

# World Journal of *Stem Cells*

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## Basic Study

## Optimal concentration of mesenchymal stem cells for fracture healing in a rat model with long bone fracture

Myung-Seo Kim, Hyun-Ju Chung, Kang-Il Kim

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## Abstract

## BACKGROUND

There is still no consensus on which concentration of mesenchymal stem cells (MSCs) to use for promoting fracture healing in a rat model of long bone fracture.

## AIM

To assess the optimal concentration of MSCs for promoting fracture healing in a rat model.

## METHODS

Wistar rats were divided into four groups according to MSC concentrations: Normal saline (C),  $2.5 \times 10^6$  (L),  $5.0 \times 10^6$  (M), and  $10.0 \times 10^6$  (H) groups. The MSCs were injected directly into the fracture site. The rats were sacrificed at 2 and 6 wk post-fracture. New bone formation [bone volume (BV) and percentage BV (PBV)] was evaluated using micro-computed tomography (CT). Histological analysis was performed to evaluate fracture healing score. The protein expression of factors related to MSC migration [stromal cell-derived factor 1 (SDF-1), transforming growth factor-beta 1 (TGF- $\beta$ 1)] and angiogenesis [vascular endothelial growth factor (VEGF)] was evaluated using western blot analysis. The expression of cytokines associated with osteogenesis [bone morphogenetic protein-2 (BMP-2), TGF- $\beta$ 1 and VEGF] was evaluated using real-time polymerase chain reaction.

## RESULTS

Micro-CT showed that BV and PBV was significantly increased in groups M and H compared to that in group C at 6 wk post-fracture ( $P = 0.040$ ,  $P = 0.009$ ;  $P = 0.004$ ,  $P = 0.001$ , respectively). Significantly more cartilaginous tissue and immature bone were formed in groups M and H than in group C at 2 and 6 wk post-fracture ( $P = 0.018$ ,  $P = 0.010$ ;  $P = 0.032$ ,  $P = 0.050$ , respectively). At 2 wk post-



fracture, SDF-1, TGF- $\beta$ 1 and VEGF expression were significantly higher in groups M and H than in group L ( $P = 0.031$ ,  $P = 0.014$ ;  $P < 0.001$ ,  $P < 0.001$ ;  $P = 0.025$ ,  $P < 0.001$ , respectively). BMP-2 and VEGF expression were significantly higher in groups M and H than in group C at 6 wk post-fracture ( $P = 0.037$ ,  $P = 0.038$ ;  $P = 0.021$ ,  $P = 0.010$ ). Compared to group L, TGF- $\beta$ 1 expression was significantly higher in groups H ( $P = 0.016$ ). There were no significant differences in expression levels of chemokines related to MSC migration, angiogenesis and cytokines associated with osteogenesis between M and H groups at 2 and 6 wk post-fracture.

## CONCLUSION

The administration of at least  $5.0 \times 10^6$  MSCs was optimal to promote fracture healing in a rat model of long bone fractures.

**Key Words:** Rat model; Femoral shaft fracture; Mesenchymal stem cells; Direct injection; Optimal concentration; Fracture healing

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**Core Tip:** This study focused on the optimal concentration of mesenchymal stem cells (MSCs) that affect fracture healing in a rat model of long bone shaft fracture. Factors related to the homing effect of MSCs, osteogenesis and angiogenesis were analyzed by *in vivo* (radiographic and histologic evaluation) as well as *in vitro* (reverse transcriptase-polymerase chain reaction and western blot analysis). Among the various concentrations used, the administration of at least  $5.0 \times 10^6$  MSCs was optimal to promote the therapeutic effect on fracture healing.

