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Evaluation of Umbilical Cord-derived Mesenchymal Stem Cells (UC-MSCs) Exosome Implantation and Platelet-Rich Fibrin (PRF) on Critical Long Bone Defects in Sprague-Dawley Rats

Ismail Hadisoebroto Dilogo[1], Mirta Hediyanti Reksodiputro[2], Radiana Dhewayani Antarianto[3], Januar Chrisant Fladimir Makabori[1,] Iqra Kousar[4,5]

[1]Department of Orthopaedic and Traumatology, Cipto Mangunkusumo General Hospital Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia

Orcid ID: 0000-0003-4060-2260

[2]Department of Otorhinolaryngology-Head and Neck Surgery, Faculty of Medicine, Universitas Indonesia, dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia

Orcid ID: 0000-0002-5926-5033

[3]Department of Histology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia

Orcid ID: 0000-0002-8578-7505

[4]Biomedical Science, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

Orcid ID: 0000-0003-4991-1799

[5]Institute of Biochemistry and Molecular Biology, Institute of Biomedical Sciences, and Research Center for Cancer Biology, China Medical University, Taichung, Taiwan.

[*]**Corresponding author:**

[*]Januar Chrisant Fladimir Makabori

Primary Affiliation: Department of Orthopedic and Traumatology Cipto Mangunkusumo Hospital; Faculty of Medicine Universitas Indonesia, Jakarta, Indonesia

Address : Pangeran Diponegoro Street Number 71, Central Jakarta, Jakarta, Indonesia

Email ID: chano.fladimir.cfm@gmail.com, Orcid ID: 0000-0003-4991-1799

[*]Iqra Kousar

Primary Affiliatio : Biomedical Science, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia, Institute of Biochemistry and Molecular Biology, Institute of Biomedical Sciences, and Research Center for Cancer Biology, China Medical University, Taichung, Taiwan

Address : Dapeng Road no.130 , Bitun district , Taichung , Taiwan

Email ID: iqrakousar806@gmail.com

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ABSTRACT

Critical bone defect is a loss of bone structure that exceeds the critical size of the bone's ability to regenerate. Autologous bone grafting is the standard therapy used in extensive bone defects. However, it is associated with various morbidities. Thus, the use of exosome obtained from umbilical cord mesenchymal stem cell (UC-MSC) and PRF is promising and can be a potential alternative to treat critical bone defects. This study was an experimental post-test only control group design that used 30 Sprague Dawley rats aged 8-12 weeks, weighing about 250-300 grams. The rats were then divided into 5 treatment groups, namely hydroxyapatite (HA) and bone graft (group I), HA, bone graft, and PRF (group II), HA, bone graft and exosome from UC-MSC (group III), HA, bone graft, PRF, and exosome from UC-MSC (group IV), and HA, PRF, and exosome from UC-MSC (group V). In each rat, a 5mm femoral bone defect was created and internally fixated using a 1.0-1.2 mm K-wire threaded. At the fourth week of follow-up, RT-PCR examination was performed to assess BMP-2 and chordin levels, as well as histomorphometry examination to measure the percentage of ossification area, fibrotic area, and void area. . One-way ANOVA and post-hoc tests were perfomred using SPSS version 26 for the analysis.In the RT-PCR examination, the highest BMP-2 gene expression was found in group I (1.0 - 1.5; median 1.2), followed by group II (0.2 - 1.2; median

0.5), group IV (0.3 - 0.7; median 0.4), group III and group V. Meanwhile, chordin gene expression was highest in group III (0 - 50), followed by the other groups with similar values. However, descriptive analysis showed no significant correlation between BMP-2 and chordin levels in critical bone defects, with p values of 0.096 and 0.690 each. Statistical analysis showed significant results for BMP-2 ($p = 0.017$) and no significant results for chordin ($p = 0.269$). Furthermore, the histomorphometry analysis for ossification, fibrotic, and void area showed no statistical significance ($p > 0.05$). The use of PRF and exosomes from SPM-TP separately showed different results, where PRF showed good results in osteogenesis and exosomes from SPM-TP showed higher results in fibritic tissue formation. However, the use of both exosomes and PRF together has not been studied for their effect on critical bone defects. In this study, the opposite results were found instead of the expected results. This may indicate that the combination of PRF and exosome from UC-MSC could possibly yield a negative effect on osteogenesis. The combination of PRF and exosome from UC-MSC did not yield positive effect on the outcomes examined in this study.

Keywords: *Critical bone defect, platelet rich fibrin (PRF), exosome from umbilical cord (UC-MSC), BMP-2, Chordin*

1. INTRODUCTION

Extensive bone defects are a major concern in the field of orthopedics and has a negative impact on patient's quality of life. Various conditions can lead to extensive bone defetcts that includes traumatic conditions, osteomyelitis, metabolic disorders, or post-tumor resection. Autologous bone grafting a standard therapy for broad bone defects, especially those measuring more than 5 cm. However, despite the robust use of bone grafts, several looming problems continues to hinder the effectiveness of the therapy. High cost, increase risk of comorbidities, infections, pain, hematoma, and fracture incidence are some outcomes associated with bone grafting. To avoid these negative outcomes the use of osteoinductive growth factors such as BMP-2 and other osteoconductive scaffolds as a potential alternative could pose a significant impact[1–3]

