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Histomorphologic Assessment of Osteoregeneration in a Rabbit Femur Model With Xenograft, Bone Morphogenetic Protein-2, Platelet-Rich Plasma, and Denosumab

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Background: This study investigated whether the combination of thermally processed bone xenograft (XG) and recombinant human bone morphogenetic protein-2 (rhBMP-2) with platelet-rich plasma (PRP) or Prolia (denosumab) can enhance bone regeneration in a rabbit femoral bone defect model.
Methods: Seventy-two rabbits with created femoral bone defects (5 mm) were divided into 4 groups: (1) XG + rhBMP-2 + Prolia; (2) XG + rhBMP-2 + PRP; (3) XG + rhBMP-2; and (4) XG. At 14, 30, and 60 days after implantation, samples were prepared for histological and histomorphometric analysis.
Results: Histomorphometrically, on day 30, there were no statistically significant differences between groups regarding the amount of newly formed bone (P = 0.2231), but a reduction in residual graft in the intramedullary space was observed in the PRP group. On day 60, more intense formation of new bone with reduced intragroup variation in the amount of newly formed bone was found in the XG + rhBMP-2 + PRP group (P < 0.001). At day 60, XG + rhBMP-2 + Prolia showed no cortical bone increase because of concurrent resorption.
Conclusions: Combined therapy using thermally processed bone graft, rhBMP-2, and PRP improves bone healing in femoral defects in rabbits. *(Plast Reconstr Surg Glob Open 2026;14:e7593; doi: 10.1097/GOX.0000000000007593; Published online 6 April 2026.)*

INTRODUCTION

Currently, a considerable number of bone defect reconstruction procedures are performed worldwide using various types of substitutes.[1–8] Bone graft substitutes are considered the most promising materials due to their osteoconductive and osteoinductive properties and their broad availability.[9–11] However, to bring the allograft closer to bone autograft in its properties, it is necessary to combine this substitute with osteoinductive agents, such as bone morphogenetic protein (BMP). In turn, the use of BMP is not unambiguous, because along with positive results, treatment outcomes are also reported in which osteoclastic bone resorption, the appearance of atypical osteoclasts, and ectopic osteogenesis occurred. An interesting agent for regulating the osteoinductive effect of BMP is Prolia, as its active ingredient, denosumab, can bind receptor activators of nuclear factor κB ligand (RANKL), thereby limiting the differentiation of osteoprogenitor cells toward osteoclastogenesis, which should help reduce the process of excessive bone tissue resorption.[12–15] Meanwhile, platelet-rich plasma (PRP) exerts a proliferative effect

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The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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on fibroblast-like and epithelial cells through numerous growth factors.[16–22] Thus, we hypothesized that using these substances to regulate cellular interaction mechanisms would lead to a balance between bone tissue synthesis and resorption, which is essential for the normal reorganization process of the defect.

The aim of this study was to conduct a comparative histological and histomorphometric assessment of new bone formation in an experimental rabbit femur model using a bone xenograft (XG) harvested according to the Marburg bone bank system and recombinant human bone morphogenetic protein-2 (rhBMP-2) in combination with local application of Prolia and PRP.

Takeaways

Question: Does the combination of heat-treated bone xenograft with recombinant human bone morphogenetic protein-2 (rhBMP-2) and platelet-rich plasma (PRP) or Prolia (denosumab) improve bone regeneration in a rabbit femoral defect model?

Findings: The use of a bone xenograft in conjunction with rhBMP-2 and PRP promoted rapid and homogeneous development of mature bone tissue, achieving cortical plate regeneration by day 60 and demonstrating minimal inflammatory response. In contrast, specimens receiving the bone xenograft, rhBMP-2, and denosumab exhibited delayed osteogenesis, eosinophilic infiltration, and areas of aseptic necrosis.

Meaning: PRP enhances the osteoinductive potential of rhBMP-2 and promotes balanced bone regeneration, whereas denosumab may impair remodeling and delay healing.