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## INTRODUCTION

Long bone fractures, such as those of the femur, tibia, and humerus, occur mainly in working-age adults [1] and are caused by high-energy trauma[2]. Long bone fractures have a high incidence of nonunion owing to the complex and specific anatomical area of the fracture[3]. Intramedullary nailing is the treatment of choice for femoral shaft fractures[4]; however, the risk of nonunion in this procedure has been reported to be up to 13%, even after nailing[5]. As femur has a significant weight-bearing role, the fracture nonunion can cause an increase in morbidity[2]. Moreover, it may be impossible for patients to return to normal daily activities after an injury[6]. Ekegren *et al*[3] reported that among fracture healing complications, the post-operative readmission rate was highest for femoral shaft fractures, followed by tibial shaft fractures. Among these, nonunion has been reported to be the most common cause. The nonunion rate has been reported to be up to 33% after intramedullary nailing in humerus and femur shaft fractures, and a relatively high nonunion rate of approximately 5%-7% has also been reported in tibial shaft fractures[7]. Thus, when nonunion occurs in long bone fractures, significant disability occurs and quality of life deteriorates, resulting in a high socioeconomic burden during treatment[3,7]. Therefore, preventing nonunion during the initial surgery of shaft fractures in long bones is important for improving patient prognosis.

Autologous cells with regeneration potential have emerged as a novel method to replace the standard method of bone repair[8-10]. Mesenchymal stem cells (MSCs) have the potential to promote both osteoinduction and osteogenesis[11]. Stem cell therapy using this type of cell has an important effect in promoting the bone-healing process[8,10]; some animal studies have reported that MSCs improve fracture healing[12]. Wilson *et al*[12] evaluated the extent of bone defect regeneration in the ramus of swine with or without MSC injection. They reported that bone healing was accelerated in a group injected with MSCs[12,13]. Obermeyer *et al*[13] reported that the administration of MSCs increased the volume and biomechanical strength of the callus in an alcohol-induced impaired fracture healing mouse model, resulting in accelerated fracture healing. Some previous studies have reported that injection of MSCs improves fracture healing; however, no studies have specifically reported the most effective concentration of MSC. Although a concentration of  $5.0 \times 10^6$  MSCs was mainly used in several previous studies[10-12], there was no rationale for this selection. Therefore, this study aimed to confirm the ability of MSCs and assess their optimal concentration to promote fracture healing in a rat model of long bone fracture. The authors hypothesized that administration of at least  $5.0 \times 10^6$  MSCs would signi-

ificantly improve fracture healing.

## MATERIALS AND METHODS

### **Animal model**

Forty-eight adult male Wistar rats (8 wk old with 200-250 g weight) were obtained from the Orient Bio Institute, Seongnam City, Gyeonggi-do, Republic of Korea. All procedures and treatments involving animals in this study followed the requirements of the Institutional Animal Care and Use Committee of the Clinical Research Institute, and the final approval was obtained from the ethics committee of Kyung Hee University Hospital at Gangdong (KHNMC AP 2020-018). The rats had free access to food and water and were bred in a controlled environment at  $21 \pm 2^\circ\text{C}$  with a 12-h/12-h light/dark cycle.

### **Long bone fracture model and MSC injection**

The femoral shaft, which is a representative long bone, was used as the fracture model in this study. Under general anesthesia, the right lower extremities of the rats were shaved and disinfected. First, the approach was performed using an anterior midline incision. After exposing the right knee joint by dislocating the patella medially, the intercondylar groove of the femur was exposed by flexion of the knee joint. An 18-gauge needle was retrogradely inserted into the center of the intercondylar groove to prevent significant displacement during the fracture. Since the proximal end of the needle protruding into the knee joint can affect the knee joint range of motion, we cut it and inserted the proximal end of the needle into the distal femur. Next, the femoral shaft was approached through a lateral approach, taking care to avoid damage to the periosteum. After applying an oscillating thin saw at a depth of 1 mm, a fracture was generated in the femoral shaft using the 3-point bending technique[14]. Sterile saline was injected into the fracture site to minimize the periosteal damage owing to heat when applying the saw. After inserting the needle tip into the fracture site, the muscular fascia was closed, the adipose-derived (AD)-MSCs were mixed with 0.3 mL sterile normal saline, and the cells were injected once directly into the fracture site. The muscular fascia was repaired before direct injection of the cell suspension to prevent AD-MSCs from flowing out. Other weight-bearing activities were unrestricted post operatively.

