Original Article

Histomorphometric and histologic evaluation of the effects of leukocyte platelet-rich fibrin (L-PRF) and nano-hydroxyapatite (nHA) on bone regeneration in rabbits

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Abstract

Introduction: The study aimed to assess the histologic and histomorphometric effects of leukocyte platelet-rich fibrin (L-PRF) and nano-hydroxyapatite (nHA) on the regeneration of calvarial bone defects in rabbits.

Methods: Four defects were created in the calvaria bone of 14 New Zealand rabbits and filled with L-PRF clot, nHA, or a combination of L-PRF and nHA. The fourth defect remained unfilled to serve as the control group. The rabbits were sacrificed either at 4 or at 8 weeks, and the specimens were evaluated for the type and degree of inflammation, foreign body reaction, new bone formation, and residual biomaterial particles.

Results: The histomorphometric analysis revealed that L-PRF significantly enhanced osteogenesis ($P<0.05$), and the number of subsequent remnant bodies in the L-PRF group was not significantly different from the control group ($P>0.05$). The results of the histologic analysis showed that the frequency of central bone regeneration significantly increased in prolonged periods ($P<0.05$). There was no significant difference in the utilized biomaterials concerning subsequent bleeding, inflammation, foreign body reaction, and lateral bone regeneration between 4 and 8 weeks of treatment ($P>0.05$).

Conclusion: The results showed that L-PRF was a suitable option for the induction of bone regeneration with fewer remnant bodies. However, future clinical studies are required to assess the efficacy of these biomaterials in the clinical setting. *(J Dent Mater Tech 2023;12(1): 43-50)*

Keywords: *Bone regeneration; leukocyte platelet-rich fibrin; nano-hydroxyapatite*

Introduction

Bone regeneration and healing is a complex combination of physiological processes through which the defect is filled by the deposition of new bone (1, 2). However, in some clinical conditions, the defects require bone augmentation (3). Several strategies can trigger bone regeneration in defects, such as bone grafting, guided bone regeneration, application of growth factors, or combining two modalities (1-3). Autogenous bone is the gold standard for bone regeneration. It is the only material with all three favorable properties of osteoinductivity, osteoconductivity, and osteogenicity (1-3). Nonetheless, using autogenous bone in the oral cavity has some limitations, such as the need for a donor site, morbidity, limited availability, and fast resorption of grafted bone (1-3). Hence, attempts are ongoing to find synthetic alternatives to autogenous bone.

Synthetic hydroxyapatite (HA) is a bone graft material with optimal biocompatibility and osteoconductivity. However, it is hardly resorbed (4, 5). The synthesis of a bioceramic type of material known as nano-HA (nHA) is now possible by recent advancements in nanotechnology. Nano-HA shows osteoconductivity, and it is well-

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resorbed and replaced with new bones (4-6). NanoBone is a nanocrystalline HA in a silica gel matrix. The advantages of nHA include osteoinductivity (5) and favorable biological properties due to the minimal size of particles and large surface area. These nanoparticles enhance protein absorption and adhesion of osteoblasts (6). Moreover, the surface of granules is very rough, which causes micro-meter and millimeter-scale porosities. Higher osteogenic properties of NanoBone compared with HA and tricalcium phosphate has been reported in the literature (7). Due to the presence of HA, which has an organic structure, bone regeneration starts shortly after its application. During the removal of the granules by osteoclasts, NanoBone is completely replaced with new bone. NanoBone also plays a role in osseointegration due to the presence of nHA crystals in a highly porous silica matrix. The silica matrix induces the formation of collagen and bone. Furthermore, NanoBone enhances the differentiation of osteoblasts and osteoclasts and induces angiogenesis (8).

