ORIGINAL PAPER

Nagoya J. Med. Sci. **87**. 421–430, 2025
doi:10.18999/nagjms.87.3.421

Comparative analysis of bone regeneration in critical-sized defects using self-assembling peptide hydrogel-178, bone morphogenetic protein-2, and calcium phosphate scaffolds in a rat femur model

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ABSTRACT

Bone regeneration is a highly demanded but challenging clinical endeavor in orthopedic surgery, necessitating the development of alternative bone grafting materials. This study aimed to evaluate the bone regenerative potential of self-assembling peptide hydrogel (0.8%), bone morphogenetic protein-2 (50 ng/μL), hydroxyapatite, and β-tricalcium phosphate, both individually and in combination with bone chips, in a rat femoral defect model. Ten-week-old female Wistar rats underwent surgical implantation of a polyetheretherketone cage into a 5-mm bony defect within the left femoral mid-shaft, maintained by an external fixator. Polyetheretherketone cages were filled with bone substitute materials alone in the first experiment and with bone substitute materials combined with bone chips in the second experiment. Radiographic and histological analyses were conducted following sacrifice at 56 weeks. While self-assembling peptide hydrogel alone exhibited moderate bone formation, with a bone-volume-to-total-volume ratio of 0.34 ± 0.09, this value was not significantly higher than that of the control group with an empty polyetheretherketone cage. Conversely, the combination of bone morphogenetic protein-2 with bone chips produced the highest level of bone regeneration, with a bone-volume-to-total-volume ratio of 0.78 ± 0.05, significantly surpassing bone chips alone ($p < 0.01$) and self-assembling peptide hydrogel with bone chips ($p < 0.05$). These findings suggest that while self-assembling peptide hydrogel holds potential as a scaffold material, particularly in minimally invasive applications, its efficacy in promoting robust bone regeneration may benefit from the inclusion of osteoinductive factors, such as bone morphogenetic protein-2.

Keywords: bone regeneration, self-assembling peptide hydrogel, BMP-2, hydroxyapatite, β-tricalcium phosphate

Abbreviations:
ALP: alkaline phosphatase
BMP-2: bone morphogenetic protein-2
β-TCP: β-tricalcium phosphate
BV/TV: bone-volume-to-total-volume
HA: hydroxyapatite

Received: September 14, 2024; Accepted: November 22, 2024
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SPG-178: self-assembling peptide hydrogel-178
PEEK: polyetheretherketone

INTRODUCTION

Bone fractures, especially in aging populations, have increased the demand for advanced bone regeneration strategies in orthopedic surgery.[1-3] Autografts remain the gold standard due to their osteogenic, osteoinductive, and osteoconductive properties.[4] However, their clinical application is limited by factors such as donor site morbidity, limited availability, and postoperative complications, including infection and chronic pain.[5,6]

To address these challenges, alternative bone graft materials have been developed. Among these, calcium phosphate-based materials, including hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP), are widely recognized for their biocompatibility and osteoconductive properties.[7-9] However, their limited osteoinductive capacity poses a significant challenge in complex cases, such as critical-sized bone defects.

Bone morphogenetic protein-2 (BMP-2), a potent osteoinductive factor, has shown promise in enhancing bone formation by promoting mesenchymal stem cell differentiation into osteoblasts.[10] Studies have demonstrated its efficacy in combination with osteoconductive scaffolds, leading to improved bone fusion rates in spinal surgeries and large bone defect repairs.[11,12] However, BMP-2 use is associated with certain risks, including ectopic bone formation and excessive inflammation.[13,14]

Self-assembling peptide hydrogels have emerged as promising scaffold materials for bone regeneration. Our previous in vivo research on a self-assembling peptide hydrogel (SPG-178) highlights its ability to promote osteoblast differentiation and enhance bone gene expression, including alkaline phosphatase (ALP) and osteocalcin.[15] Additionally, its combination with allogeneic bone chips has been shown to increase bone formation in critical-sized defects.[16] This suggests SPG-178 could serve as a valuable alternative to traditional bone graft materials, particularly when autografts or BMP-2 treatments are infeasible due to patient-specific risk factors.

