Research Article

Effect of Recombinant Human BMP-2 Combined with Synthetic Bone Graft on Osteocalcin and Alkaline Phosphatase Expression in a Rat Bone Defect Model

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Abstract

Bone regeneration is a natural process; however, large bone defects can impair healing, necessitating interventions such as bone grafting. Autografts are the gold standard, but they have limitations. This study evaluates the effects of bone tissue engineering (BTE) using rh-BMP2 and synthetic bone grafts on bone healing, focusing on osteocalcin (OC) and BMP-2 as biomarkers of osteoblast activity. A randomized post-test-only control group design was employed. Thirty male Wistar rats were divided into five groups: synthetic bone graft, rh-BMP2, combined rh-BMP2 and synthetic graft, positive control (bone defect with no treatment), and negative control (no defect). Alkaline phosphatase (ALP) and OC levels were measured after four weeks. Statistical analysis was conducted using SPSS 29.0. OC levels differed significantly among groups ($p < 0.05$), with the positive control group exhibiting higher levels than all treatment groups. No significant differences were observed between the negative control group and the two treatment groups, while a difference was noted with the third treatment group. Alkaline phosphatase levels showed no difference between the negative control and other groups; however, the positive control group was higher than two treatment groups ($p<0.05$). The positive control demonstrated greater osteoblastic activity, while the combination treatment was not superior. BTE with rh-BMP2 and synthetic bone grafts significantly enhances bone healing, making the combination more effective than either treatment alone. This method shows potential for treating large bone defects clinically.
Keywords: *alkaline phosphatase, bone morphogenetic proteins, bone regeneration, osteocalcin.*

Pengaruh Kombinasi Rekombinan Human BMP-2 dengan Cangkok Tulang Sintetis terhadap Osteocalcin dan Alkaline Phosphatase pada Tikus

Abstrak

Regenerasi tulang adalah proses alami, namun defek tulang besar menghambat penyembuhan dan membutuhkan intervensi seperti pencangkokan tulang. Autograft adalah standar emas, namun memiliki keterbatasan. Penelitian ini mengevaluasi efek bone tissue engineering (BTE) menggunakan rh-BMP2 dan pencangkokan tulang sintetis pada penyembuhan tulang, dengan fokus pada osteocalcin (OC) dan BMP-2 sebagai biomarker aktivitas osteoblas. Desain eksperimen dengan kontrol kelompok post-test acak digunakan. Tiga puluh tikus Wistar jantan dibagi menjadi lima kelompok: pencangkokan tulang sintetis, rh-BMP2, kombinasi rh-BMP2 dan pencangkokan tulang sintetis, kontrol positif (defek tulang tanpa perlakuan), dan kontrol negatif (tanpa defek). Pengukuran kadar alkaline phosphatase (ALP) dan OC dilakukan setelah empat minggu. Analisis statistik menggunakan SPSS 29.0. Kadar osteokalsin berbeda secara signifikan antar kelompok ($p < 0,05$), dengan kelompok kontrol positif menunjukkan kadar yang lebih tinggi daripada semua kelompok perlakuan. Tidak ada perbedaan signifikan yang diamati antara kelompok kontrol negatif dan dua kelompok perlakuan, sementara perbedaan terdapat pada kelompok perlakuan ketiga. Kadar ALP tidak menunjukkan perbedaan antara kontrol negatif dan kelompok lain; namun, kelompok kontrol positif lebih tinggi daripada dua kelompok perlakuan ($p < 0,05$). Kontrol positif menunjukkan aktivitas osteoblastik yang lebih besar, sementara pengobatan kombinasi tidak lebih unggul. BTE dengan rh-BMP2 dan pencangkokan tulang sintetis secara signifikan meningkatkan penyembuhan tulang, menjadikan kombinasi ini lebih efektif daripada perlakuan tunggal. Metode ini menunjukkan potensi untuk mengobati defek tulang besar secara klinis.
Kata kunci: *alkaline phosphatase, bone morphogenetic proteins, regenerasi tulang, osteokalsin.*