In recent years, growth factors have been used, along with bone substitutes, to improve the outcome of bone regeneration (7). Platelet-rich fibrin is the second generation of platelet-rich plasma (PRP) and has numerous advantages over PRP (11, 12). It is derived from the patient's blood without using any anticoagulant (12). Leukocyte-PRF is a type of PRF that is rich in platelets and leukocytes and has more advantages than PRF. It triggers faster activation of growth factors and reinforces the healing process (9). A simple, fast, and affordable preparation procedure with no biochemical involvement is among the advantages of L-PRF over other similar products. Moreover, it has a physiologically functional fibrin network that can retain and gradually release growth factors, cytokines, and leukocytes. The fibrin matrix degrades within 7 to 10 days (11). The advantages of applying L-PRF include enhanced wound healing and bone maturation, the graft's stability, wound sealing, hemostasis, and improved handling of graft material (10-12). Evidence shows that bone substitutes, along with growth factors in L-PRF, can effectively enhance bone density, induce new bone formation, decrease pocket depth in bone defects, and increase attachment gain (13-16). Many clinicians prefer the use of L-PRF combined with graft materials or bone substitutes (14).

This study aimed to assess the histologic and histomorphometric effects of L-PRF, nHA (NanoBone®), and a combination of both on the regeneration of calvarial bone defects in rabbits.

Materials and methods

Animals

The research was conducted under the rigorous animal care guidelines established by Shahed University of Medical Sciences, Tehran, with the animal protocol provided by Shahid Beheshti University, Tehran, Iran. The study employed healthy albino rabbits of the New Zealand breed, which were procured from Seband Institute and acclimated for a week under standardized temperature and humidity conditions, with regular light/dark cycles, in separate cages within an animal room**.**

Convenience sampling was utilized to select a total of 14 rabbits, all of similar age and weight, averaging between 2-3 kg. A veterinarian ensured the rabbits' systemic health and monitored their nutritional intake throughout the study. The rabbits received a standard diet and gained weight, which was indicative of their overall well-being.

Surgery

Before the surgical procedure, the operating table underwent rigorous disinfection with sodium hypochlorite. Then, ketamine hydrochloride (10%, 44 mg.kg-1) and Xylazine (2%, 7 mg.kg-1) were injected intramuscularly into the superior-lateral quadriceps muscle for anesthesia induction. The head's incision site was carefully shaved and scrubbed with betadine (7%) to prevent infection. A perforated surgical drape was placed over the surgical site, and the area was disinfected again with betadine (7%). Lidocaine (2%) was used to induce local anesthesia and control bleeding **.**An anteroposterior incision, 1 cm in length, was made with a surgical scalpel (#45). The area's skin, periosteum, and muscles were gently retracted using a molt periosteal elevator (#9) to expose the parietal and frontal bones. Four calvaria defects were created using a microsurgical motor (3i, USA), a contra-angle hand-piece (SGM-ER20i 20:1, NSK, Japan), and an eight-millimeter trephine bur (Implantium Co., Korea). Two defects were created in the parietal bone at the sides of the sagittal suture, and two were created anterior to the midline of the other two defects**.**

To avoid overheating, normal saline was used as external irrigation during the trephine bur procedure. Care was taken not to damage the dura mater or fibrous attachments adhered to the skull's internal surface. The defects were rinsed with saline, and biomaterials were inserted. The first defect was filled with L-PRF, the second with nHA (NanoBone; Artoss GmbH, Germany), and the third with a combination of both. The fourth defect remained unfilled as a control**.**

The order of biomaterial insertion in the first rabbit was randomized, and a code was assigned for each sample.

For subsequent rabbits, the allocation of biomaterials to defects changed clockwise to eliminate any positional effect. To avoid contaminating the meninges, the biomaterials were carefully inserted into the defects. After applying the biomaterials, the periosteum was sutured with 4/0 Vicryl sutures. The calvarial skin was then sutured with 3/0 nylon sutures, as depicted in Figure 1.

During the first week postoperatively, the rabbits received enrofloxacin (10%, 5 mg.kg-1) and ketoprofen (10%, 1%) intramuscularly twice and once a day, respectively. After the rabbits recovered in a warm location, they were returned to their home cages.

Sample preparation

The animals were randomly divided into two groups for euthanization, one at 4 weeks and the other at 8 weeks. Euthanasia was administered through an intravenous infusion of 0.22 mL.kg-1 sodium pentobarbital, resulting in complete cessation of heartbeat and respiration within 5 min. Mydriasis was also observed in all animals. The mandible was then separated from the calvaria, and each calvaria was fixed in a container containing 10% formalin.