Building on our previous findings, this study aims to evaluate the bone regeneration capabilities of SPG-178, BMP-2, HA, and β-TCP, both individually and in combination with bone chips, in a rat femoral defect model. We hypothesize that SPG-178, particularly when combined with bone chips, will serve as a viable scaffold for supporting bone regeneration while minimizing the risks associated with conventional treatments. Futhermore, a comprehensive evaluation of these combinations can inform the development of safer and more effective bone regenerative therapies that address the limitations of current materials and approaches in orthopedic surgery.

MATERIALS AND METHODS

Animals

Twenty-seven female Sprague-Dawley rats, aged 10 weeks and weighing between 300 and 350 g, were used in this study. All procedures were conducted in accordance with the guidelines of the National Research Council's Guide for the Care and Use of Laboratory Animals (1996) and were approved by the Institutional Animal Care and Use Committee of Nagoya University. The rats were housed in a temperature-controlled environment with a 12-h light/dark cycle and had ad libitum access to food and water. Isoflurane was used for anesthesia, and postoperative pain

management was achieved with subcutaneous buprenorphine (0.05 mg/kg) every 12 h for 48 h. Considerable efforts were made to minimize pain and distress throughout the study.

A power analysis based on previous studies determined a sample size of 27 rats to ensure sufficient statistical power ($\beta = 0.8$, $\alpha = 0.05$) for detecting significant differences among experimental groups.

Bone defect model and polyetheretherketone cage

A critical-sized bone defect (5 mm in length) was created in the midshaft of the femur of each rat under anesthesia. An external fixator was used to stabilize the defect, and a polyetheretherketone (PEEK) cage (outer diameter, 5 mm; inner diameter, 3 mm; height, 5 mm) was inserted into the defect. The PEEK cage, chosen for its high biocompatibility, resistance to degradation, and mechanical stability compared to other polymers, provided consistent structural support throughout the bone healing process. The wound was closed using 5-0 nylon sutures following cage insertion, and the rats were monitored for 56 days postoperatively.

Experimental groups

Two primary experiments were conducted to evaluate the effects of bone substitute materials on bone regeneration. In the first experiment, the PEEK cages were filled with bone substitute materials alone: SPG-178 hydrogel (0.8%), BMP-2 (50 ng/μL), HA, and β-TCP. In the second experiment, bone chips (0.02 g per cage) were integrated with each bone substitute material, and BMP-2 was applied to both sides of the chip using a micropipette to ensure even distribution. SPG-178 hydrogel was prepared by dissolving the peptide in sterile water to a concentration of 0.8%, whereas BMP-2 was reconstituted to a concentration of 50 ng/μL using sterile saline. Bone chips were harvested from fresh allograft sources, cleaned with saline, and sterilized by autoclaving at 120 °C for 20 minutes. The control group had empty PEEK cages, while experimental groups had cages filled with the respective materials.

Imaging and quantitative analysis

At 56 days postoperatively, digital X-ray imaging and micro-computed tomography (CT) were performed to assess bone regeneration within the defect. Three-dimensional reconstruction software was used to calculate the bone-volume-to-total-volume (BV/TV) ratios. Additional metrics, including trabecular thickness, separation, and number, were quantified to provide a comprehensive understanding of bone structure. Histological analysis was performed on undecalcified bone sections. Von Kossa and alizarin red stains were used to evaluate mineralization, ALP staining was used to assess osteoblast activity, and tartrate-resistant acid phosphatase staining was utilized to evaluate osteoclast activity and bone resorption.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test for pairwise comparisons between experimental groups. ANOVA was selected under the assumption of normally distributed data and equal variances between groups. These assumptions were verified using the Shapiro-Wilk test for normality and Levene's test for homogeneity of variances. All data were presented as means ± standard deviation. Statistical significance was set at a $p < 0.05$, and effect sizes with 95% confidence intervals were calculated to provide a better understanding of the clinical relevance of the findings.

RESULTS

In the first experiment, the bone regeneration potential of SPG-178, BMP-2, HA, and β-TCP was assessed using PEEK cages in critical-sized defects of rat femurs. Radiological and micro-CT analyses revealed no significant differences in bone formation among the groups or between all materials and the controls (Fig. 1). SPG-178 demonstrated moderate bone formation, with a BV/TV ratio of 0.34 ± 0.09, although this result was not significantly higher than that of the control group. Similarly, the results for the other materials did not show significant osteogenesis BMP-2 (BV/TV, 0.30 ± 0.07), HA (BV/TV, 0.25 ± 0.23), β-TCP (BV/TV, 0.23 ± 0.13) compared to controls.