Other promising alternatives include mesenchymal stem cells (MSC) and its derivatives (secretome and exosome). MSC can differentiate into osteogenic and chondrogenic lineages. In critical bone defects, stem cell therapy has shown the ability to promote bone regeneration by increasing osteogenic proliferation and differentiation and enhance healing and restoration. . However, varying results in available in the literature limit its standardization. Preclinical findings translated into human clinical trials are still limited, therefore, results obtained in animal studies may not accurately represent clinical outcomes in human subjects.[1,4,5]

Exosomes derived from umbilical cord mesenchymal stem cells (UC-MSC) are nanometer-sized vesicles, carrying a variety of bioactive molecules, such as proteins, lipids, and RNA, and plays an important role in intercellular communication. A systematic review compiled by Tan, et al that included 23 studies on the effects of MSC derivative exosomes for bone regeneration in experimental animal models of bone defect diseases such as osteonecrosis and osteoporosis stated that MSC exosomes have a good effect on bone regeneration by supporting proliferation, migration, osteogenesis, and angiogenesis. PRF is a concentrate of platelets collected on a single fibrin membrane, containing all blood components that play a role in healing and immunity. There is no research yet that compares the use of UC-MSC exosomes and PRF as autologous grafts, and the combination of both, in large bone defects in experimental animals.[6–8].

2. MATERIALS AND METHODS

Ethics Statement

The research protocol of the study was approved from the ethics committee of the Faculty of Medicine, Universitas Indonesia (KET- 933 /UN2.F1/ETIK/PPM.00.02/2022). All rats used in this study received appropriate care and treatment in accordance with the guidelines outlined in the Government of the Republic of Indonesia's Regulation No. 95 of 2012 (Veterinary Public Health and Animal Welfare) and the National Guidelines on Health Research Ethics established by the Health Research Ethics Committee, Ministry of Health, Republic of Indonesia.

Experimental Design

The experiment was carried out by dividing the rats into 5 groups through a randomization table, namely I. HA + bone graft as control, II. HA + bone graft + PRF, III. HA + bone graft + UC-MSC exosomes, IV. HA + bone graft + PRF + UC-MSC exosomes, and V. HA + PRF + UC-MSC exosomes of various origins as treatments, and HA granules were prepared as 0.1 gram per rat. All the rats were placed in the same cages during the observation period. All the rearing parameters and feeding regimens were regulated in homogeneous conditions.

Preparation of Animal Models

Animals

The study included a sample of thirty male Sprague Dawley rats. Sample selection was based on body weight (BW), sex,

bone maturity, and the findings of clinical, radiological, and laboratory exams. The study involved male rats that were fully developed and was in good health, with a weight ranging from 250 to 300 grams. The sample selection was carried out by a veterinarian who holds a certification in basic laboratory animal training and possesses over 10 years of expertise in conducting research involving laboratory animals.

Acclimatization process and treatment of experimental animals

Acclimatization of experimental animals was done for 3 days and use as a standard for animals to their original condition after their transport to a new location (body weight, pulse rate, and activity conditions).63 In this study, it was decided to acclimatize for 7 days to optimize the condition of the experimental animals before treatment. Animal care was in accordance with international animal care protocols. Animals were given food and water ad libitum in the form of commercial pellets. Pellets were also spread on the floor of the cage to facilitate the food gathering instinct of the rats.

Anaesthesia process in experimental animals

Following acclimatization and randomization, Sprague Dawley rats were anesthetized using ketamine hydrochloride 75mg/kg and xylazine hydrochloride 5mg/kg. Both were administered intraperitoneally (the dose was adjusted to the weight of each rat).

Fracture treatment, bone defect creation and bone graft harvesting

after ansthetizing the rats, the hair on the surgical field (right thigh) is shaved clean. Aseptic and antiseptic were performed using 10% Providon iodine. A longitudinal incision was made on the lateral thigh. The vastus lateralis and biceps femoris muscles were retracted, leaving the femur bone exposed. Osteotomy was performed to obtain a 5 mm defect confirmed using a caliper. The bone was then fixed retrogradely using a 1.3-1.4 mm kirschner wire. In the same rat, an incision was made on the iliac wing of the rat to retrieve the bone graft. The bone defect was then treated according to the group. Postoperatively, rats were given ceftriaxone 28mg per rat, administered intramuscularly to reduce the risk of infection (Figure 1 and 2).

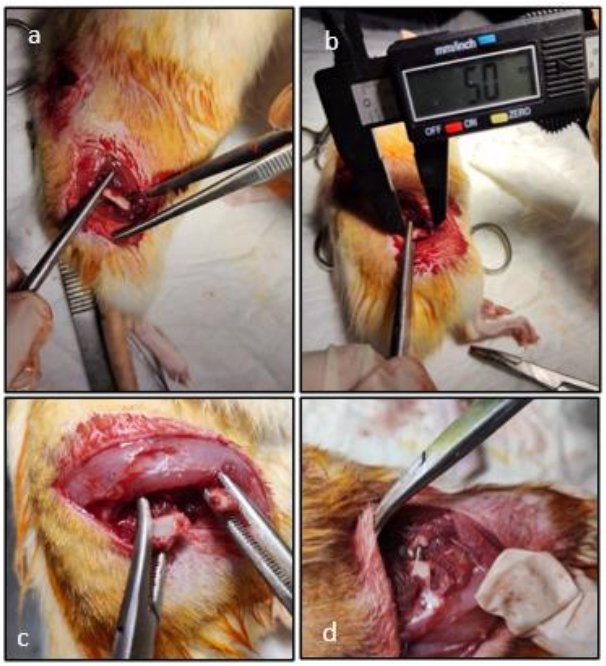

Figure 1. An exposure of the femur was performed by cutting the intermuscular septum between the anterior and posterior compartments. The muscle was retracted medially to enhance the exposure (a), following which the length of the bone to undergo osteotomy, a total of 5mm, was measured (b). An osteotomy of the femur was then carried out on the research sample (c). Post-osteotomy, a retrograde placement of a 1.3-1.4mm Kirschner wire was performed (d).

