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ORIGINAL ARTICLE

Bone Marrow - Mesenchymal Stem Cell and Platelet Rich Fibrin: A Promising Step of Growth Plate Injury Treatment in Rabbit

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Abstract

Background/Aim: Paediatric physeal injuries account for about 30 % of all bone injuries, often affecting growth plates. Current treatments emphasise prevention, as regenerating the damaged physis is challenging. This study aimed to analyse whether combining bone marrow mesenchymal stem-cell (BM-MSC) and platelet-rich fibrin (PRF) can regenerate physis bone.

Methods: The twenty-four New Zealand white rabbits, aged 6 weeks, were acclimatised for 1 week. BM-MSC and PRF were then prepared. A physis injury was induced in the proximal tibia of the rabbits, then divided into four groups: control, BM-MSC treatment, PRF treatment, combination of BM-MSC and PRF treatment. After 4 weeks, rabbits were sacrificed and evaluated. The bony bar diameter was measured using haematoxylin-eosin (H&E) staining, while the expression of tumour necrosis factor-alpha (TNF-α), vascular endothelial growth factor (VEGF) and SRY-box transcription factor 9 (SOX-9) was evaluated using immunohistochemistry (IHC).

Results: Histology showed that the BM-MSC and PRF combination led to better regeneration than the control, BM-MSC alone, or PRF alone in terms of osteochondral union. SOX-9 indicators showed significant differences between the control vs BM-MSC groups ($p = 0.099$); BM-SC vs PRF groups ($p = 0.032$). TNF-α indicators showed no significant differences at all. VEGF indicators showed significant differences between the control vs BM-MSC and PRF groups ($p = 0.008$); PRF vs BM-MSC and PRF groups ($p = 0.021$).

Conclusion: Administration of BM-MSC, PRF or a combination of BM-MSC and PRF showed comparable effectiveness in osteochondral union based on histological outcomes. Conversely, PRF alone exhibited the highest effectiveness in IHC analysis. However, none of these results were statistically significant.

Key words: Bone marrow; Health outcomes; Mesenchymal stem cell; Platelet-rich fibrin; Physeal injury; Tibia; Product innovation.

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Introduction

Paediatric physeal injuries generally account for about 30 % of all bone injuries, often affecting growth plates near major joints.[1] They are more common among boys who are active in sports. These injuries focus on damaging the cartilage in the growth plates, causing abnormal bone growth and potentially leading to problems such as low back pain and osteoarthritis. The current

treatment focuses on prevention, as regeneration of the damaged physis is still difficult to achieve.[2] Various regenerative therapies, including the use of bone marrow-mesenchymal stem cells (BM-MSC) and platelet-rich fibrin (PRF) are being developed.[3, 4]

The MSCs derived from bone marrow or adipose tissue induce bone regeneration by promoting osteogenesis and angiogenesis. MSCs enhance bone formation, exhibit mechanical properties similar to native bone and modulate the immune response to facilitate healing in bone defects and physeal injuries.[5]

The use of PRF, a platelet derivative, offers superior regeneration outcomes and avoids platelet-rich plasma (PRP)'s limitations. PRP production necessitates anticoagulants and bovine thrombin, potentially inducing antibodies and coagulopathies. PRP's liquid form may disperse from injury sites, reducing effectiveness. PRF, in gel form, is easier to apply, prevents dispersion and serves as a scaffold for physis defects.[6, 7]

Tumour necrosis factor-alpha (TNF-α), vascular endothelial growth factor (VEGF) and SRY-box transcription factor 9 (SOX-9) are crucial for physis bone healing. TNF-α initiates the inflammatory response, recruiting immune cells. VEGF promotes angiogenesis, ensuring blood supply and nutritional delivery. SOX-9 regulates chondrogenesis, guiding cartilage formation and bone repair. These factors coordinate to enhance physis regeneration during bone healing.[8, 9]

A comprehensive comparison of BM-MSCs alone, PRF alone and the combination of MSCs and PRF elucidates their individual and synergistic regenerative potential, underlying mechanisms, clinical significance, therapeutic interactions and inherent limitations, ultimately contributing to more effective tissue repair and regeneration strategies.[10, 11] However, research on their effectiveness in physeal injuries, specifically in osteochondral union promotion and bony bridge prevention in fibrous union is limited: This study aimed to explore whether the combination of BM-MSC and PRF can successfully regenerate physeal bone.