### **Preparation of MSCs**

Human AD-MSCs (Jointstem; R-Bio, Seoul, Korea) were used in this study[15,16]. Three weeks before injection, human adipose tissue was collected by lipoaspiration using the tumescent technique. The aspirated tissue was digested with collagenase I to obtain AD-MSCs, and the digested tissue was centrifuged after removing cellular debris. The obtained pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen)-based medium containing 0.2 mmol/L ascorbic acid and 10% fetal bovine serum, and the cell suspension was recentrifuged. When the cells reached 90% confluence by resuspension and recentrifugation, they were passaged[16]. AD-MSCs at passage 3 were used in this study. AD-MSCs were prepared under Good Manufacturing Practice conditions at the Stem Cell Research Center of RNL BIO. The expanded cells were then tested for number, viability, purity, identity, and fungal, bacterial, endotoxin, and mycoplasma contamination, as suggested by the Code of Federal Regulations Title 21, before further use. Cultured AD-MSCs showed a survival rate of over 80% between 2 and 8 °C for 72 h[15]. Immediately before injection,  $2.5 \times 10^6$ ,  $5.0 \times 10^6$ , and  $10.0 \times 10^6$  AD-MSCs were counted using a hemocytometer[17]. The prepared AD-MSCs were injected into rats within one day of arrival at the animal laboratory.

### **Categorization according to the concentration of administered MSCs**

After breeding for one week, the rats were randomly divided into four groups ( $n = 6$  in each group): Rats injected with normal saline (C),  $2.5 \times 10^6$  (L),  $5.0 \times 10^6$  (M), and  $10.0 \times 10^6$  (H) groups. Several studies have reported the injection of  $5 \times 10^6$  MSCs into animal models[10-12]. In particular, Wilson *et al* [12] injected  $5.0 \times 10^6$  MSCs based on the study by Hou *et al*[18] that concluded that  $> 3.0 \times 10^6$  MSCs should be injected for bone healing. Therefore, a concentration of  $5.0 \times 10^6$  MSCs were used as a reference in this study. The highest concentration was set as  $10.0 \times 10^6$ , according to a previous study that reported that the effective dose for fracture healing was between  $2.0 \times 10^6$  and  $10.0 \times 10^6$  MSCs[19]. Moreover,  $2.5 \times 10^6$  cells, an intermediate concentration between normal saline and  $5.0 \times 10^6$  cells, was set as the lowest concentration.

At 2 and 6 wk post-fracture, the rats were sacrificed to harvest femur specimens, and the intramedullary needle was removed. Six weeks post-fracture has been reported as an important time point for fracture healing in previous studies[20-22]; hence, 6 wk after fracture was chosen in this study to evaluate the late phase of fracture healing. Wang *et al*[20] reported that the expression of transforming growth factor-beta 1 (TGF- $\beta$ 1), a chemokine that has an important effect on MSC migration, peaked at 2 wk post-fracture. Moreover, it is known that the renewal phase, in which MSCs proliferate and differentiate, usually occurs 7-10 d post-fracture[23]. In this study, the expression levels

**Table 1** Fracture healing scores according to the histological findings

Score	Histological findings
1	Fibrous tissue
2	Predominantly fibrous tissue with small amount of cartilage
3	Equal mixture of fibrous and cartilaginous tissue
4	Predominantly cartilage with small amount of fibrous tissue
5	Cartilage
6	Predominantly cartilage with small amount of immature bone
7	Equal mixture of cartilage and immature bone
8	Predominantly immature bone with small amount of cartilage
9	Union of fracture fragments by immature bone
10	Union of fracture fragments by mature bone

of factors related to MSC migration were also analyzed. Referring to the above studies, the early phase was set at 2 wk post-fracture.

### Assessment of fracture healing (in vivo studies)

**Radiologic evaluation through micro-computed tomography:** Micro-computed tomography (micro-CT) was used to evaluate the volume of the newly formed callus [bone volume (BV)] and the percentage of BV [(PBV), calculated as BV/tissue volume]. A 6-mm long section centered on the fracture site was analyzed. Preexisting cortical bone and medullary canal volumes were excluded according to the method described by Wang *et al*[20]. The femur specimens were scanned using three-dimensional micro-focus micro-CT (Sky-Scan 1172™, Skyscan, Kontich, Belgium) at 10 µm resolution, 440 ms exposure, 0.4° rotation step, 80 kV, and 167 µm with a 0.5 mm aluminum filter.