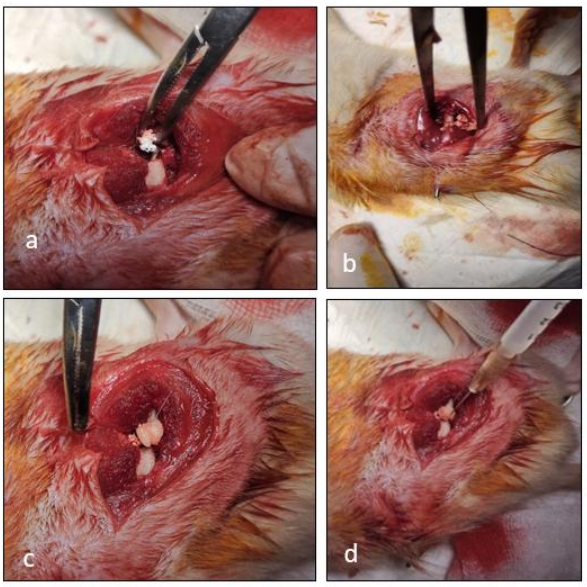

Figure 2. After the osteotomy and fixation with Kirschner wires in the rats, 0.1mg of HA granules per rat was administered (a), followed by the application of bone graft on the bone samples (b). PRF (c) and SPM-TP Exosome (d) were then administered according to the treatment groups; this procedure was not carried out in the control group.

Determination of rats

Rats were determined at the end of week 4 using Ketamine and Xylazine intraperitoneally and cardiac aspiration. Bone tissue was then prepared by decalcification, followed by osteotomy of the femur using a mini grinder, with a saw diameter of 22mm cut longitudinally from the proximal to distal part of the callus and each tissue was fixed using 10% buffered formalin for 24 hours. Tissue preparation, histomorphometric examination were performed at the Department of Histology and SCTE FKUI. The tissue preparation process consisted of dehydration with 70%, 80%, 95% alcohol immersion for 3 hours each. Then continued with the Clearing process, the preparation was soaked with Xylol solution twice for 3 hours (Figure 3).

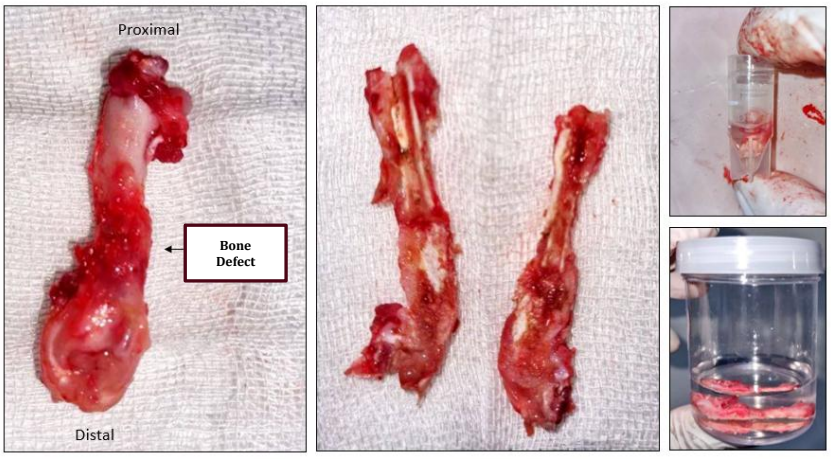

Figure 3. The clinical appearance of the rat femur post-disarticulation. The callus was identified as the tissue to be examined in histomorphometry and qRT-PCR.

Exosome Preparation

The preparation of exosomes was carried out following a protocol according to Copyright certificate number EC002022103579 entitled Protocol for Isolation and Characterization of EVs Stem Cells. (Copyright Registration Number IP Database: 000419323.

Study Parameters

Clinical parameters, including activities, signs of infection, signs of wound healing, paralysis, and appetite, were examined daily. In addition, bodyweight was also measured. Histopathologic examination was performed to measure the percentage area of chronic fibrosis, inflammation, void, and osseous tissue, which was compared between subjects in groups using J images (Figure 4). qRT-PCR assay was performed to assess osteogenic signalling pathways of total mRNA extraction to Zymo kit instructions. RNA concentration and purity were calculated using a nanodrop spectrometer and the RNA was stored at 800°C after calculating its concentration and purity. The CT values are then processed using the Livak Formula aimed to determine the relative expression of target genes such as; BMP2 and chordin against the housekeeping gene in the form of GAPDH. The Livak formula used, as follows with the provisions of ΔCT in the form of CT values from samples on day 7, 14, 21 (time X), and ΔCT calibrator in the form of CT values from MSC culture samples (time 0).

