Original Article

Crosslinking Strategies for DBM Putty

Ionic versus Covalent Crosslinking of Xenograft Demineralized Bone Matrix Putty: Comparative in Vitro Stability and Osteoblast Compatibility for Spinal Fusion Applications

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ABSTRACT

Objective: To compare an ionic crosslinker, aluminium hydroxide [$Al(OH)_3$] gel, and a covalent crosslinker, paraformaldehyde (PFA), in xenograft DBM putty with respect to in vitro stability and osteoblast compatibility.
Study Design: An in vitro experimental study
Place and Duration of Study: This study was conducted at the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia, between November 2025 and April 2026.
Methods: An in vitro experimental study was conducted using bovine DBM putty composed of 70% DBM, 25% carboxymethylcellulose, and 5% crosslinker. Specimens were crosslinked with either $Al(OH)_3$ gel or PFA and sterilized by gamma irradiation. Stability was evaluated through swelling ratio, quantitative cohesiveness, and enzymatic degradation testing. Biocompatibility was assessed using 7F2 osteoblast viability and adhesion assays. Twenty-five specimens were analyzed per group.
Results: Swelling ratio was comparable between the $Al(OH)_3$ gel and PFA groups (408.4% vs 444.4%, p = 0.068). PFA demonstrated lower mass loss during cohesiveness testing (26.0% vs 36.3%, p = 0.026) and lower enzymatic degradation (10% vs 25%, p = 0.025). Osteoblast adhesion was higher in the $Al(OH)_3$ gel group than in the PFA group (44.7% vs 38.4%, p = 0.019). Cell viability did not differ significantly between groups (31.7% vs 30.0%, p = 0.210).
Conclusion: PFA was associated with greater cohesiveness and lower enzymatic degradation, whereas $Al(OH)_3$ gel supported greater osteoblast adhesion. Both formulations demonstrated similarly low cell viability, highlighting the need for further formulation optimization.
Key Words: Demineralized Bone Matrix, Spinal Fusion, Bone Transplantation, Biomaterials, Osteoblasts

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INTRODUCTION

Spinal fusion remains a fundamental procedure for the treatment of degenerative, traumatic, and deformity related spinal disorders. Despite advances in surgical techniques and instrumentation, pseudarthrosis continues to represent an important cause of treatment failure and revision surgery. Fusion outcomes depend not only on mechanical stabilization but also on the biological performance of the graft material used to support new bone formation.[1–4]

Autologous bone graft remains the clinical standard because it provides osteoconductive, osteoinductive, and osteogenic properties. However, donor site morbidity, limited graft availability, and additional operative requirements have encouraged the development of alternative bone graft substitutes. Demineralized bone matrix (DBM) has attracted considerable interest because demineralization preserves a collagen rich extracellular matrix and bioactive proteins that contribute to bone regeneration.[5–8]

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Putty form DBM offers practical advantages for spinal fusion, including ease of handling, adaptability to irregular defects, and improved graft containment during implantation. Nevertheless, the collagen-based structure of DBM putty may be associated with excessive swelling, limited cohesiveness, and accelerated degradation under physiological conditions. These limitations may reduce graft retention and shorten the period during which the scaffold can support bone healing.[9,10]

Crosslinking has emerged as a strategy to improve the stability of collagen-based biomaterials by strengthening interactions within the matrix structure. Covalent crosslinking may improve resistance to degradation and material integrity, whereas ionic crosslinking may better preserve cell material interactions.[11–14] However, comparative evidence evaluating ionic and covalent crosslinking approaches in putty form DBM remains limited. This study compared xenograft DBM putty crosslinked with aluminium hydroxide [$Al(OH)_3$] gel as an ionic crosslinker and paraformaldehyde (PFA) as a covalent crosslinker. The formulations were evaluated through in vitro assessments of swelling ratio, cohesiveness, enzymatic degradation, osteoblast viability, and osteoblast adhesion to characterize the balance between material stability and biological compatibility.

METHODS

Study Design: This in vitro experimental study was conducted at the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia, between November 2025 and April 2026. The study compared xenograft demineralized bone matrix (DBM) putty crosslinked with either aluminium hydroxide [$Al(OH)_3$] gel or paraformaldehyde (PFA). The evaluated outcomes included swelling ratio, quantitative cohesiveness, enzymatic degradation, osteoblast viability, and osteoblast adhesion.

Ethical Approval: The study protocol was approved by the Ethics Committee of the Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia (No. 347/EC/KEPK/FKUA/2025).

Preparation of DBM Putty: All procedures were performed in accordance with ASTM F2212 guidelines for DBM characterization and relevant ISO standards for biological evaluation and sterilization of medical devices. Fresh bovine long bones were cleaned of residual soft tissue and immersed in 70% ethanol for 24 h, followed by 3% hydrogen peroxide for an additional 24 h to remove residual tissue and lipids. The specimens were subsequently demineralized in 0.6 N hydrochloric acid at 4°C for 24–48 h, washed repeatedly with phosphate buffered saline (PBS) until a neutral pH was achieved, and dried at 37–45°C. The resulting DBM was ground and sieved to obtain particles measuring 100–500 μm.