Histological analysis supported these observations (Fig. 2). Hematoxylin and eosin (HE) staining showed reduced bone formation in HA and β-TCP, whereas BMP-2 exhibited substantial new bone formation. HA and β-TCP were not stained black by HE staining, and both of them were considered degraded. Von Kossa and alizarin red staining confirmed the presence of enhanced mineralization in BMP-2, with dense calcium deposits throughout the defect. Additionally, ALP staining indicated increased osteoblastic activity in BMP-2, correlating with radiological findings of robust bone formation.

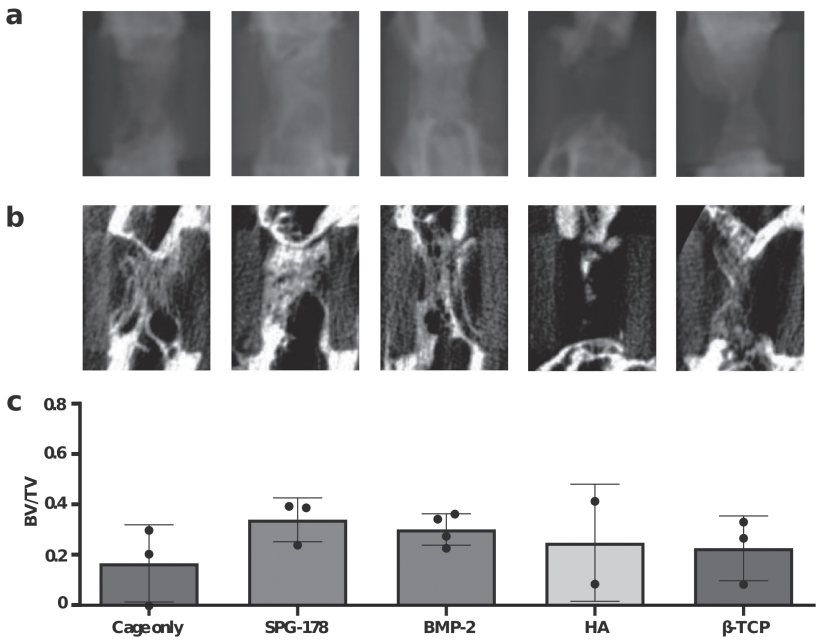

Fig. 1 X-ray and quantitative micro-CT analysis of bone repair using cell-seeded scaffolds filled only with bone substitute material

Fig. 1a: X-ray
Fig. 1b: CT
Fig. 1c: Quantitative micro-CT analysis
CT: computed tomography
BV/TV: bone-volume-to-total-volume
SPG-178: self-assembling peptide hydrogel-178
BMP-2: bone morphogenetic protein-2
HA: hydroxyapatite
β-TCP: β-tricalcium phosphate