METHODS

Preparation of Bone XGs

In this study, a bone XG from the head of the femur from a living donor (after arthroplasty) was used in accordance with the national legislation of Kazakhstan.[23,24] The XG, namely the head of the femur, was obtained from patients who underwent arthroplastic surgery (hip joint endoprosthesis). The femoral head was taken in the operating room and subjected to mechanical cleaning of soft tissues, cartilage, and ligaments under sterile conditions. The container was then closed and placed in the Lobator heat treatment device. The processing cycle was 94 minutes.[25] Upon completion of the cycle, sterility was evaluated via a designated inspection port in the container. The bone XG was then stored in a freezer at a temperature of −80°C following the protocol.

Preparation of rhBMP-2

A total of 150 µg of rhBMP-2 (Cusabio, Houston, TX) was mixed with 3 mL of saline solution to form the rhBMP-2 solution (concentration, 50 µg/mL). Subsequently, 100 µL (5 µg) of the aforementioned solution was meticulously introduced into 0.5 g of bone graft using a micropipette, precisely 30 minutes before application.[22]

Preparation of PRP

PRP was obtained by double centrifugation according to a previously published method.[26]

Preparation of Prolia

Prolia (denosumab) solution was carefully injected into 0.5 g of bone graft using a micropipette exactly 30 minutes before application.

Animals and Surgical Procedures

The experiment was carried out at the university using male non–breed rabbits. At baseline, the animals were 6–7 months of age, with a mean body weight of 1931 ± 83 g. Animals were randomly assigned to 4 equal groups (n = 24 each) and acclimated for 2 weeks. All procedures involving the animals adhered to the protection guidelines of Directive 2010/63/EU for animals used in scientific research[27] and were approved by the university animal care committee under protocol number 27, dated September 27, 2020.

Three hours before, the rabbits received an intramuscular injection of gentamycin 0.1 mL/kg (Mapichem, Switzerland). Animals were anesthetized with an intramuscular injection of Zoletil 0.1 mg/kg (Virbac) and Rometar 5 mg/kg (Bioveta, Czech Republic). Following hip skin disinfection, an incision was made in the distal femur. The periosteum was then lifted, and a 5-mm diameter unicortical bone defect was created in the distal femoral metaphysis using a drill.[28]

Bone defects in the first group were treated using thermally processed bone grafts with rhBMP-2 and Prolia (XG+rhBMP-2+Prolia). In the second group, defects were treated with thermally processed bone grafts with rhBMP-2 and autologous PRP (XG+rhBMP-2+PRP). In the third group, treatment was carried out using thermally processed bone grafts with rhBMP-2 (XG+rhBMP-2). In the fourth group, treatment was carried out using thermally treated bone grafts (XG).

After surgery, each animal received intramuscular injections of gentamycin 0.1 mL/kg (Mapichem) and ketonal 0.04 mL/kg (Sandoz, Slovenia) for 3 days. At 14, 30, and 60 days, 8 animals from each group (24 rabbits at each time period) were euthanized according to ethical standards by intravenous administration of a lethal dose of Zoletil (50 mg/mL), and the distal femur was harvested.

Histopathologic Examination

The samples were fixed in 10% neutral formalin (24 h), were decalcified (Biodec R, Bio-Optica Milano SPA, 24 h), underwent standard histological processing, and were embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin to assess morphology and inflammation, and Masson trichrome stain to assess newly formed bone and its remodeling.[29]

Hematoxylin and Eosin Staining Process

The tissue slices were submerged in Mayer hematoxylin for a quarter of an hour, followed by a 5-minute rinse

with water. Thereafter, the sections were subjected to a minute-long eosin staining.