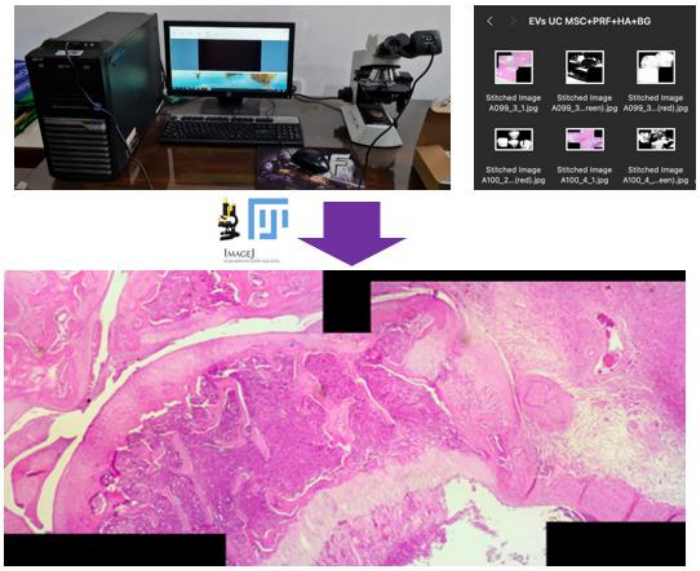

Figure 4. The prepared samples were evaluated under a light microscope and photographed using a camera and Optilab microscope, with image stitching done using Fiji Image J.

Statistical Analysis.

The data obtained was analyzed using the SPSS v23 application. The characteristics of the experimental animals used are shown first. Data is presented in the form of numerical variables, attached in graphs, tables, or figures. The normality of the data was tested using the Shapiro Wilk test followed by one-way ANOVA test for normal distribution and Kruskal-Wallis test for non-normal distribution. Levene's test was used to determine the homogeneity of the data. Post-hoc tests were conducted to assess comparative relationships between groups singly (each group).

3. RESULTS

Animal Characteristics

Thirty male Sprague Dawley rats were used as subjects in this study. Each rat was randomly placed into fifteen cages with a closed system, with two rats in each cage. Before the action was taken, each rat underwent acclimatization for one week. At the beginning of the study, each rat was weighed, and every single rat from each cage was given a marker. In this study, none of the rats experienced mortality during the pre- and post-action observation periods. Most of the samples showed good response in subjective assessment of activity and diet. However, those with bone graft removal tended to have lower activity and feeding patterns than those without bone graft removal (group V) (Table 1). However, during necropsy, some rats were found to have infected bone segments, characterized by the presence of pus (Figure 5).

Figure 5. Infection process found during necropsy

Table 1. Mean Body Weight between Groups

Groups	Body Weight (gram)*
I: HA and Bone Graft	269 ± 20,1
II: HA, Bone Graft, and PRF	265,3 ± 20,8
III: HA, Bone Graft, and UC-MSC Exosome	227,3 ± 23,2
IV: HA, Bone Graft, PRF, and UC-MSC Exosome	237 ± 54,8
V: HA, PRF, and UC-MSC Exosome	255±21,6

Histomorphometric Analysis

The percentage of reinforcement area, fibrosis area, and void area can be seen in Table 2. the results shows that group V produced the smallest reinforcement area (25.61 [SD ± 6.23]) and the largest fibrosis area (64.78 [SD ± 10.61]). Meanwhile, the largest reinforcement area can be seen in group II (36.99 [SD ± 12.91]), and the smallest fibrosis area in group I (53.66 [SD± 9.07]). The data were then checked for normality with the Kolmogorov-Smirnov test and normal data distribution was obtained. Furthermore, the data were analyzed by one-way ANOVA test. The study did not find any significant results for the analysis of fibrosis area, reinforcement, and void area (p value > 0.005) in the test group. Furthermore, the results shows that group V produces the smallest reinforcement area and the largest fibrosis area that can be seen in Figure 6..

Table 2. Histomorphometric Analysis and Percentage Area of Each Assessment Area

Groups	Bone Deposition Percentage (SD)	Fibrosis Area Percentage (SD)	Void Area Percentage (SD)
I: HA and Bone Graft	33.14 (7.05)	53.66 (9.07)	40.09 (39.51)
II: HA, Bone Graft, and PRF	36.99 (12.91)	57.82 (13.58)	17.03 (24.87)
III: HA, Bone Graft, and UC-MSC Exosome	32.95 (7.48)	60.04 (11.35)	26.97 (21.71)

IV: HA, Bone Graft, PRF, and UC-MSC Exosome	36.16 (22.79)	63.03 (17.32)	15.65 (6.57)
V: HA, PRF, and UC-MSC Exosome	25.61 (6.23)	64.78 (10.61)	30.69 (38.52)
p value	0.581*	0.591*	0.196*