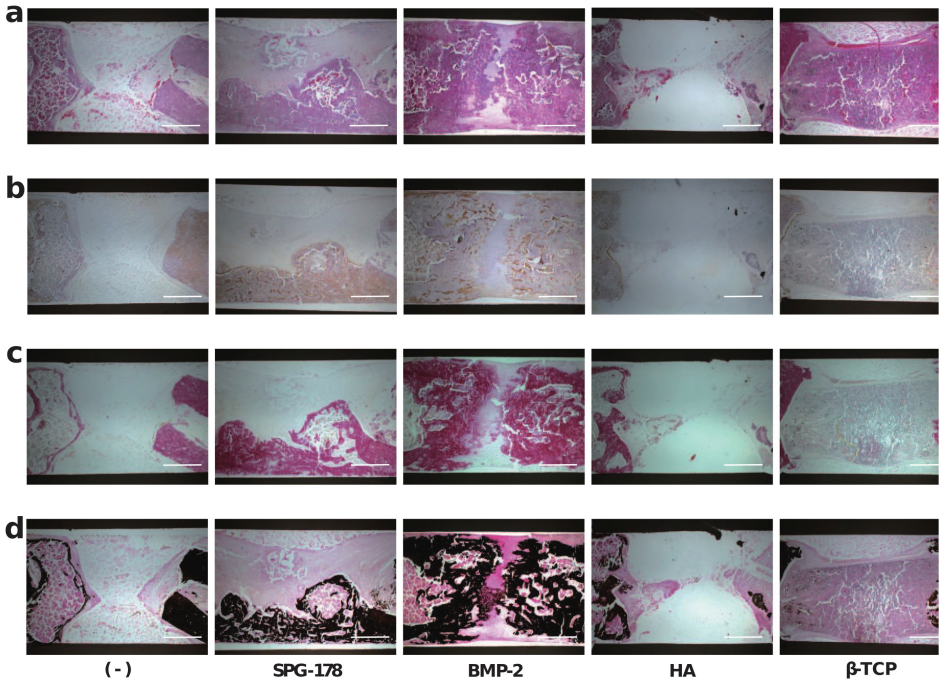

Fig. 2 Histological analysis of bone repair in response to cell-seeded scaffolds filled only with bone substitute material

Fig. 2a: HE
Fig. 2b: ALP
Fig. 2c: Alizarin red
Fig. 2d: Von Kossa
HE: hematoxylin and eosin
ALP: alkaline phosphatase
SPG-178: self-assembling peptide hydrogel-178
BMP-2: bone morphogenetic protein-2
HA: hydroxyapatite
β-TCP: β-tricalcium phosphate

Despite moderate bone formation in SPG-178, significant portions of the defect were unhealed, indicating incomplete bone regeneration. This suggests that SPG-178 alone offered limited osteoinductive capacity compared to BMP-2.

Given the results of the first experiment, the second phase of the study focused on integrating bone chips with the bone substitute materials to assess their combined bone regeneration abilities. This experiment is significant because the use of integrated bone materials is commonly practiced in clinical settings.

Results from the second experiment revealed that combining BMP-2 with bone chips produced the highest level of bone regeneration, with a BV/TV ratio of 0.62 ± 0.06, significantly surpassing bone chips alone ($p < 0.01$) and SPG-178 with bone chips ($p < 0.01$; Fig. 3). Radiographic and micro-CT images confirmed near-complete healing of the defect in this group, with findings of newly formed bone seamlessly bridging the gap.

Conversely, combining SPG-178 with bone chips did not significantly improve bone regeneration compared to bone chips alone (BV/TV, 0.20 ± 0.17; $p = 0.45$). Likewise, combining HA

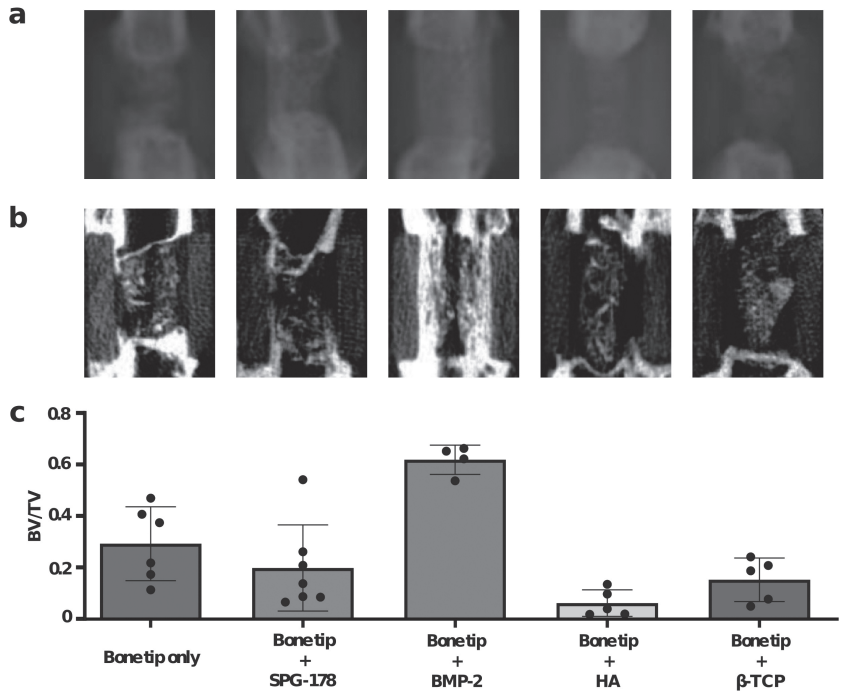

Fig. 3 X-ray and quantitative micro-CT analysis of bone repair using cell-seeded scaffolds filled with bone substitute material and bone chips

Fig. 3a: X-ray
Fig. 3b: CT
Fig. 3c: Quantitative micro-CT analysis
Abbreviations are explained in the list to Fig. 1.