Masson Trichrome Staining Procedure

Sections were stained with Masson trichrome using a commercial kit (Trichrome Stain [Masson] Biovitrum TU 9398-001-89079081-2012) according to the manufacturer's instructions. Microscopic examination was performed at ×400 magnification on a Zeiss AxioLab 4.0 microscope using AxioVision 7.2 software (Windows). Morphometric analysis was performed by 2 independent researchers blinded to the animals' group assignments, using the American Society for Bone and Mineral Research terminology.[30]

The closure of the cortical plate defect with newly formed bone tissue as well as the tissue composition of the defect zone and intramedullary space (%) were assessed (Fig. 1). Morphometric analysis of these areas was performed, as described by us earlier.[31]

Ethical Approval

The study was conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (2010) and approved by the local ethics committee of Karaganda Medical University (No.27.27.09.2020).

Statistical Analysis

All experimental values are presented as means and SDs. Comparisons between 2 groups were made using the χ^2 test with Yates continuity correction and the Mann-Whitney test, whereas multiple comparisons were performed using the Pearson χ^2 test. Statistical analysis of the research results was performed using IBM SPSS Statistics 20.0 and STATISTICA 10. A *P* value of less than 0.05 was deemed to indicate statistical significance.

RESULTS

Cortical Plate

Histological and Morphometric Analysis of Newly Formed Bone Tissue

Supplemental Digital Content 1 and Figure 2 present the results of the histopathologic assessment of the regeneration of the bone defect and the tissue composition of the intramedullary space in the projection of the surgical intervention. (**See table, Supplemental Digital Content 1**, which displays the comparative histopathologic assessment of the regeneration of the bone defect and the tissue composition of the intramedullary space in the projection of surgical intervention after 14 d [A], after 30 d [B], and after 60 d [C], **https://links.lww.com/PRSGO/E747**.)

The histological and morphometric analysis of newly formed bone tissue at the site of the cortical plate defect showed that on day 14 in the XG+rhBMP-2+Prolia group, the area of the bone defect was characterized by the development of separate heterogeneous bundles of new bone tissue. These bundles contained lacunae with osteocytes and a rich supply of vascular channels. On day 30 in the XG+rhBMP-2+Prolia group, the bone tissue consisted of chaotically arranged bone trabeculae that merged into lamellar structures, comprising 68.3% of the length and