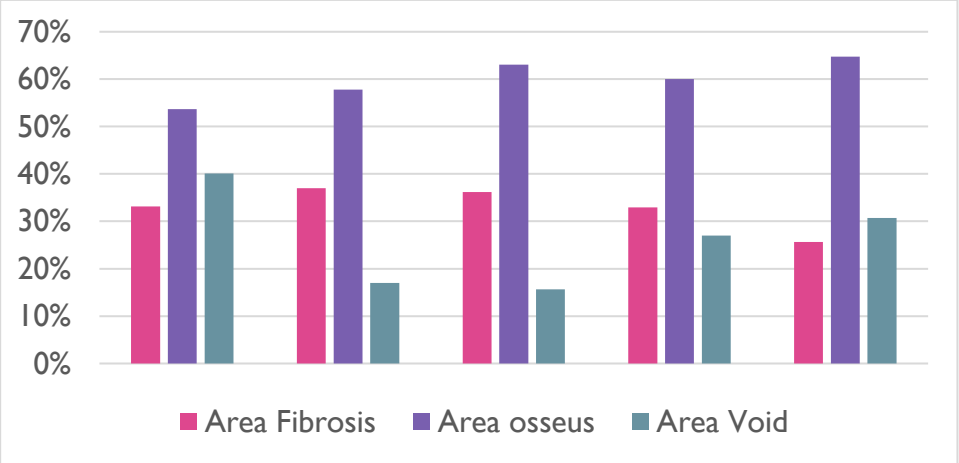

Figure 6. Comparison chart showing the percentage of area for each group.

In the histomorphometric examination, two areas were examined, namely the green area interpreted as the reinforcement area and the red area interpreted as the fibrosis area (soft callus). From figure 7, a broader fibrosis area was observed in the exosome group (group III), while a wider reinforcement area was seen in the control group (group I).

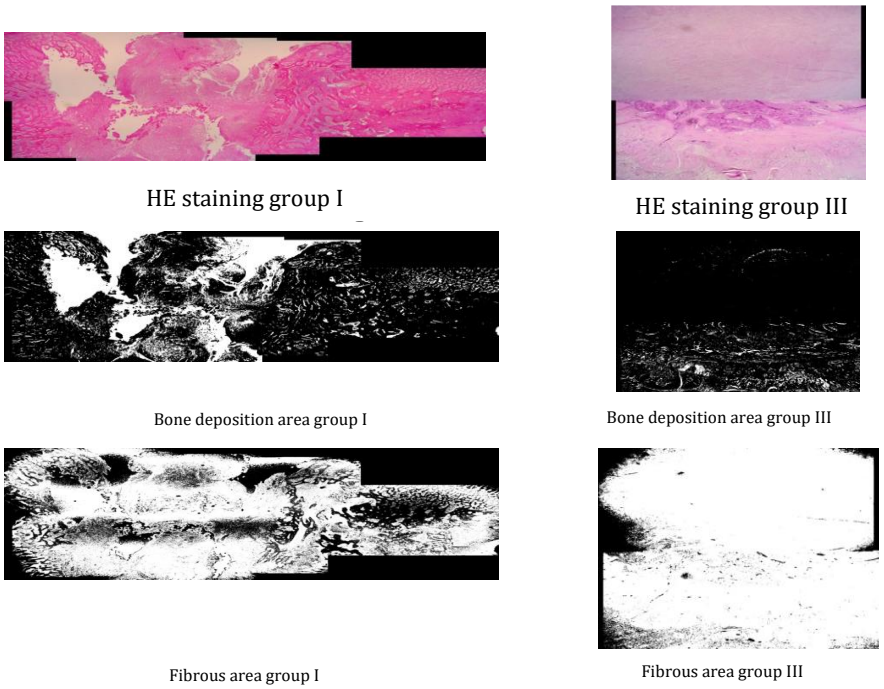

Figure 7. The results of histomorphometric examination of bone tissue in Sprague Dawley rats comparing group I and group III.

The current study also investigated the effect of PRF usage in the treatment group compared to the control group. The study showed in an average percentage of fibrosis (57.827 ± 13.82) that was higher in the intervention group than in the control group (53.665 ± 9.07)(Figure 8). From figure 8, a wider fibrosis area and reinforcement area were observed in the PRF group (group II) compared to the control group.

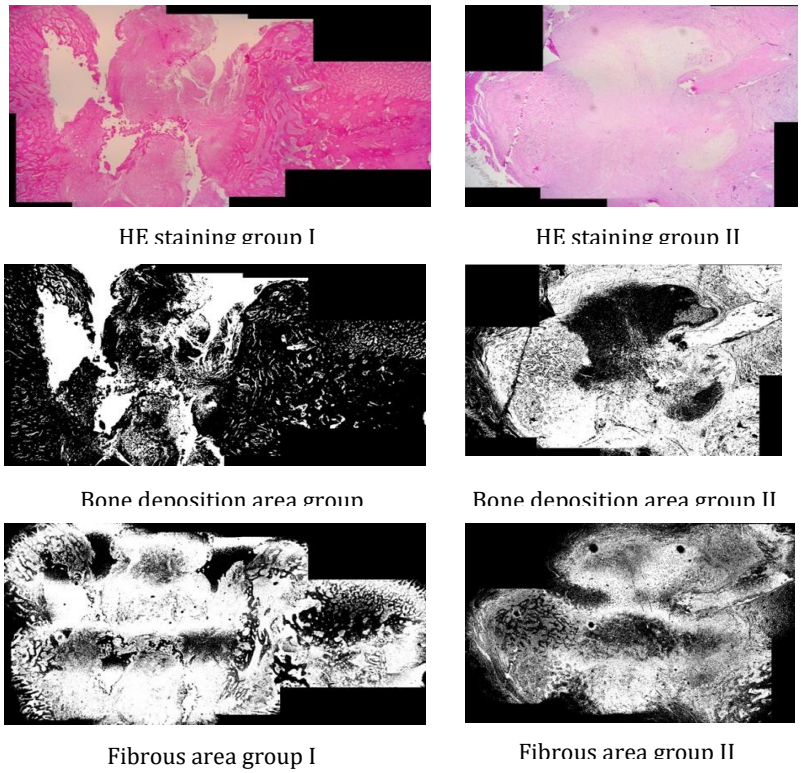

Figure 8. The results of the histomorphometric examination of bone tissue in Sprague Dawley rat groups I and II.