or β-TCP with bone chips did not enhance bone formation. Interestingly, HA combined with bone chips appeared to inhibit bone regeneration, as indicated by a reduced BV/TV ratio of 0.06 ± 0.05. This finding is consistent with previous research suggesting that HA may slow down bone formation due to its slower degradation rate and interference with the natural remodeling process.[17,18]

Histological analysis further supported these results (Fig. 4). HA and β-TCP were not stained black by HE staining, and both of them were considered degraded. Von Kossa and alizarin red staining revealed extensive mineralization in BMP-2 with bone chips, while the remaining bone materials with bone chips demonstrated incomplete mineralization and lower levels of new bone formation. ALP staining also indicated significantly higher osteoblastic activity in BMP-2 with bone chips compared to the other bone materials, confirming the enhanced bone regeneration observed in radiological and histological analyses.

Collectively, these results indicate that while SPG-178 alone or in combination with bone chips provided moderate support for bone regeneration, BMP-2, particularly when combined with bone chips, significantly enhanced bone formation and defect healing in critical-sized defects. These findings underscore the critical importance of osteoinductive factors, such as BMP-2, to achieve optimal bone regeneration in clinical applications.

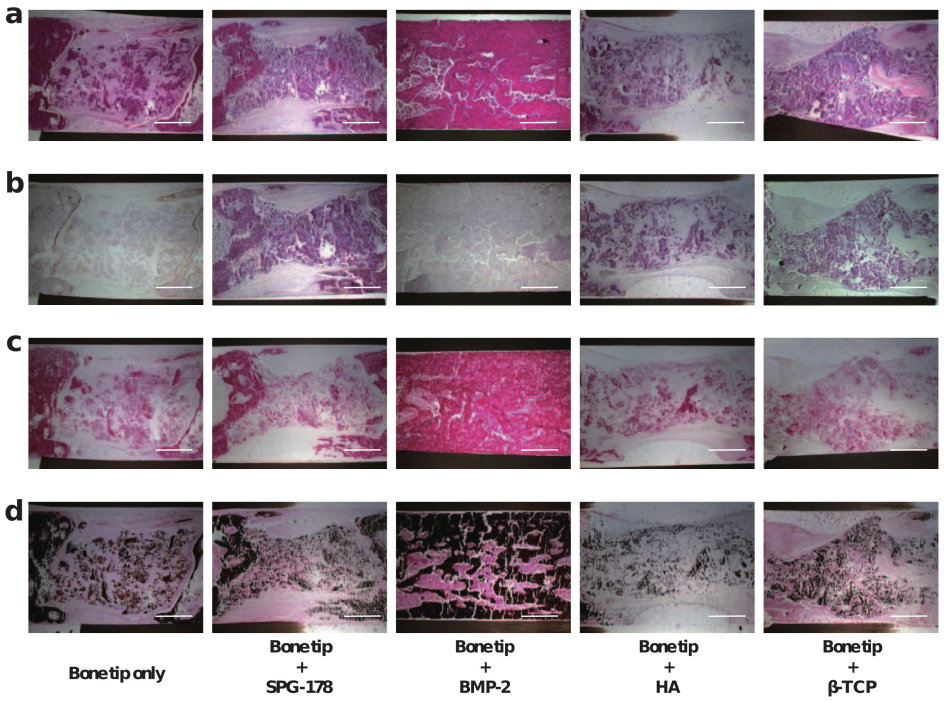

Fig. 4 Histological analysis of bone repair in response to cell-seeded scaffolds filled with bone substitute material and bone chips

Fig. 4a: HE
Fig. 4b: ALP
Fig. 4c: Alizarin red
Fig. 4d: Von Kossa
Abbreviations are explained in the list to Fig. 2.

DISCUSSION

This study builds upon our previous research on SPG-178 and further highlights the significance of osteoinductive agents in promoting bone regeneration, particularly in critical-sized defects. Our findings confirm that while SPG-178 shows promise as a biocompatible scaffold, it requires additional osteoinductive support such as BMP-2 to achieve optimal bone healing.