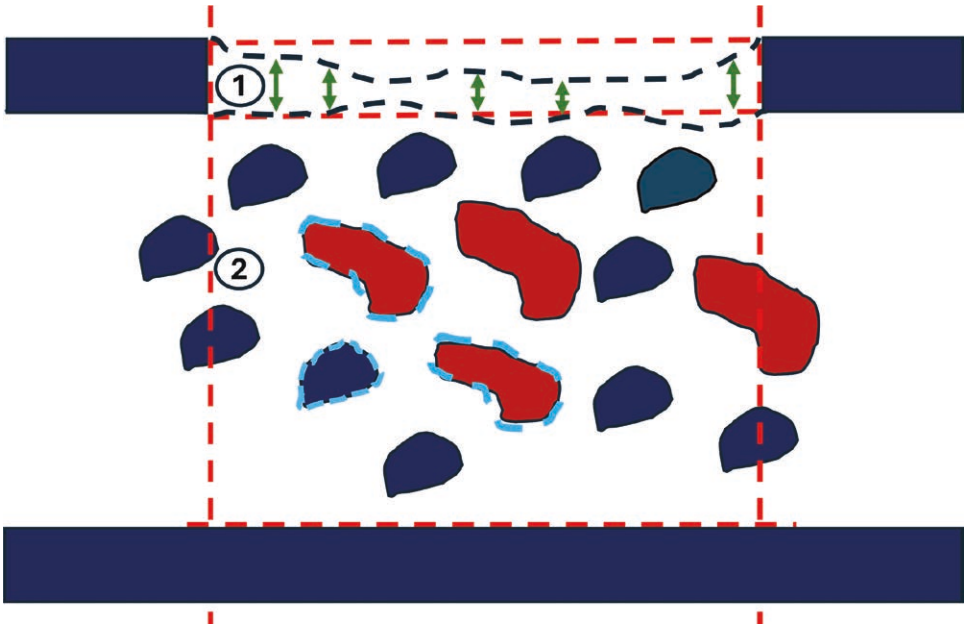


Fig. 1. Scheme of morphometric analysis. Designations: (1) cortical plate; (2) intramedullary space; red dotted lines indicate boundaries of morphometric analysis areas; blue dotted lines indicate boundaries of bone tissue subject to semi-quantitative assessment; green arrows indicate perpendicular measurements of the thickness of newly formed bone.

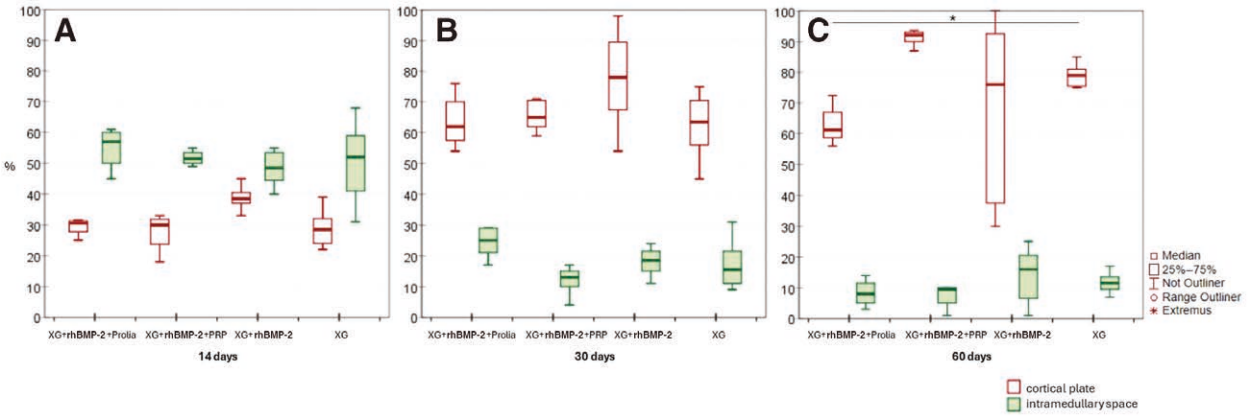


Fig. 2. Comparative histopathologic assessment of the percentage of bone tissue in the cortical plate and intramedullary space on days 14 (A), 30 (B), and 60 (C) in the study groups.