Subsequently, this study evaluated the effects of using exosomes from umbilical cord mesenchymal stem cells combined with PRF in the treatment group compared to the control group. The study concluded that the average percentage of fibrosis (63.03 ± 17.32) was higher in the intervention group than in the control group (53.665 ± 9.07) as shown in Figure 9.

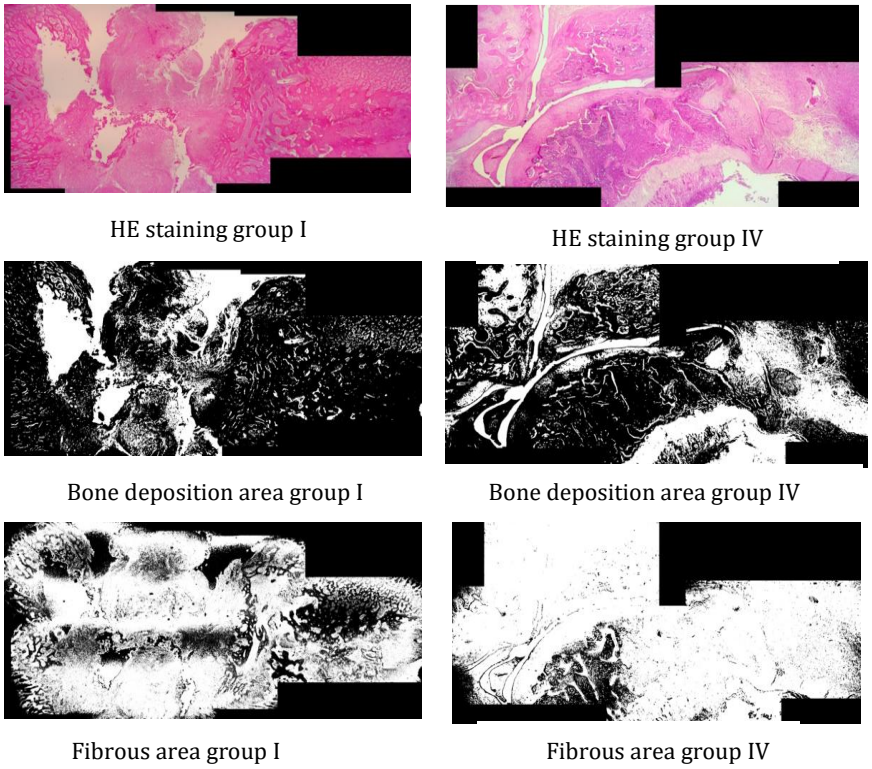

Figure 9. The results of the histomorphometric examination of bone tissue in Sprague Dawley rat groups I and IV.

Furthermore, the effects of using exosomes from umbilical cord mesenchymal stem cells combined with PRF without the application of bone graft in the treatment group compared to the control group were also evaluated. The results showed that the average percentage of fibrosis (64.78 ± 10.61) was higher in the intervention group than in the control group (53.665 ± 9.07) as shown in Figure 10.