After the skin and soft tissue were removed, the frontal bone, parietal bone, and superior rim of the orbit were decalcified for 45 days using 10% nitric acid. The process was constantly monitored to make sure the bone was soft enough to be cut. After 45 days, the specimens were immersed in a 20% sodium carbonate solution for 5 min to neutralize any residual acid and to enhance staining quality. A transverse section was made at the longest diameter of the defect, and the samples were placed in cassettes and further fixed with 10% formalin for 21 h. The cassettes were then dehydrated using 70%, 90%, and 100% ethyl alcohol, followed by the preparation of paraffin blocks for each specimen. The sections were made at the center and around the largest diameter of defects using a microtome. A minimum of four consecutive sections were made with 4 µm thickness. Finally, the specimens were stained with hematoxylin and eosin and observed under a light microscope (Nikon Eclipse E400, Japan) at 40X, 100X, 200X, and 400X magnifications.

Microscopic evaluation of the surgical site

Histomorphometric analysis

A pathologist histologically and histo-morphometrically evaluated the bone defects. The pathologist was blinded to the group allocation of defects. The frequency of the newly formed bone and the remaining biomaterial particles were recorded. It was calculated on the specimens at the largest diameter of the defects on a digital image (E450; Nikon, Japan) taken at 40X magnification using Iranian Histomorphometric Analysis software (v1, SBMU, Iran) (Fig 2). The osteogenesis frequency and the remaining biomaterial in each group were recorded at two intervals.

Histologic Analysis

The type of inflammation was recorded as acute or chronic bleeding. The degree of inflammation was recorded as no inflammation (<10%), mild inflammation (10% to 30%), moderate inflammation (30% to 50%), and severe inflammation (>50%). Foreign body reaction was recorded as the presence or absence of giant cells. Moreover, newly formed bone (+ or -) and remnants of biomaterial particles (+ or -) were recorded.

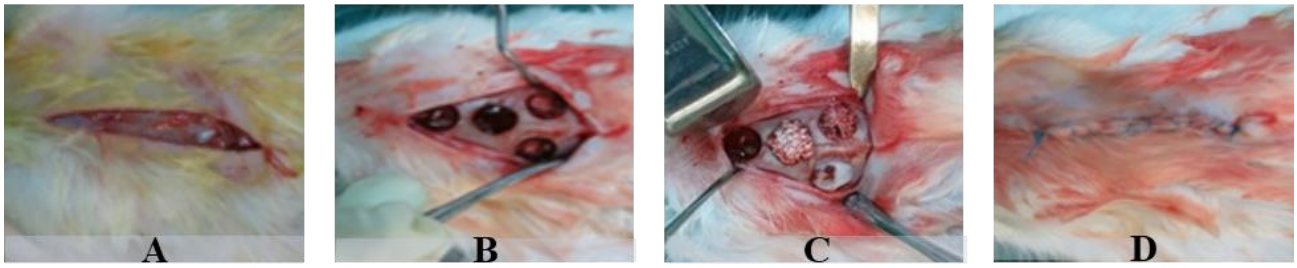

Figure 1. Surgical procedure, including anteroposterior incision (A), preparation of defects (B), filling of defects with biomaterials (C), and suture of the periosteum (D)