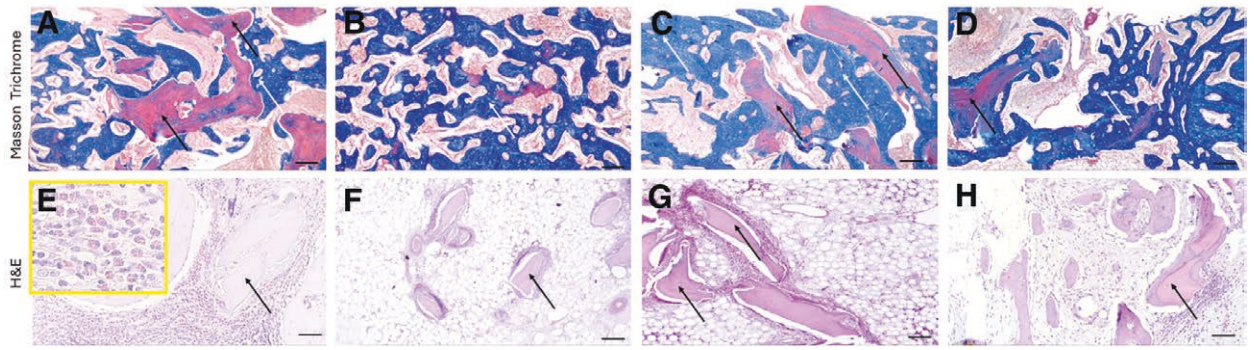


Fig. 3. Representative micrographs of the defect area of the cortical plate and intramedullary space on the 60th day after filling the defect with bone XG with rhBMP-2 and Prolia or PRP. A, E(1), E(2), XG + rhBMP-2 + Prolia: in the cortical defect area, formation of new bone tissue (white arrows) with a heterogeneous structure and varying degrees of mineralization is observed, with the preservation of significant fragments of the XG (black arrows). In the intramedullary space, small fragments of bone XG (black arrows) are surrounded by diffuse eosinophil infiltration. B, F, XG + rhBMP-2 + PRP: complete closure of the defect with newly formed bone tissue with restoration of normal thickness and continuity. Adipose tissue and bone marrow in the intramedullary space reflect positive remodeling of the intramedullary space in the defect. C, G, XG+rhBMP-2: cortical plate defect zone filled with newly formed bone tissue, with uneven regeneration of newly formed bone tissue (white arrows) forming thick bone trabeculae that gradually replace the defect and integrate with bone XG (black arrows) fragments. Small fragments of lysed bone XG (black arrows) are present in the intramedullary space. D, H, XG: in the cortical plate defect area, newly formed bone tissue (white arrows) is observed. The histoarchitecture of the cortical plate remains heterogeneous, with small fragments of the XG (black arrows) surrounded by mineralized bone tissue. Fragments of the XG with signs of lysis are present in the intramedullary space. A–D, Histochemical staining of collagen proteins with Masson trichrome, 100× magnification, scale bar 200 μm. 3E, F–H, H&E staining, 40× magnification, scale bar 500 μm. H&E, hematoxylin and eosin.

59% of the width of the cortical plate. At day 60, moderate mineralization, weak transverse growth, and areas of aseptic necrosis were observed in the XG+rhBMP-2+Prolia group. In the XG+rhBMP-2+PRP group, the defect was almost completely (91.3 ± 2.4%) filled with mature bone tissue, covering 89.5% of the length and 93.1% of the thickness (Fig. 3).

Comparative Characteristics of Inflammatory and Necrotic Patterns

On day 14, a solitary reactive cellular infiltrate was observed at the defect site in all groups without any necrotic changes. On day 30 in the XG+rhBMP-2+Prolia group, diffuse infiltration by eosinophils was noted in one-third of the cases (3 of 8; 37.5%), along with scattered infiltration by lymphocytes and macrophages. On day 60, in all cases surrounding the XG, an eosinophilic infiltrate was observed: diffuse in 7 cases (87.5%) and focal in 1 case (12.5%). The bone tract exhibited moderate mineralization, weak transverse growth, and areas of aseptic necrosis (Fig. 3A). On days 30 and 60 in the XG+rhBMP-2+PRP and XG+rhBMP-2 groups, there were no signs of inflammatory cellular infiltration, indicators of infection (such as polymorphonuclear cells, lymphocytes, macrophages, or multinucleated cells), or necrosis of tissues in any of the surgically created defects (Figs. 3B–D).

Intramedullary Space

On day 14, in the XG+rhBMP-2+Prolia and XG+rhBMP-2+PRP groups, approximately one-third of the intramedullary space was occupied by bone marrow, 65% by bone and cartilage tissue, and 2% by fibrous tissue. In the XG+rhBMP-2 group, bone marrow constituted 37% of the intramedullary space, with bone tissue at 51.8% and cartilage tissue at 9.3% (**Supplemental Digital Content 1**, **https://links.lww.com/PRSGO/E747**). Eosinophilic infiltration persisted around the graft fragments (Fig. 3E).