DBM putty was prepared using a formulation adapted from previously reported DBM based composites. The final composition consisted of 70% DBM, 25% carboxymethylcellulose (CMC), and 5% crosslinker. For the ionic crosslinking group, 1% $Al(OH)_3$ gel was incorporated into the DBM CMC mixture and incubated for 1–2 h before PBS washing. For the covalent crosslinking group, 2% PFA was added and incubated for 2 h at 4°C, followed by repeated PBS washing to reduce residual crosslinker. The resulting putty was molded into cylindrical specimens measuring approximately 10 mm in diameter and 5 mm in height. Prior to testing, all specimens were sterilized using 15 kGy gamma irradiation at the Radiation Technology Laboratory, National Research and Innovation Agency, Indonesia.

Stability Assessment: Physical stability was evaluated using swelling ratio, quantitative cohesiveness, and enzymatic degradation assays.

For swelling ratio testing, dried specimens were immersed in PBS (pH 7.4) at 37°C for 24 h. The swelling ratio was calculated from the difference between wet and dry weights and expressed as a percentage of the initial dry weight.

Quantitative cohesiveness was assessed by measuring mass loss after immersion in PBS at 37°C for 2 h. Lower mass loss indicated greater cohesiveness and structural integrity of the putty formulation.

Enzymatic degradation was evaluated by incubating specimens in type II collagenase solution (125 U/mL) at 37°C for 24 h. Following incubation, specimens were washed, dried, and reweighed. Degradation was expressed as the percentage reduction in dry weight relative to baseline.

Biocompatibility Assessment: Biocompatibility was evaluated using 7F2 osteoblast cells in accordance with ISO 10993-5 recommendations. For cell viability testing, material extracts were prepared by incubating 0.2 g of putty in 1 mL of Dulbecco's Modified Eagle Medium (DMEM) for 24 h at 37°C. Extracts were subsequently applied to 7F2 osteoblast cultures seeded at a density of 1×10^4 cells/well. Cell viability was quantified using the MTT assay after 24 h of exposure and expressed relative to untreated controls.

For cell adhesion testing, putty disks measuring approximately 5 mm in diameter and 2 mm in thickness were sterilized by ultraviolet exposure and placed in culture wells containing DMEM supplemented with 10% fetal bovine serum. Osteoblasts were seeded at a density of 1×10^5 cells/well and incubated for 24 h. Nonadherent cells were removed by PBS washing, and adherent cells were quantified using the MTT assay with absorbance measured at 570 nm.

Sample Size and Statistical Analysis: Sample size was estimated using Cohen's d methodology assuming a large effect size (d = 0.8), a significance level of 0.05,

and a statistical power of 80%, resulting in a minimum of 25 specimens per group for each outcome measure.

Data were analyzed using IBM SPSS Statistics version 29. Normality was assessed using the Shapiro–Wilk test and homogeneity of variance using Levene's test. Normally distributed variables were compared using the independent t test, whereas nonnormally distributed variables were analyzed using the Mann–Whitney U test. Statistical significance was defined as $p < 0.05$.

RESULTS

A total of 25 specimens were evaluated in each group for all outcome measures (Total sample size = 250). Enzymatic degradation data were not normally distributed and were analyzed using nonparametric methods, whereas all other variables met assumptions for parametric analysis.

The mean swelling ratio across all specimens was 426.4%. The $Al(OH)_3$ gel group demonstrated a lower swelling ratio than the PFA group (408.4 ± 62.8% vs 444.4 ± 73.3%), although the difference was not statistically significant ($p = 0.068$).

Quantitative cohesiveness differed significantly between groups. The $Al(OH)_3$ gel group exhibited greater mass loss than the PFA group (36.3 ± 15.8% vs 26.0 ± 15.9%; $p = 0.026$), indicating lower cohesiveness and reduced structural integrity following PBS immersion.

Similarly, enzymatic degradation was significantly greater in the $Al(OH)_3$ gel group than in the PFA group, with median degradation rates of 25% (range, 0–48%) and 10% (range, 0–40%), respectively ($p = 0.025$). These findings indicate greater resistance to collagenase mediated degradation in the PFA crosslinked formulation.

Table No.1: Stability and biocompatibility outcomes of xenograft DBM putty crosslinked with aluminium hydroxide gel and paraformaldehyde

Outcome	$Al(OH)_3$ gel (n = 25)	PFA (n = 25)	p value
Swelling ratio (%)	408.4 ± 62.8	444.4 ± 73.3	0.068
Mass loss (%)	36.3 ± 15.8	26.0 ± 15.9	0.026
Enzymatic degradation (%)†	25 (0–48)	10 (0–40)	0.025
Cell viability (%)	31.7 ± 5.7	30.0 ± 4.0	0.210
Cell adhesion (%)	44.7 ± 8.8	38.4 ± 9.6	0.019

Data are presented as mean ± standard deviation unless otherwise indicated.