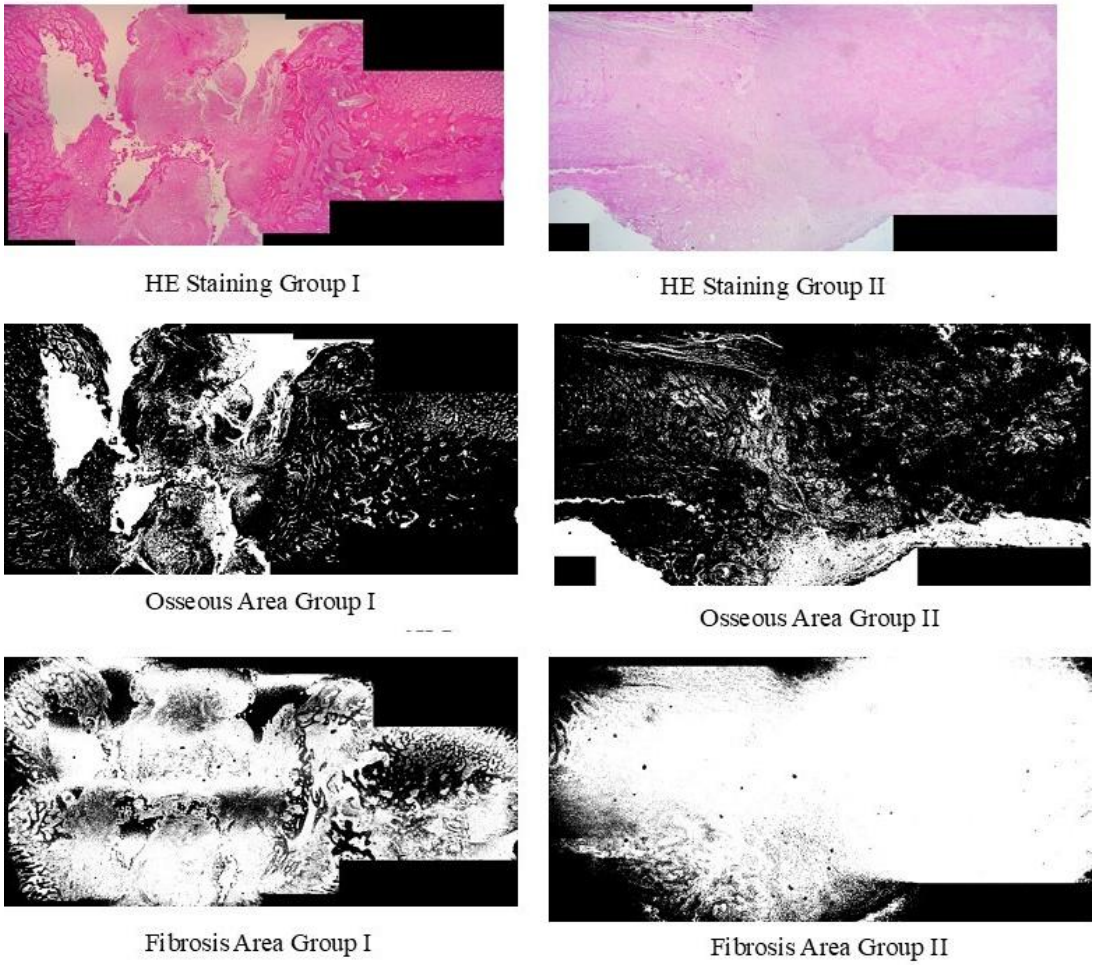

Figure 10. The results of the histomorphometric examination of bone tissue in Sprague Dawley rat groups I and V.

qRT-PCR Analysis for BMP-2 and Chordin Expression

In this study, it was found that the value of BMP-2 in the control group (HA and bone graft) had the highest value (1.21 ± 0.25), while, the smallest value was obtained in group IV (administration of HA, bone graft, PRF and UC-MSC exosomes) which was 0.16 ± 0.072 (Table 3). A statistically significant result was found for BMP-2 values of the intervention group (p value = 0.017). Furthermore, Bonferroni post-hoc test was performed to assess the relationship between intervention groups. The results of the post-hoc test can be seen in the table 4. In addition, a significant difference in BMP-2 values between group III and group V (p = 0.026), group IV and group I (p = 0.026), and group V and group I (p = 0.04) was found. (Table 4).

Table 3. Comparative analysis of BMP-2 values of each intervention group

Groups	Mean (SD)	P value
I: HA and Bone Graft	1.21 (0.25)	0.017
II: HA, Bone Graft, and PRF	0.56 (0.52)	

III: HA, Bone Graft, and UC-MSC Exosome	0.36 (0.19)
IV: HA, Bone Graft, PRF, and UC-MSC Exosome	0.16 (0.072)
V: HA, PRF, and UC-MSC Exosome	0.34 (0.42)

Table 4. Post-hoc test between intervention groups on BMP-2 scores

Group Intervention		I	II	III	IV	V	P value*
I	Mean diff.		0.64	0.84	1.04	0.86	0.017
	P value		0.287	0.21	0.28	0.24	
II	Mean diff.	-0.64		0.19	0.41	0.22	
	P value	0.28		1.00	1.00	1.00	
III	Mean diff.	-0.84	-0.19		0.21	0.025	
	P value	0.21	1.00		1.00	0.026	
IV	Mean diff.	-1.04	-0.41	-0.21		-0.18	
	P value	0.026	1.00	1.00		1.00	
V	Mean diff.	-0.86	-0.22	-0.025	1.833		
	P value	0.04	1.00	1.00	1.00		

In this study, it was found that the value of chordin in the control group (HA and bone graft) had the smallest value (1.11 ± 0.21), with the highest value obtained in group IV (administration of HA, bone graft, PRF and UC-MSC exosomes) which amounted to 33.47 ± 63.01 (Table 5). The study did not find any significant difference in the chordin scores in the intervention group (p value = 0.269).

Table 5. Chordin Level Comparison between Groups

Group	Mean (SD)	P value *
I: HA and Bone Graft	1.11 (0.21)	0.269
II: HA, Bone Graft, and PRF	4.06 (5.64)	
III: HA, Bone Graft, and UC-MSC Exosome	2.23 (1.73)	
IV: HA, Bone Graft, PRF, and UC-MSC Exosome	33.47 (63.01)	
V: HA, PRF, and UC-MSC Exosome	2.06 (2.68)	

4. DISCUSSION

In the RT-PCR examination, the highest BMP-2 gene expression was found in group I (1.0 - 1.5; median 1.2). Meanwhile, chordin gene expression was highest in group III (0 - 50), followed by the other groups with similar values. However,

descriptive analysis showed no significant correlation between BMP-2 and chordin levels in critical bone defects, with p values of 0.096 and 0.690 each. Statistical analysis showed significant results for BMP-2 ($p = 0.017$) while chordin ($p = 0.269$) and histomorphometry analysis for ossification, fibrotic, and void area showed no statistical significance ($p = 0.591$, $p = 0.581$, $p = 0.196$, respectively).