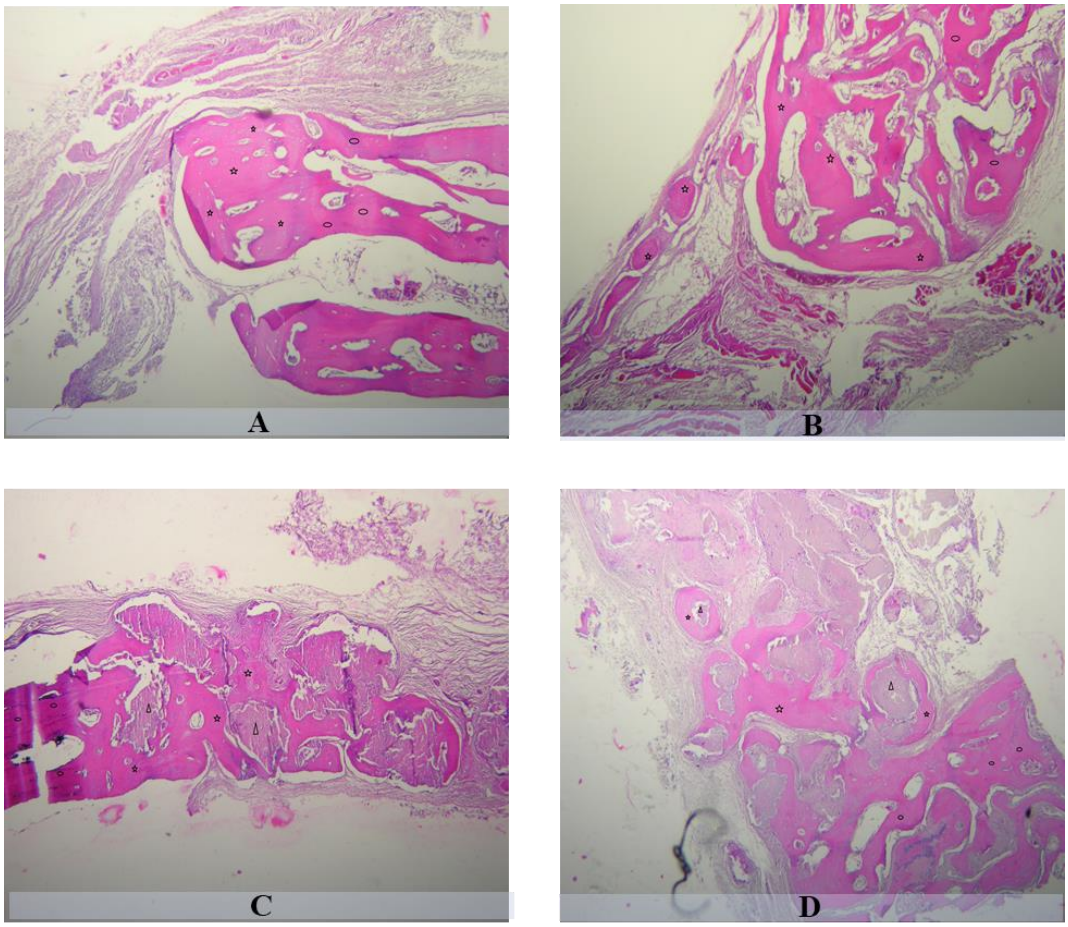

Figure 2. Histological micrographs from control (A), L-PRF (B), nHA (C), and L-PRF+nHA (D) groups
The new bone has been marked with asterisks (*), and the biomaterial remnant with a bullet (○).

Statistical Analysis

The recorded data were analyzed through two-way ANOVA and Tukey's post hoc mean comparison tests. Non-parametric values were subjected to a chi-square test. All statistical analyses were conducted using IBM SPSS 21.0 software. The p-values less than 0.05 were considered significant.

Results

Histomorphometric Analysis

The statistical analysis showed that osteogenesis was significantly different in the calvarial bone when the defect was treated with different biomaterials ($P<0.05$). However, osteogenesis was not significantly different between the healing periods of 4 and 8 weeks ($P>0.05$). Post hoc analysis showed that filling defects with L-PRF significantly improved osteogenesis compared to the control group ($P<0.05$), whereas the application of nHA or the combination of L-RPF and nHA had no significant effect on osteogenesis compared to the control group ($P>0.05$; Fig 3).

ANOVA showed that the frequency of the remnant body was significantly different among various biomaterials ($P<0.01$). The results of post hoc analysis revealed that when the defects were filled with L-PRF, the frequency of the remnant body was not significantly different from the control group ($P>0.05$; Fig 4). However, when the defect was filled with nHA or with the combination of L-RPF and nHA, the frequency of the remnant body was significantly higher than that of the control group ($P<0.05$; Fig 4).