† Data are presented as median (range).

The mean cell viability across all specimens was 30.8%. No significant difference was observed between the $Al(OH)_3$ gel and PFA groups (31.7 ± 5.7% vs 30.0 ± 4.0%; $p = 0.210$). Notably, cell viability remained below the ISO 10993-5 threshold for noncytotoxic materials in both formulations.

In contrast, osteoblast adhesion differed significantly between groups. The $Al(OH)_3$ gel group demonstrated greater cell adhesion than the PFA group (44.7 ± 8.8% vs 38.4 ± 9.6%; $p = 0.019$), suggesting a more favorable surface environment for early osteoblast attachment.

A summary of all stability and biocompatibility outcomes is presented in Table 1.

DISCUSSION

This study compared ionic and covalent crosslinking strategies in xenograft demineralized bone matrix (DBM) putty for spinal fusion applications. The principal finding was a tradeoff between structural stability and cellular response. PFA crosslinking improved cohesiveness and resistance to enzymatic degradation, whereas $Al(OH)_3$ gel supported greater osteoblast adhesion. Swelling behavior and cell viability were comparable between formulations. These findings highlight the challenge of balancing scaffold persistence and biological performance during the development of DBM based graft substitutes.[15,16]

Although swelling ratio did not differ significantly between groups, marked differences were observed in cohesiveness and enzymatic degradation. The lower mass loss and reduced collagenase mediated degradation observed in the PFA group indicate that covalent crosslinking generated a more stable matrix structure. This observation is consistent with previous studies demonstrating that crosslinking strengthens intermolecular interactions within collagen-based biomaterials and improves resistance to degradation.[17,18] In the context of spinal fusion, preservation of scaffold integrity may be advantageous because premature graft degradation could reduce osteoconductive support before sufficient new bone formation occurs.

The biological findings warrant careful consideration. Despite demonstrating superior stability, PFA did not support greater osteoblast adhesion. Instead, adhesion was significantly higher in the $Al(OH)_3$ gel group, suggesting that ionic crosslinking may better preserve surface characteristics that facilitate early cell attachment. Surface chemistry plays a critical role in regulating cell material interactions, and modifications that improve scaffold stability may simultaneously alter cellular attachment behavior.[19] Because osteoblast attachment precedes proliferation, differentiation, and matrix deposition, this finding may have important implications for the biological performance of DBM based graft substitutes.

No significant difference in cell viability was observed between groups. However, both formulations demonstrated viability values below the ISO 10993-5 threshold for noncytotoxic materials. This represents the most important limitation identified in the present study. The finding suggests that neither formulation achieved satisfactory cytocompatibility under the tested conditions. Previous studies have reported that aluminium containing biomaterials and aldehyde based crosslinkers may influence osteoblast function in a concentration dependent manner.[20,21]

The present findings emphasize that improvements in physical stability alone are insufficient for the development of clinically useful bone graft substitutes. An ideal scaffold for spinal fusion should maintain structural integrity while simultaneously supporting favorable cellular responses. While covalent crosslinking enhanced cohesiveness and degradation resistance, ionic crosslinking favored osteoblast attachment. Neither formulation achieved an optimal balance between these characteristics, indicating that further refinement of formulation composition and crosslinker concentration is required.

Several limitations should be acknowledged. The absence of an uncrosslinked DBM control group limits assessment of the absolute contribution of crosslinking. In addition, only in vitro outcomes were evaluated, and osteogenic differentiation, mineralization, mechanical properties, and in vivo fusion performance were not assessed. Future studies should investigate optimized formulations and validate these findings in preclinical spinal fusion models.

Overall, PFA crosslinking improved cohesiveness and resistance to enzymatic degradation, whereas $Al(OH)_3$ gel supported greater osteoblast adhesion. However, neither formulation achieved satisfactory cytocompatibility. Further optimization is required before either approach can be considered for in vivo evaluation.

CONCLUSION

PFA crosslinking improved cohesiveness and resistance to enzymatic degradation, whereas $Al(OH)_3$ gel promoted greater osteoblast adhesion. However, neither formulation achieved satisfactory cytocompatibility. Further optimization is required to improve the balance between scaffold stability and biological performance before in vivo evaluation for spinal fusion applications.

Author's Contribution:

Concept & Design or acquisition of analysis or interpretation of data:	Andi Bahauddin Fahmi, Lukas Widhiyanto, Dwikora Novemberi Utomo
Drafting or Revising Critically:	Heri Suroto, Romaniyanto, Atika
Final Approval of version:	All the above authors
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