after 4 weeks, the percentage of new bone in 6 mm-defect was not different from 8 and 11 mm-defect. Furthermore, the authors suggested that four small defects could be used when early phase healing response of several materials are to be investigated.[23] In the present study, four defects were created in single animal to simultaneously compare several materials whilst avoiding inter-individual variation.

Several *in vitro* studies on lactoferrin and bone allowed direct contact of lactoferrin with bone cells.[1,4,24-26] However, to the best of our knowledge, this is the first animal study that used lactoferrin in direct contact with the defect. In previous animal studies, lactoferrin was administered orally or as subcutaneous injection.[4,12,27,28] With regard to inflammatory reaction, the results of the present study showed that most specimens (30 out of 32) showed no sign of inflammation and the addition of lactoferrin to Bio-Oss in the defects had no effect on inflammatory reactions of specimen. Regarding the inflammatory response to Bio-Oss, the result of this study is in accordance with other studies that reported no inflammatory reactions in defects filled with Bio-Oss.[29,30] Furthermore, this result confirms the previous findings, reported that lactoferrin modulates the inflammatory process, by regulating the proliferation and differentiation of immune cells and preventing the release of cytokines from monocytes.[31]

The percentages of remaining material were not significantly different between the study groups. However, B group showed slightly more, but not statistically significant, remaining material. This may be related to a fact that the surgical defect in B group was filled only with ABB and initially the volume of ABB (Bio-Oss) in B-group was higher than other groups. Therefore, this is not surprising that more material remained in B group after 1 month. The results of this study showed a slight more bone formation in BLV group (2.5% more new bone formation) in comparison with B group. However, this slight improvement was not statistically significant. The result of the present study is in accordance with that of Guo *et al*. study. To evaluate the effects of lactoferrin on bone loss in osteoporosis, lactoferrin was administered orally in ovariectomized rats. The authors indicated that although oral administration of lactoferrin attenuates estrogen-dependent trabecular bone loss in ovariectomized rats, this treatment did not alter the thickness of cortical bone in femoral diaphysis. The authors assumed that lactoferrin may be a more effective protecting agent against bone loss on the trabecular than cortical bone.[28] Noteworthily, calvaria consist mostly of cortical bone and this could partially explain the finding of this study that showed no detectable influence of lactoferrin in bone regeneration in experimentally induced bone defects in rabbit calvaria.

The current results are not in line with some other studies. Studies by Blais *et al*. showed that oral administration of lactoferrin decreased bone loss and bone resorption markers in ovariectomized rats[12] and this improvement was associated with modulation of immunological functions such as antigen presentation properties, T cell activation and down-regulation of cytokine molecules.[27]

In addition, Cornish *et al*. showed that subcutaneous injection of bovine lactoferrin for five consecutive days in mouse calvaria resulted in local bone formation after 10 days.[4] The influence of lactoferrin on bone growth might be apparent at earlier stages of healing. Since the specimens were prepared after 1 month in this study, the possible effects of lactoferrin cannot be evaluated in shorter intervals.

In the present study, lactoferrin was applied with a vehicle, in order to prevent washout of lactoferrin and to provide better sustain release properties. Noteworthily, although not significant difference, BV group showed more bone formation compared to B group (15.02% vs. 13.44%). No study was found to investigate the possible effects of tragacanth on bone regeneration. Tragacanth is a natural non-toxic gum. When solubilized in water, tragacanth forms viscous solutions. Tragacanth is among the most acid-resistant gums. The viscosity of tragacanth remains quite stable over a broad range (pH 2-10). Because of its high acid stability, the gum is widely used in acidic conditions in pharmaceutical industry.

Gum tragacanth is also an excellent emulsifying agent for oil-in-water emulsions. Tragacanth acts as a stabilizer for active ingredients of pharmaceutical products.[32] More new bone formation in defects filled with tragacanth may be due to the ability of this ingredient in stabilization of blood clot. The initial clot adhesion and wound stabilization is one of the most critical factors in wound healing sequence, which leads to predictable bone formation. The initial blood clot is a rich source of growth factors and signaling molecules that recruit clearing cells to the wound site.[33]

CONCLUSION

Within the limitations of this study, it can be concluded that application of bovine lactoferrin in experimentally induced bone defect cannot accelerate new bone formation in rabbit calvaria.

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