Histologic Analysis

Chi-square analysis showed a significant difference in bone regeneration between the treatment periods ($P<0.05$; Fig 5). The frequency of central bone regeneration increased significantly in prolonged periods. At 4 weeks after treatment, the osteogenesis rate was 25%, whereas, at 8 weeks after treatment, it increased to 53% (Fig 5). There was no significant difference between different biomaterials regarding subsequent bleeding, inflammation, foreign body

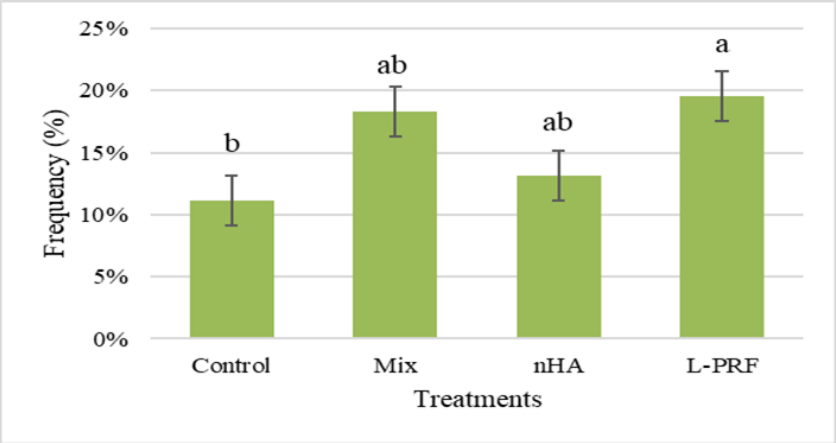

Figure 3. Effect of different materials on osteogenesis. The groups that have been defined with different letters showed statistically significant differences at P<0.05. (nHA: nano-hydroxyapatite; L-PRF: leukocyte platelet-rich fibrin)

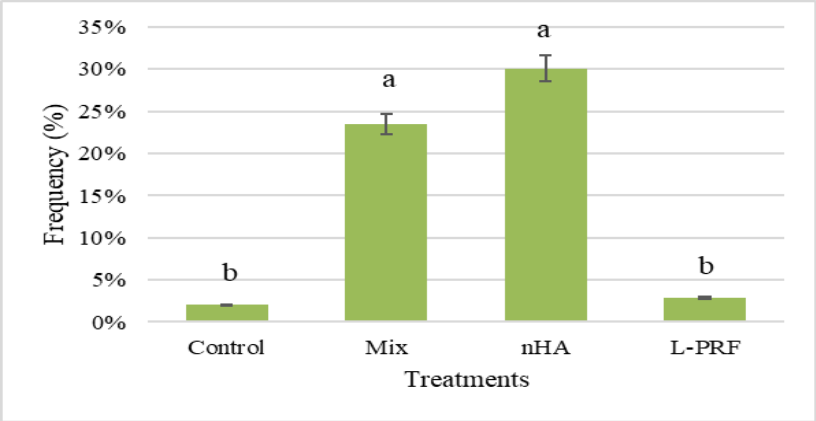

Figure 4. The frequency of remnant bodies after treatment with various biomaterials. The groups that have been defined with different letters showed statistically significant differences at P<0.05. (nHA: nano-hydroxyapatite; L-PRF: leukocyte platelet-rich fibrin)

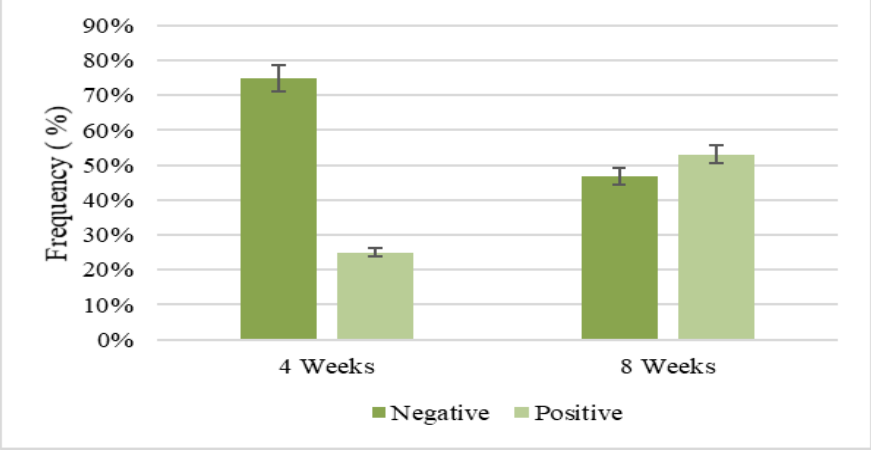

Figure 5. Comparison of central bone regeneration after treatment with different biomaterials between the two intervals

reaction, and lateral bone regeneration between the treatment periods of 4 weeks and 8 weeks (P>0.05).