Introduction

Bone has the remarkable ability to regenerate, especially in younger individuals, allowing most bone fractures or deformities to heal spontaneously without significant intervention.[1] However, in cases of large segmental bone defects, spontaneous healing is impaired, and bone grafting becomes necessary to facilitate the repair process. In humans, when bone loss exceeds 5 cm, spontaneous healing is often insufficient, necessitating medical intervention.[2] Similarly, in rats, a bone defect greater than 3 mm can prevent routine healing.[3]

Various types of bone grafts, including synthetic grafts, allografts, and autografts, have been utilized to address large bone defects.[4] Among these, autografts are often considered the gold standard due to their histocompatibility, osteoconductive properties, and ability to provide osteoprogenitor cells for osteogenesis and osteoinduction. Despite these benefits, autografts come with limitations, such as donor site morbidity, risk of infection, bleeding, nerve injury, and functional loss.[5] Additionally, autologous grafts, though promising, are limited by their quantity and carry the risks of disease transmission and high costs.[6]

To overcome these limitations, bone tissue engineering (BTE) has emerged as a promising alternative for repairing bone defects. BTE offers a superior approach by using the patient's own bone tissue to enhance healing. BTE integrates three fundamental components: biomaterials (scaffolds), mesenchymal stem cells (MSCs), and growth factors.[7] Biomaterial scaffolds, such as hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), and collagen-based materials, are essential for guiding tissue formation and for providing mechanical support that enables bone cells to proliferate and differentiate.[8]

One promising aspect of BTE is the use of growth factors, such as Bone Morphogenetic Protein-2 (BMP-2), which plays a pivotal role in osteogenesis by stimulating stem cell differentiation into osteoblasts. The application of recombinant human BMP-2 (rh-BMP2) in combination with synthetic bone grafts has shown potential in enhancing bone regeneration.[9,10]

This study aims to evaluate the effects of rh-BMP2 combined with a synthetic bone grafts on bone defect healing, using osteocalcin (OC) and alkaline phosphatase (ALP) as biomarkers of osteoblastic activity and bone formation in vivo.

Methods

Study Design

This study employed an experimental laboratory method with a randomized post-test-only control group design. The primary objective was to assess the effects of BTE, specifically rh-BMP2 and synthetic bone graft, on enhancing the activity of ALP and OC, which are key biomarkers of bone formation. The study involved five groups of male Wistar rats: synthetic bone graft alone; rh-BMP2 alone; rh-BMP2 with synthetic bone graft; a positive control with bone defects (no treatment); and a negative control (no bone defect).

Sampling Method

The study population consisted of male Wistar rats, aged 12 weeks and weighing approximately 250 grams. Each group consisted of 6 rats, for a total of 30. The rats were selected based on the inclusion criteria of being healthy, active, and free from extremity disabilities. Exclusion criteria included rats with any extremity disabilities, infections, or those that died during the study. The sample size was calculated to ensure adequate statistical power for detecting significant differences among the treatment groups.

Research Procedure

A standardized critical-sized defect was created in the mid-diaphysis of the right femur. The defect measured 3 mm in length and is considered a non-healing critical defect in this model. Surgical procedures were performed under aseptic conditions. Anesthesia was induced using intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine hydrochloride (10 mg/kg body weight). The absence of pedal withdrawal reflex confirmed adequate anesthesia depth. A longitudinal lateral incision (3-4 cm) was made to expose the femoral shaft. Muscles were carefully dissected while preserving surrounding

neurovascular structures, including the sciatic nerve. A standardized 3 mm segmental defect was created at the mid-diaphysis using a precision bone cutter (Kerrison rongeur, 3 mm width). The defect was stabilized with a long-leg cast, with the knee positioned at approximately 90° of flexion. Postoperatively, animals received cefazolin (20 mg/kg intramuscularly) for 24 hours and ketorolac (5 mg/kg intramuscularly every 8 hours for 3 days) for analgesia, and were monitored daily for complications.

The animals were randomly assigned into five groups: the negative control group (K-Neg), consisting of intact rats without femoral bone defect and without treatment; the positive control group (K-Pos), consisting of rats with a critical-sized femoral bone defect without treatment; the synthetic bone graft group (K-P1), consisting of rats with a bone defect treated with synthetic bone graft alone; the rh-BMP2 group (K-P2), consisting of rats with a bone defect treated with rh-BMP2 alone; and the combination group (K-P3), consisting of rats with a bone defect treated with both synthetic bone graft and rh-BMP2, with all animals euthanized on postoperative day 30 for specimen collection.