Methods

Study design and sampling

This study employed a true experimental method with a post-test only control group design. Experimental and control groups were created randomly to ensure equivalence. The experimental group received the treatment and after a specific period, the dependent variable was measured in both groups to compare differences. There were four groups: a control group, treatment group 1 (injected with PRF only), treatment group 2 (injected with BM-MSC only) and treatment group 3 (injected with a combination of PRF and BM-MSC). After four weeks, the rabbits were sacrificed to measure levels of TGF-α, SOX-9 and VEGF. The subjects were the distal femoral physes of adult New Zealand white rabbits with inclusion criteria of male gender, age 6-9 weeks, no physical deformities and being healthy and active. Exclusion criteria included being younger than 6 weeks or older than 9 weeks and any illness or infection during the study.

Simple random sampling was used to select 24 rabbits, with 6 in each group. New Zealand white rabbits were chosen because they are ideal for physeal injury studies due to their predictable growth, manageable size and bone structure similar to human growth plates. Their availability and widespread use in research ensure consistent, reproducible results. Extensive literature and established protocols further support their application in orthopaedic research, providing a robust framework for experimental design and data interpretation.

Research procedure

This procedure was adapted from Yin et al, who used a rabbit model in their research.[12]

Animal preparation

Twenty-four 6-week-old New Zealand white rabbits were purchased and acclimated for one week before the procedure. They were housed in 50 x 70 cm cages, fed 300 g of pellets daily and given water *ad libitum*. Their cages were cleaned daily. On surgery day, the rabbits were anesthetised with intravenous ketamine (25 mg/kg) and xylazine (2.5 mg/kg), maintained with 1.5–3.0 % isoflurane. Vital signs were monitored and the surgical area was prepared and disinfected.

PRF preparation

About 5 mL of venous blood was drawn from the rabbit's ear and placed into sterile 6 mL vacuum tubes without any anticoagulant. The tubes were then placed in a centrifuge and spun at 2700 rpm for 12 min. This process resulted in three layers in the tube: the bottom layer containing red blood cells, the top layer containing cellular plasma and the middle layer containing a fibrin clot. The top plasma layer was removed and the middle layer was taken 2 mm below the dividing line.

BM-MSC preparation

Bone marrow sampling. Rabbits were anesthetised with ketamine (40 mg/kg) and xylazine (10 mg/kg). The rabbits were then sacrificed. The femur bones were collected and sectioned for BM-MSC culture.

BM-MSC culture. Bone marrow samples were diluted with MSC growth medium and distributed across multiple culture dishes. The dishes were incubated at 37 °C with 5 % CO_2 and 2 % O_2 (hypoxia) for 4-5 days. The medium was replaced every 3-4 days to remove dead cells. MSC colonies started forming within 5-7 days, with some cells continuing to divide. By days 12-14, small colonies were visible. Cells were washed and sub-cultured using trypsin or EDTA to detach them. The cells were then washed off the surface and divided into two dishes. If needed, MSCs were concentrated by centrifugation and resuspended in fresh growth medium. The cells were counted using a haemocytometer and placed into tubes at the final concentration. This process allowed for the isolation and expansion of MSCs for further study.

BM-MSC characterisation and differentiation. The process involved extracting bone marrow, culturing the mononuclear cells in DMEM medium with 10 % foetal bovine serum and subculturing when the cells reached 80 % confluence. The cells were then washed, counted and verified for cell phenotype. The final product was a suspension of 1.0-2.5 × 10^6 BM-MSCs per 2 mL, ready for transplantation.

Physeal injury model

The injury model was applied to the right proximal tibia of rabbits. A 3 cm incision was made to expose the physis. The soft tissue was cleared to reveal the medial collateral ligament. A 1 mm drill bit was used to create a 5 mm deep defect perpendicular to the tibial axis. The defect was irrigated with sterile saline and the wound closed with sutures. The rabbits were then allowed to resume normal activity. This model mimics a common injury in humans and allows for the study of bone healing and regeneration.

PRF and BM-MSC implantation

The PRF and BM-MSC were implanted into the defect area, which was then closed with a muscle flap followed by wound closure. All animals were returned to their cages and monitored until the evaluation procedure.