Although SPG-178 exhibited moderate bone regeneration, especially when combined with bone chips, its efficacy as a standalone material was limited compared to BMP-2. This aligns with prior studies suggesting that SPG-178 primarily functions as a supportive matrix rather than a potent bone-forming agent on its own. The role of SPG-178 as a scaffold was further confirmed by the absence of significant bone formation when used alone or with bone chips in the absence of BMP-2. Despite its moderate osteoconductive properties, the lack of sufficient osteoinductive potential limited the degree of bone healing in these experimental groups. This observation emphasizes that while SPG-178 provides a stable environment for bone regeneration, it is dependent on additional factors, such as BMP-2, for more substantial healing.

Several studies on the efficacy of SPG-178 have been reported for skull defects in mice, tibial bone defects, and posterior spinal fusion in rabbits.[16,19] Advantages of self-assembling peptides include their being 100% chemically synthesized materials, minimizing cytotoxicity and risks of

biological contamination or undefined factors.[15,20] These materials are expected to have clinical applications in post-fracture pseudoarticulation and intervertebral spinal fusion.

Conversely, BMP-2 demonstrated superior bone healing capabilities, especially when combined with bone chips. BMP-2 promoted robust bone formation, evidenced by findings of high BV/TV ratios, extensive mineralization, and increased osteoblastic activity in this study. This is consistent with extensive clinical evidence on spinal fusion surgeries, where BMP-2 has been successfully applied to enhance fusion rates in anterior and posterior lumbar fusions, as well as complex multilevel spinal procedures.[21,19] Despite its advantages, this material is not without risks. Studies have shown that its application may lead to certain complications, such as ectopic bone formation, inflammation, and excessive bone growth.[13,14] Nevertheless, our study showed that BMP-2 provides a potent osteoinductive stimulus for successful bone healing in the management of critical-sized defects. BMP-2 has been reported in several studies in combination with drug delivery systems due to complications associated with supra-physiologic doses when applied clinically.[22,23] Lee et al[22] developed a collagen-HA scaffold incorporated with BMP-2 and alendronate-loaded poly (lactic-co-glycolic acid) microspheres for the sequential release of drugs to control bone remodeling. Tateiwa et al showed that the combination treatment with rhBMP-2 and a retinoic acid receptor γ antagonist-loaded nanoparticle resulted in a significantly better spinal fusion rate and bone formation than rhBMP-2 alone.[23] Further studies on the combination of SPG-178 and BMP-2 are warranted.

In clinical applications, HA is often used as a bone graft extender rather than a primary graft material. However, its performance as a standalone bone graft substitute has produced mixed results.[24] HA has been reported to support bone healing when used in conjunction with growth factors or osteoinductive agents, such as BMP-2.[25] Furthermore, in certain clinical models, standalone HA use has been associated with delayed bone healing or fibrous tissue formation.[26] This may explain our findings of reduced bone formation in HA, underscoring the challenges of using standalone HA in complex bone healing scenarios and the need for osteoinductive supplementation for optimal results.

Histological analysis confirmed these findings. Combining BMP-2 with bone chips resulted in significantly greater osteoblastic activity, indicated by enhanced ALP staining and extensive mineralization, whereas SPG-178 and HA showed reduced bone formation and mineralization. This emphasizes the established notion that these materials require osteoinductive agents for sufficient bone healing. The histological analysis, particularly ALP staining and mineralization assessments, further confirms the importance of the osteoinductive properties of BMP-2. The stark differences in new bone formation between BMP-2 and other bone materials highlights the former's key role in stimulating osteogenesis in critical bone defects.

This study had a few limitations. First, the number of samples per model was limited. Additionally, because a rat model was used, the results of this study cannot be directly applied to humans. Second, the secondary effects of the materials have not been fully investigated.

In conclusion, while SPG-178 holds promise as a scaffold material, particularly in minimally invasive applications, its potential for promoting significant bone regeneration requires supplementation with osteoinductive factors like BMP-2. Similarly, despite the widespread use of HA for its osteoconductive properties, this material appeared to be less effective in promoting bone healing without additional osteoinductive support. Future research should focus on optimizing the combination of bone grafting materials with osteoinductive agents to maximize their potential in spinal fusion and other orthopedic settings. The ongoing development of composite scaffolds combining both osteoconductive and osteoinductive materials may offer a more comprehensive approach to bone regeneration in complex clinical cases, where traditional single-material approaches may prove insufficient.

DISCLOSURE STATEMENT

The authors declare that there are no conflicts of interest related to this study and no specific funding was received to support this work.

MISCELLANEOUS ACKNOWLEDGEMENT

We would like to thank Editage (www.editage.com) for English-language editing.

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