Discussion

The present study investigated the histomorphometric and histologic effects of L-PRF, nHA, and a combination of them on bone regeneration in rabbits. The histomorphometric results showed that L-PRF significantly improved bone regeneration compared to the control group. Moreover, when the defects were filled

with L-PRF, the frequency of the remnant body was not significantly different from the control group. The histologic analysis exhibited that the frequency of central bone regeneration increased significantly in prolonged periods. Moreover, there was no significant difference between the biomaterials for subsequent bleeding, inflammation, foreign body reaction, and lateral osteogenesis between 4 and 8 weeks of treatment.

The outcomes of this study are in agreement with the results of Shah et al. (15) who revealed the superior outcome of L-PRF application compared with deproteinized freeze-dried bone allograft and suggested L-PRF application to treat bone defects. In contrast to the findings of this study, Oliveira et al. (10) evaluated the effect of L-PRF and Bio-Oss on bone regeneration in 5 mm rat calvarial defects. They showed that L-PRF positively affected bone regeneration only when combined with Bio-Oss. Elgendy et al. (13) showed that nHA, in combination with L-PRF, had clinical advantages over the use of nHA alone. Nacopaulos et al. (17) evaluated the effect of L-PRF in combination with synthetic materials for bone regeneration in rabbits. They showed higher cortical and subcortical bone formation when PRF was combined with synthetic materials. Fetner et al. (18) found that the application of L-PRF combined with simulated body fluids (SBF) significantly enhanced bone regeneration compared to the application of each alone (18).

Recently, growth factors have shown promising results in bone regeneration (7). PRP and L-PRF are growth factors that serve as biological mediators. The use of PRF has some advantages over PRP. It does not require the addition of an anticoagulant agent and its subsequent neutralization. Thus, its preparation is simple and does not require biochemical procedures. L-PRF improves the regenerative process in bone defects and elevates inflammatory response (20). Moreover, L-PRF has been reported as a valuable biomaterial for bone grafting (21, 22) and leads to faster bone healing (23). L-PRF-dependent bone regeneration is accomplished by aggravation and acceleration of bone regeneration pathways that are usually responsible for continuous tissue regeneration (24).

Bone regeneration is a complex combination of physiological procedures involving the interaction of the immune system and growth factors (1, 2). Despite the reportedly positive effects of L-PRF and PRP on bone regeneration, their exact mechanism of action has not been well understood. Researchers presume that thrombin and PRP release high growth factors into the interstitial tissues, which soon become inactive. Materials such as thrombin receptor activator peptide-6 and bone substitutes are more effective than thrombin in maintaining higher levels of growth factors, which is critical for initiating a cascade of cellular events, leading to osteogenesis (25). It has been reported that PRF enhances the healing process by triggering the synthesis and release of growth factors.

It should be noted that the cortical bone of calvaria in rabbits is physiologically similar to an atrophic mandible. The regeneration of bone in rabbits is approximately 3 to 4 times faster than in humans. For this purpose, rabbits are a suitable animal model for short-term studies (26) and are commonly used to assess the effects of biomaterials before their use in larger animals (27). However, a rabbit model cannot well simulate the conditions in the human body due to differences in geometry, biomechanics, and clinical properties between animal models and the clinical environment. Hence, the generalization of the results of this study to the clinical setting should proceed with caution.

Conclusion

The results showed that L-PRF was suitable for the induction of bone regeneration with fewer remnant bodies. However, future clinical studies are required to assess the efficacy of these biomaterials in the clinical setting.

Ethical Considerations

All experiment procedures were conducted according to the rules of experimental animal ethics at Shahed University of Medical Sciences. The ethical code number for this project was IR.Shahed.REC.1395.44.

Conflicts of Interest

The authors report no relevant financial conflicts, and there is no conflict of interest.

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