Evaluation procedure

Animals were sacrificed in the fourth week and an evaluation was conducted by measuring the diameter of the bony bar using histological examination with haematoxylin-eosin (H&E) staining at 8 x magnification. Histological findings were evaluated with a histological grading scale modified from Wakitani et al with amount of the new bone 0 = < 25 %; +1 = 25-50 %; +2 = 50-75 %; +3 = 75-100 %; +4 = > 100 %, the mean amount of the repaired bone compared with the surrounding bone. Additionally, an evaluation was performed using immunohistochemistry (IHC) staining to assess the expression of TNF-α, VEGF and SOX-9. The concentrations of TNF-α, VEGF and SOX-9 were measured from the injured physeal tissue using enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

The recorded data were described, categorised and analysed using SPSS 27.0.0 for Windows (*IBM* Chicago, IL, USA). Appropriate parametric or non-parametric test were performed: Shapiro Wilk test, independent t-test, Mann-Whitney U-test. Significance was set at $p < 0.05$.

Results

A total of 24 preparations underwent histology examination with H&E staining, then observed under a microscope with 400 x magnification to identify the presence of fibrous union, osteochondral union and bone union (Table 1) (Figure 1).

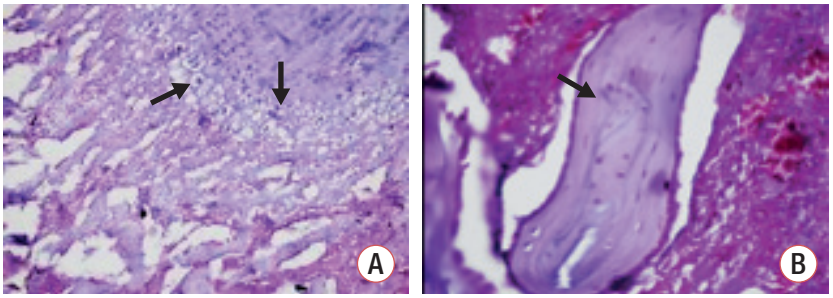
An immunohistochemistry examination was carried out on 24 preparations using ELISA, with indicators of physical bone regeneration examined in the form of transcription factors SOX-9, TNF-α and VEGF (Figure 2).

Table 1: Histological score of groups

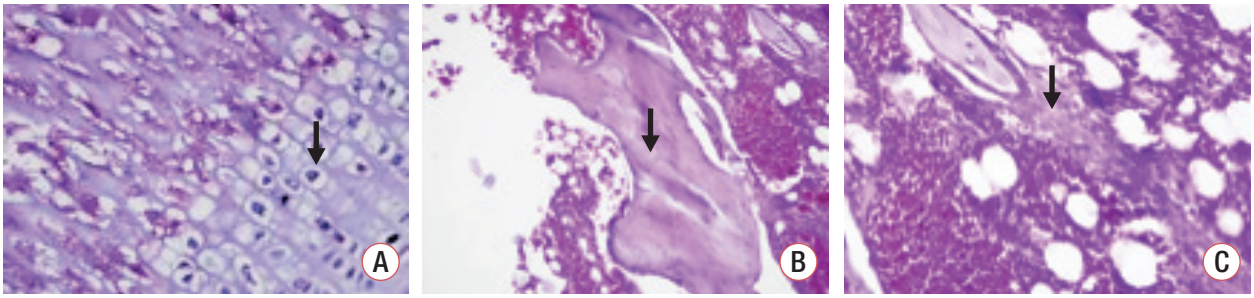
Sample	N	Fibrous union	Osteochondral union	Bone union
Control group	1	0	+1	+1
	2	0	+1	+1
	3	0	+1	+1
	4	0	+1	+2
	5	0	+1	+1
	6	0	+1	+1
BM-MSC group	1	+1	+1	+2
	2	+1	+1	+3
	3	+1	+1	+3
	4	0	+1	+2
	5	0	+1	+1
	6	0	+1	+2
PRF group	1	0	+1	+1
	2	0	+1	+1
	3	0	+1	+1
	4	0	+1	+1
	5	+1	+1	+2
	6	+1	+1	+2
BM-MSC and PRF group	1	+2	+1	+1
	2	+2	+1	+1
	3	+1	+1	+3
	4	+1	+1	+1
	5	+1	+1	+1
	6	+1	+1	+2