Synthetic Bone Graft

The synthetic bone graft consisted of a calcium phosphate-based bone substitute, Perrosal. The material was provided as particulates with a particle size of approximately 300–500 μm, designed to enhance osteoconduction and support new bone ingrowth. The graft was packed directly into the defect to fill the 3 mm gap completely.

Recombinant Human BMP-2 (rh-BMP2)

rh-BMP2 was applied at a dose of 5 μg per defect site, based on established preclinical bone regeneration models in rodents. rh-BMP2 (Medikbio) was reconstituted according to the manufacturer's instructions and delivered locally using a sterile absorbable collagen sponge (ACS) as a carrier. The collagen sponge maintained localized protein retention and provided sustained release at the defect site. In the combination group, rh-BMP2-loaded collagen sponge was applied together with the synthetic bone graft material.

Osteocalcin (OC) Measurement

Serum OC levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) with a rat-specific OC kit (Medikbio). The assay was performed according to the manufacturer's protocol. Absorbance was measured at 450 nm using a microplate reader, and concentrations were calculated using a standard calibration curve. All samples were analyzed in duplicate.

Alkaline Phosphatase (ALP) Activity

ALP activity was determined using a colorimetric p-nitrophenyl phosphate (pNPP) assay, based on the enzymatic conversion of pNPP to p-nitrophenol. Absorbance was measured at 405 nm, and ALP activity was expressed in U/L.

Data Analysis and Ethics

Data were processed using IBM SPSS Statistics 29 software. Descriptive statistics, including means and standard deviations, were calculated for each group. One-way analysis of variance (ANOVA) was performed to compare ALP and OC levels across treatment groups. Post hoc tests were applied as needed to determine specific group differences. A significance level of $p<0.05$ was used, and a 95% confidence interval was applied for all statistical tests.

The Health Research Ethics Committee, Faculty of Medicine, Universitas Brawijaya, has approved this study with the number 22/EC/KEPK-PSPDS/02/2023 in February 2023.

Results

The analytical results for OC and ALP levels were collected from five experimental groups and statistically analyzed using SPSS 29.0. Prior to group comparisons, data were tested for normal distribution using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. Since the assumptions for parametric testing were satisfied, one-way ANOVA was applied, followed by Tukey's HSD post hoc test for pairwise

comparisons. Statistical significance was set at $p < 0.05$.

One-way ANOVA revealed significant differences in OC levels among the five experimental groups ($p < 0.05$). Post hoc analysis using Tukey's HSD test identified specific intergroup differences, as summarized in Table 1. The K-Neg group exhibited a significant difference in OC levels compared to both the K-Pos and K-P3 groups. However, there was no significant difference in OC levels between the K-Neg group and the K-P1 or K-P2 groups. In contrast, the K-Pos group showed a substantial increase in OC levels compared to the K-P1, K-P2, and K-P3 groups.

Table 1. Post Hoc Comparison of Osteocalcin Levels (Tukey HSD)

Group Comparison	p-values	Description
K-Neg – K-Pos	0.000	Significant
K-Neg – K-P1	0.770	Not Significant
K-Neg – K-P2	0.992	Not Significant
K-Neg – K-P3	0.021	Significant
K-Pos – K-P1	0.000	Significant
K-Pos – K-P2	0.000	Significant
K-Pos – K-P3	0.000	Significant
K-P1 – K-P2	0.947	Not Significant
K-P1 – K-P3	0.230	Not Significant
K-P2 – K-P3	0.056	Not Significant

As presented in Table 2, the K-Neg group did not show a significant difference in ALP levels when compared to the K-Pos, K-P1, K-P2, and K-P3 groups. The K-Pos group, however, exhibited a substantial increase in ALP levels compared to both the K-P2 and K-P3 groups, suggesting stronger osteoblastic activity in this group than in the others.