On day 30, in the XG+rhBMP-2+Prolia group, about one-quarter of the intramedullary space was occupied by the bone XG, around 4% by fibrous tissue, and about 70% by bone marrow. In the XG+rhBMP-2+PRP group, the bone XG accounted for about 12%, with positive remodeling of the intramedullary space in the defect area and restoration of normal anatomical structure.

On day 60, no remarkable features were observed in the intramedullary space in the groups (Figs. 3F–H). Tissue ratios are shown in Supplemental Digital Content 1 (**https://links.lww.com/PRSGO/E747**).

DISCUSSION

The results showed that the combination of PRP with thermally processed bone XGs and rhBMP-2 enhances bone recovery. Specifically, in the group where the defect was filled with bone XG combined with rhBMP-2 and PRP, a greater volume of newly formed bone tissue was observed at 60 days compared with the group receiving bone XG with rhBMP-2 and Prolia ($P = 0.000155$). Additionally, the results were more homogeneous, with reduced intragroup variations in the amount of newly formed bone tissue, achieving a relatively consistent and uniform distribution of mature bone tissue across all time points. The combination of PRP with rhBMP-2 led to the formation of a wider cortical plate that filled the entire defect area and integrated well with the bone tissue at the defect margins, indicating a higher quality of bone recovery.

In the present study, a group in which the defect was filled only with bone XG without the addition of osteoactive components was used as a control. The effectiveness of a similar approach was previously confirmed by us in a model of femoral bone defect in rabbits, which served as the basis for including this group in the study as a comparative control.[26,32]

Notably, none of the cases using PRP with rhBMP-2 showed the negative results reported in other studies.[32–37] Throughout all stages of the experiment, the observed histomorphologic pattern indicated a normal process of osteoregeneration and remodeling of the XG in the defect area in the PRP with rhBMP-2 group. These results suggest that PRP may be a suitable means for balanced bone formation when using rhBMP-2, potentially leading to more predictable positive outcomes.

This likely reflects a synergistic effect of PRP–rhBMP-2. Specifically, PRP enhances the microenvironment by providing a high concentration of growth factors that stimulate the proliferation and differentiation of mesenchymal stem cells, accelerate angiogenesis, and modulate the inflammatory response, creating favorable conditions for osteogenesis in the early stages, as previously demonstrated.[32,38] Meanwhile, rhBMP-2 supports and amplifies osteoinductive processes during the later stages of bone mineralization, collectively minimizing fluctuations at various stages of regeneration and promoting uniform and consistent formation of new bone tissue in the defect area. Additionally, PRP suppresses osteoclast differentiation induced by RANKL,[39,40] leading to reduced bone resorption that may develop with rhBMP-2 use.[41,42] It can be hypothesized that the combination of PRP with rhBMP-2 mitigates mutual negative effects through synergistic action on the microenvironment, supporting both direct and reverse cell-tissue interactions.

Additionally, in the group using bone grafts combined with PRP and rhBMP-2, a greater reduction in residual graft in the intramedullary space was observed by day 30 compared with the other groups ($P < 0.01$). The results of this study demonstrated statistically significant differences in the rate of XG resorption in the intramedullary space, indicating the potential of PRP with rhBMP-2 as an important tool for improving bone remodeling processes.

In cases of defect closure with the bone XG and rhBMP-2 combined with Prolia, no significant increase in bone formation in the cortical plate was observed, likely due to substantial resorption occurring simultaneously with new bone formation. Although the results at 30 days did not show statistically significant differences between the study groups regarding bone tissue formation in the defect area of the cortical plate, at 60 days, the group using Prolia showed a decrease in active osteogenesis and focal aseptic necrosis, with a statistically significant reduction in the area of defect closure with newly formed bone compared with the PRP group ($P < 0.001$).