Amount of the new bone, 0 = < 25 %; +1 = 25-50 %; +2 = 50-75 %; +3 = 75-100 %; +4 = > 100 %; BM-MSC: bone marrow-mesenchymal stem cells; PRF: platelet-rich fibrin;

1. Control group



2. BM-MSC group



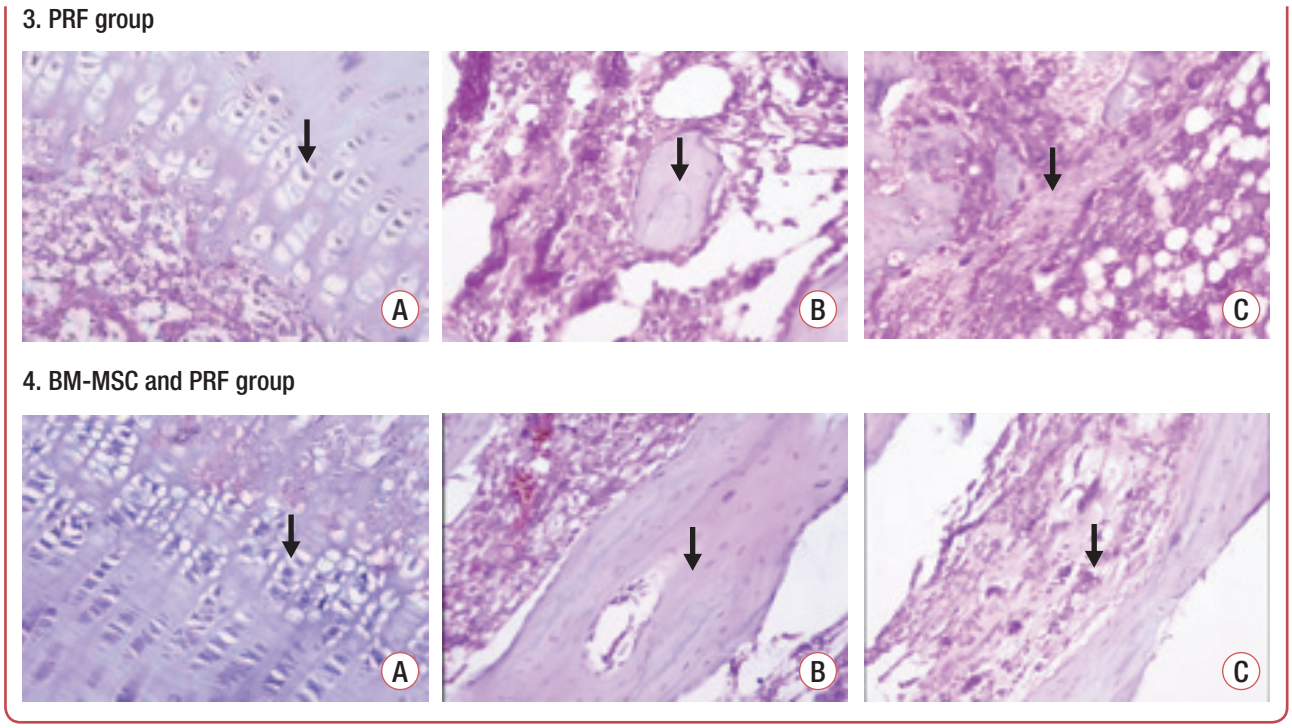


*Figure 1: Pathohistological examination: 1. Control group; 2. Bone marrow-mesenchymal stem cells (BM-MSC) group; 3. Platelet-rich fibrin (PRF) group; BM-MSC and PRF group: (**A**) Osteochondral union; (**B**) Bone union; (**C**) Fibrous union (magnification 400 x).*