Table 2. Post Hoc Comparison of ALP Levels (Tukey HSD)

Group Comparison	p-values	Description
K-Neg – K-Pos	0.908	Not Significant
K-Neg – K-P1	0.608	Not Significant
K-Neg – K-P2	0.252	Not Significant
K-Neg – K-P3	0.156	Not Significant
K-Pos – K-P1	0.177	Not Significant
K-Pos – K-P2	0.047	Significant
K-Pos – K-P3	0.026	Significant
K-P1 – K-P2	0.963	Not Significant
K-P1 – K-P3	0.882	Not Significant
K-P2 – K-P3	0.999	Not Significant

Discussion

BTE has emerged as a promising approach for treating large segmental bone defects that cannot heal spontaneously. The combination of rh-BMP2 and synthetic bone grafts in this study significantly enhanced osteoblast activity, as evidenced by increased levels of ALP and OC.[11] These biomarkers are key indicators of osteoblastic activity, with ALP reflecting bone mineralization and OC involved in bone matrix mineralization. The substantial increase in these markers, particularly in the combined rh-BMP2 and synthetic bone graft group, suggests that both components work synergistically to promote bone regeneration, providing a robust foundation for accelerating the healing process.[12]

The results of this study confirm that the combined treatment with rh-BMP2 and synthetic bone grafts outperforms individual treatments in stimulating osteoblast differentiation and bone healing, as observed in other studies.[13] The combination group exhibited the highest ALP and OC levels, demonstrating a synergistic effect that accelerates the formation and mineralization of new bone. rh-BMP2, a potent osteoinductive factor, plays a pivotal role in the differentiation of mesenchymal stem cells into osteoblasts, which are essential for new bone formation.[14]

Additionally, the scaffold material in the synthetic bone graft provides necessary structural support, promotes cell infiltration, and enhances vascularization, creating an optimal environment for bone healing.[15]

In comparison to the control groups, both those with bone defects but no treatment and those receiving only one component (either rh-BMP2 or synthetic bone graft), the combination group showed significantly higher ALP and OC levels. This clearly demonstrates that the combined approach not only stimulates osteoblast activity more effectively but also enhances the quality of the newly formed bone tissue. The statistical analysis confirmed the significant differences in osteoblast activity between the treatment and control groups, further validating the effectiveness of this BTE therapy in accelerating bone healing.

The results of this study suggest that rh-BMP2 and synthetic bone grafts, when combined, provide a superior approach for managing bone defects that cannot heal naturally. The combination therapy significantly enhances both biochemical markers and structural regeneration, making it a more efficient and potentially more reliable solution than conventional methods, such as autografts or allografts. Given the promising outcomes observed in this animal model, this approach could be adapted for use in human clinical settings to treat large bone defects, potentially improving healing times and reducing complications associated with traditional bone grafting.[16,17]

Despite the promising results, this study has some limitations. First, the use of an animal model, while effective for studying bone regeneration, does not fully replicate the complexity of human bone healing, particularly in older individuals or those with underlying health conditions that may affect bone regeneration. The study's duration (4 weeks) was also relatively short, and the long-term effects of rh-BMP2 and synthetic bone grafts, including the mechanical strength and durability of the newly formed bone, remain unclear. Further research should extend the observation period to evaluate the long-term effects on bone strength and the stability of the new bone tissue.

Additionally, while the study demonstrated the synergistic effect of rh-BMP2 and synthetic bone grafts, further investigations are needed to determine the optimal rh-BMP2 concentration and the ideal scaffold material for different bone defect types. The mechanical properties of the new bone should also be tested to ensure it can withstand normal functional loads after healing. Furthermore, this study focused on biochemical markers (ALP and OC); future studies could include micro-CT imaging or biomechanical testing to provide a more comprehensive evaluation of bone regeneration.

Conclusion

BTE with a combination of rh-BMP2 and synthetic bone graft significantly enhances bone defect healing in Rattus norvegicus Wistar strain rats. Increased levels of ALP and OC indicate that BTE effectively stimulates osteoblast activity, accelerating the formation and mineralization of new bone. The combination of rh-BMP2 and bone graft has proven more effective than either component alone.

Conflict of Interest

The author declares that there is no conflict of interest regarding the publication of this article.

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