The decrease in active osteogenesis from 30 to 60 days in the rhBMP-2+Prolia group was accompanied by diffuse eosinophilic infiltration in the tissues of the bone defect and intramedullary space. The pronounced eosinophilic infiltration in the Prolia and rhBMP-2 group may be related to a complex immune-mediated response caused by the interaction between Prolia and rhBMP-2 components in the specific microenvironment of the bone defect. Osteoclasts and T cells formed in the acute phase of bone tissue damage play a key role, participating in the initial phase of the reparative inflammatory process aimed at resorption of damaged cells and tissues. Prolia as an inhibitor of RANKL causes a decrease in the quantitative and qualitative activity of osteoclasts, which leads to a violation of bone remodeling; on the other hand, Prolia also affects the line of T cells expressing RANKL, modulating the local immune microenvironment and changing the cytokine profile in the bone defect zone.

The results of using a combination of an osteoinductive agent (rhBMP-2) and an osteoclast inhibitor (Prolia) showed that such a combination leads to inhibition and suppression of the formation of a normal reparative and reactive microenvironment in the acute phase of the inflammatory response in uncomplicated bone defects. This condition is accompanied by the development of

pronounced eosinophilia associated with a delay in the maturation of mature bone tissue and a delayed closure of the bone defect compared with the group in which RANKL inhibitors were not used.

The biological role of the detected eosinophilia is unclear. A classic allergic reaction is unlikely, because it was not observed in other XG groups or in our previous studies.[26,32,43] Eosinophilia in this case may be a side effect associated with delayed bone defect repair. It may reflect a compensatory shift in the local immune microenvironment associated with changes in the cytokine profile in response to inhibition of osteoclast activity and impaired bone remodeling, which requires further study to better understand the mechanisms of interaction between RANKL inhibitors, osteoinductive agents, and XGs in the context of bone regeneration. There is a number of studies in the literature on locally applied bisphosphonates in combination with systemic RANKL inhibitors, which have shown their antiresorptive effects in animal models[44] and clinical practice.[45,46] However, according to the results of the present study, no statistically significant difference was found in the amount of newly formed bone between the group receiving locally applied Prolia in combination with BMP-2 and the control group with BMP-2. The effectiveness of locally applied Prolia in combination with BMP-2 was not demonstrated in this study; therefore, further research is needed to compare the systemic and local efficacy of Prolia in combination with BMP-2. The advantage of this study is the use of a standardized rabbit femoral defect model and comprehensive histological and morphometric assessment at early and late stages of regeneration.

A limitation is the use of only histological methods. In addition, the lack of open defect comparison groups without XG and without osteoinductive substances, as well as a group with XG + PRP without BMP-2, limited the assessment of the contribution of BMP-2 compared with natural healing. Despite these limitations, the results of this study provide valuable information about the potential benefits of PRP in combination with rhBMP-2. One advantage of the combination of rhBMP-2 and PRP is that PRP suppresses RANKL-induced osteoclast differentiation, reducing bone resorption. Another benefit of using PRP with rhBMP-2 is that it appears to decrease the incidence of aseptic bone necrosis, which can slow down regeneration, while also facilitating earlier recovery of the intramedullary space in bone. Thus, the side effects of rhBMP-2 can be managed with PRP, as demonstrated in this study. Consequently, using PRP alongside rhBMP-2 may enhance treatment outcomes.

CONCLUSIONS

This study demonstrated that the combination of rhBMP-2 with PRP leads to improved bone healing compared with the combination of Prolia with rhBMP-2 and rhBMP-2 alone. The addition of PRP to the XG and rhBMP-2 combination not only enhances the osteoinductive properties of this complex but also significantly reduces the heterogeneity of the results, allowing for more stable positive outcomes. The results obtained with the addition of Prolia to the XG and rhBMP-2 combination were uniform; however, these results cannot be interpreted as positive due to the halt in osteoregeneration and the slowdown in remodeling of the bone XG observed as early as day 30, indicating the need for further studies.

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DISCLOSURES

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ETHICAL APPROVAL

The study was conducted according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (2020) and approved by the local ethics committee of Karaganda Medical University (No.27.27.09.2020).

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