Stem cell therapy has demonstrated benefit in preclinical studies for a variety of conditions through anti-inflammatory, anti-apoptotic, anti-oxidative stress, anti-fibrotic, immunomodulatory, and proangiogenic mechanisms. In humans, the use of stem cells has been shown to be safe and effective in some cases. For example, stem cell therapy has shown effectiveness in treating diabetic foot ulcers, by increasing wound healing rates and reducing amputation rates. In critical bone defects, stem cell therapy has demonstrated the ability to enhance bone regeneration by increasing proliferation and osteogenic differentiation and promoting healing and restoration of function.[1,9–11]

Research by Zhang et al in 2019 showed positive results of UC-MSC exosome implantation with hyaluronan, HA and heparin-filled hydrogels in a rat model of femoral diaphyseal fracture stabilized with femur bone intramedula wire to improve fracture healing through increased neoangiogenesis via the HIF-1 signaling pathway.[12] A literature review by Wang et al in 2022 also supported and summarized similar studies with positive results on the application of MSC TP exosomes in combination with hydrogels.[13] The negative results in this study may be due to differences in the method of UC-MSC exosome implantation. This study dripped UC-MSC exosomes onto a combination of HA + bone graft or PRF + HA + bone graft. UC-MSC exosomes dripped onto HA + bone graft or PRF + HA + bone graft are likely to spread unevenly and are not retained for sufficient time to enter osteoprogenitor cells contained in the bone graft, bone marrow, or periosteum so that they quickly disappear without providing a regenerative effect.

UC-MSC exosomes in this study combined with HA + bone graft + PRF showed a higher fibrosis area, as well as a lower reinforcement area compared to the HA + bone graft and HA + bone graft + PRF control groups. This is in line with the results of the osteogenic signaling pathway examination with low BMP-2 gene expression and high Chordin gene expression. This study is the first to combine UC-MSC exosomes and PRF to treat critical bone defects with HA and bone graft as controls.[14,15] A systematic review and meta-analysis by Eidhaq, et al that included 11 quantitative studies and 24 qualitative studies stated that PRF when combined with other materials such as bone marrow-derived mesenchymal stem cells and hydroxyapatite will increase its osteogenic ability, whereas when used alone the results are still variable. However, the use of PRF alone is said to increase ALP levels, increase RUNX2 and OCN, and decrease TRAP levels.[16]

Although there are no studies that directly combine PRFs and exosomes for bone defect regeneration, both have compatible mechanisms of action and support each other. A systematic review of the effects of mesenchymal stem cell-derived exosomes by Tan, et al explained that there were positive effects of MSC exosomes on cell survival, proliferation, migration, osteogenesis and angiogenesis. The therapeutic effectiveness of MSC exosomes was demonstrated in animal models exhibiting bone abnormalities and conditions such as osteonecrosis and osteoporosis. MSC exosomes also support blood vessel formation in newly formed bone. The exact mechanism by which MSC exosomes exert their therapeutic effects is still being investigated, but it is believed that MSC exosomes can deliver their cargo to target cells, activate signaling pathways, and regulate gene expression to promote tissue repair and regeneration. Nevertheless, certain studies exhibit unclear or minimal risk in terms of bias and insufficient reporting, underscoring the necessity for uniformity in the measuring and reporting of outcomes.[17]

However, contrary to the results in this study, the group of animals that received the combined intervention of UC-MSC and PRF exosomes showed lower BMP-2 levels and higher chording levels compared to the control group. The statistically significant result for BMP-2 levels reinforced this result. This could indicate a possible unfavorable effect of the combination of UC-MSC and PRF exosomes. However, this could also be due to a variety of other factors. Several studies testing the usefulness of exosomes for repairing critical bone defects are still debating the optimal dose of administration. (citation needed) Apart from the dose, the process of isolation and characterization of exosomes may also be one of the factors affecting these results. Tan, et al in their study mentioned that the ultracentrifugation method to isolate exosomes is the most commonly used method. In addition, exosome isolation tools are also commercially available, which can reduce the morphological variation of exosomes. However, exosome preparation still has possible variability that cannot be determined from downstream outcome studies, such as the storage method of isolated exosomes.[17,18]

5. CONCLUSION

In this study, administration of mesenchymal stem cell exosomes from the umbilical cord combined with platelet-rich fibrin (PRF), bone graft, and hydroxyapatite did not result in a significant increase in callus growth in critical long bone defects in Sprague Dawley rats compared to the control group. Therefore, the combined use of UC-MSC and PRF exosomes to treat critical bone defects in long bones is not recommended. However, further research is needed to determine what factors may influence these results. Factors such as the origin of the exosomes, dosage, method of isolation, time and duration of exposure, and method of administration should be considered for future studies. However, the use of PRF alone provided a good increase in callus growth in critical bone defects, but future studies are recommended.

Declarations

Funding Statement: The authors received no financial support for the research, authorship, and/or publication of this article.

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethics Approval: Ethics approval was obtained from the Ethic Committee of Faculty of Medicine, Universitas Indonesia, with ethic number of KET-933/UN2.F1/ETIK/PPM.00.02/2022.

Availability of data and material: The data used to support the findings of this study are available from the corresponding author upon request.

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