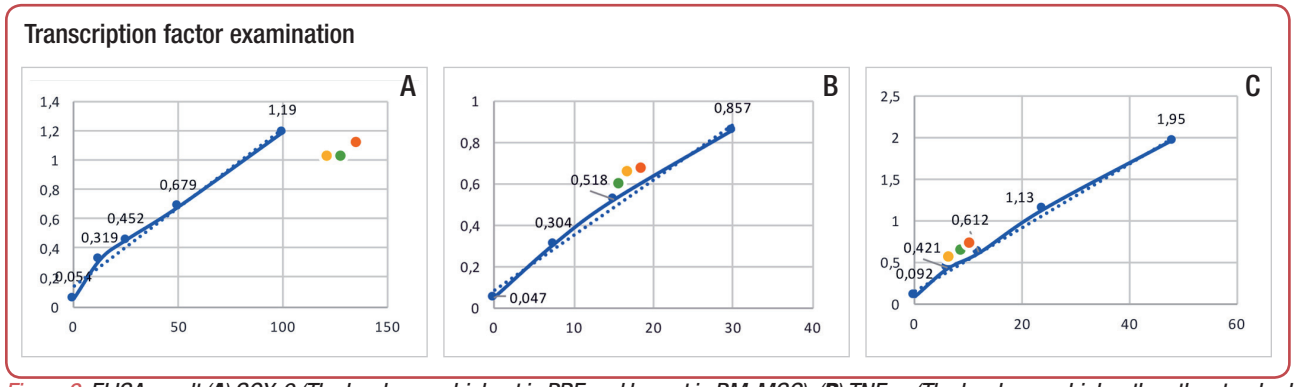


*Figure 2: ELISA result (**A**) SOX-9 (The levels were highest in PRF and lowest in BM-MSC); (**B**) TNF-α (The levels were higher than the standard OD curve, indicating an increase in TNF-α in all samples); and (**C**) VEGF (The levels were higher than the standard OD curve, indicating an increase in VEGF in all samples. The largest increase was observed in the PRF sample, while the smallest increase was in the BM-MSC and PRF sample). The yellow dot represents BM-MSC; Orange dot represent PRF; Green dot represent BM-MSC and PRF; TNF-α: tumour necrosis factor-alpha; VEGF: vascular endothelial growth factor; SOX-9: SRY-box transcription factor 9; BM-MSC: bone marrow-mesenchymal stem cells; PRF: platelet-rich fibrin;*

Comparison analysis in this study showed, most Shapiro-Wilk p-values were above 0.05, except for PRF data with the TNF-α indicator. For the SOX-9 indicator, the p-values for independent t-tests were: control vs BM-MSC (p = 0.099), control vs PRF (p = 0.202), control vs BM-MSC and PRF (p = 0.348), BM-MSC vs PRF (p = 0.032), MSC vs BM-MSC and PRF (p = 0.727) and PRF vs BM-MSC and PRF (p = 0.105). For TGF-α, Mann-Whitney and t-test p-values were non-significant. For VEGF, significant differences were observed in control vs BM-MSC and PRF (p = 0.008) and PRF vs BM-MSC and PRF (p = 0.021) (Table 2).

Table 2: Comparison between groups of factors crucial for physis bone healing

Compared groups	SOX-9		TNF-α		VEGF	
	Mean ± SD	p-value	Mean ± SD	p-value	Mean ± SD	p-value
Control	0.918 ± 0.65	0.099	0.501 ± 0.08	0.288	1.067 ± 0.12	0.152
BM-MSC	0.923 ± 0.18		0.541 ± 0.10		1.218 ± 0.08	
Control	0.918 ± 0.65	0.202	0.501 ± 0.08	0.262	1.067 ± 0.12	0.791
PRF	1.113 ± 0.14		0.571 ± 0.07		1.345 ± 0.15	
Control	0.918 ± 0.65	0.348	0.501 ± 0.08	0.368	1.067 ± 0.12	0.008*
BM-MSC + PRF	0.958 ± 0.16		0.559 ± 0.07		1.110 ± 0.15	
MSC	0.923 ± 0.18	0.032*	0.541 ± 0.10	0.423	1.218 ± 0.08	0.242
PRF	1.113 ± 0.14		0.571 ± 0.07		1.345 ± 0.15	
MSC	0.923 ± 0.18	0.727	0.541 ± 0.10	0.730	1.218 ± 0.08	0.310
BM-MSC + PRF	0.958 ± 0.16		0.559 ± 0.07		1.110 ± 0.15	
PRF	1.113 ± 0.14	0.105	0.571 ± 0.07	0.873	1.345 ± 0.15	0.021*
BM-MSC + PRF	0.958 ± 0.16		0.559 ± 0.07		1.110 ± 0.15	

** Independent t-test, $p < 0.05$, 95 % confidence interval; SD: standard deviation; TNF-α: tumour necrosis factor-alpha; VEGF: vascular endothelial growth factor; SOX-9: SRY-box transcription factor 9; BM-MSC: bone marrow-mesenchymal stem cells; PRF: platelet-rich fibrin;*

Discussion

A study by Wong et al demonstrated that a single intra-articular MSC injection into injured distal femur growth plates in rats enhanced growth plate repair and reduced limb-length discrepancies, despite histological bone-bridge formation.[13] Research by Guo et al showed a potential of MSCs, which facilitate growth plate repair and mitigate bone-bridge formation, influenced by MSC quantity, pre-existing conditions, growth factors, chondrocyte-MSC interactions and scaffold roles.[14] PRF promotes bone regeneration by significantly increasing osteoblast alkaline phosphatase activity within 72 hours post-PRF application, with low complication risks and simple preparation.[15] It was hypothesised that the combination of BM-MSCs and PRF is considered optimal as it combines the osteogenic potential of BM-MSC with the growth factors of PRF and its gel form can serve as a scaffold for BM-MSC. However, presented study showed no significant differences in histological outcomes. A close examination of the histological results for osteochondral union reveals that all samples produced similar outcomes. This union better represents the cartilage condition in the physis and describes non-bony bridge formation.

Additionally, administering a combination of BM-MSC and PRF with the indicator SOX-9, TNF-α and VEGF yielded lower outcomes than PRF alone. When BM-MSCs were combined with PRF; SOX-9, TNF-α and VEGF results improved over controls but were similar to PRF. In bone regeneration, BM-MSC did not significantly outperform PRF. This aligns with Wang et al study, where a single intraarticular BM-MSC injection into 40 rat distal femurs with growth plate injuries triggered growth plate repair, reduced limb-length discrepancy, but did not histologically inhibit bone-bridge formation.[10]

For physeal injuries, MSCs reduced bone-bridge formation and improved tissue repair. Factors included MSC count, preconditions, growth factors, interactions and scaffolds.[16] PRF has a straightforward manufacturing process with no risk of immunological rejection or infection from additives. In presented study, there were observations of osteochondral union, bone union and a few fibrous unions. When PRF was administered with SOX-9, TNF-α and VEGF indicators, better outcomes were seen compared to controls. PRF also outperformed BM-MSC administration, with a significant difference observed in the SOX-9 indicator. This aligns with research indicating that administering PRF to bone defects can enhance bone regeneration, as evidenced by increased alkaline phosphatase activity from osteoblasts 72 h after PRF administration. PRF offers a low risk of complications and its manufacturing process is relatively simple.[16] PRF treatment yielded higher Pax7 expression scores than the control at both two and four weeks, benefiting muscle healing.

PRF enhances bone union, cortical growth and graft integration in rabbits. However, research on PRF is limited, with no studies on its effects on physeal injuries.[17, 18]

In general, in presented study, the outcomes of treatment with BM-MSC alone, PRF alone and combination BM-MSC with PRF showed no significant differences, contrasting with previous studies that reported MSC-PRF combination as having the best results in muscle.[19] This is also in contrast with a systematic review of 24 studies, which demonstrated that combining PRF with other elements showed more promising results than using PRF alone. Several other conducted studies also show improved muscle regeneration outcomes when using a combination of MSC compared to using biomaterials alone. Besides, usage of BM-MSC and PRF significantly enhanced physis bone healing, with TNF-α initiating the inflammatory response, VEGF promoting angiogenesis and SOX-9 regulating chondrogenesis.[15, 19] However, no studies have specifically focused on the effects of BM-MSC and PRF on physeal injuries compared to BM-MSC alone and PRF alone.

This study has several limitations that should be addressed in future research that subject rabbit results may not fully replicate human paediatric physeal injuries. However, four weeks may be too short to assess long-term bone regeneration. Radiological evaluation with an X-ray or CT-scan is needed to assess bone healing post-physeal injury.

Conclusion

Administration of BM-MSC, PRF or a combination of BM-MSC and PRF showed comparable effectiveness in osteochondral union based on histological outcomes. Conversely, PRF alone exhibited the highest effectiveness in immunohistochemical analysis. However, none of these results were statistically significant.

Ethics

Ethical Clearance was granted by the Animal Care and Use Committee (ACUC), Universitas Airlangga, decision No 2.KEH.175.12.2022, dated 27 December 2022.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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