**Histological evaluation:** Decalcification was performed using a rapid decalcifier solution (RDO, Apex Engineering Products Corporation) at room temperature for three days. The RDO solution was replaced daily. The decalcification process and endpoint were assessed using a surgical blade and radiographic analysis, wherein in the opacity of the tissue suggested incomplete decalcification. The femur specimen was sagittally sectioned to a thickness of 3 µm and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich) for histological analysis. The slides were visualized using an Olympus CX41 microscope (Olympus Co., Tokyo, Japan). Fracture healing was evaluated using a histological scoring tool for fracture healing[24] (Table 1).

### Assessment of fracture healing (in vitro studies)

**Western blot analysis:** Rat femur specimens were ground in liquid nitrogen and incubated with lysis buffer containing 140 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/L phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, and 1% (w/v) Nonidet P-40 in 20 mmol/L Tris-HCl (pH 7.4). Protein fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels and electrotransferred onto PVDF membranes (Bio-Rad, Hercules, CA, United States). The membranes were then blocked with Tris-buffered saline buffer consisting of 1% nonfat dry milk and 1% bovine serum albumin for 1 h. Next, membranes were then incubated overnight at 4 °C with primary antibodies against stromal cell-derived factor 1 (SDF-1) (Abcam, Cat # ab18919, 1:3000), TGF-β1 (Abcam, Cat # ab215715, 1:3000), vascular endothelial growth factor (VEGF) (Santa Cruz, SC-7269, 1:2000) and β-actin (Santa Cruz Biotechnology; 1:1000). The membranes were developed for 1 h peroxidase-conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology). The blots were visualized using a ChemicDoc XRS system (Bio-Rad), and protein concentrations were quantified using the Quantity One imaging software (Bio-Rad). All experiments were performed in triplicates.

**Real-time quantitative polymerase chain reaction:** Total RNA from the rat femur specimens was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. RNA was reverse transcribed using Superscript II reverse transcriptase (Life Technologies) at 42 °C *via* random hexamer priming. The quantitative polymerase chain reaction (qPCR) conditions were as follows: Pre-denaturation at 95 °C for 5 min, denaturation at 95 °C for 10 s, and annealing at 60 °C for 30 s for a total of 40 cycles, followed by fluorescence signal detection during annealing. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal reference for normalization. The reactions were performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, United States). The sequences of the primers used for reverse transcriptase-qPCR (RT-qPCR) are listed in Supplementary Table 1.

**Statistical analysis:** Based on the concentration of MSCs, one-way analysis of variance and *post hoc* analyses were performed to evaluate the differences in micro-CT, histological scores of fracture healing, and mRNA and protein expression (as evidenced by RT-qPCR and western blot results, respectively). Statistical significance was set  $P = 0.05$ , with 95% confidence interval. SPSS version 21.0 software (SPSS, Inc., Chicago, Illinois, United States) was used for all statistical analyses.

## RESULTS

### *In vivo studies*

**Fracture healing as evaluated via micro-CT analysis:** At 2 and 6 wk post-fracture, BV was significantly higher in group M and H than in group C ( $P = 0.048$ ,  $P = 0.040$  and  $P = 0.023$ ,  $P = 0.009$ , respectively). There was no significant difference in BV between M and H groups ( $P = 0.999$  and  $P = 0.887$ ). There was no significant difference in PBV between four groups at 2 wk post-fracture. However, PBV was significantly increased in groups M and H compared to that in group C ( $P = 0.004$  and  $P = 0.001$ , respectively) and group L ( $P = 0.026$  and  $P = 0.003$ , respectively) at 6 wk post-fracture. There was no significant difference in the PBV between groups M and H ( $P = 0.425$ ) (Table 2, Figures 1 and 2).

**Histological scores of fracture healing evaluated using H&E staining:** The formation of fibrous, cartilaginous, and immature bones was evaluated using histological scores. At 2 and 6 wk post-fracture, there was no significant difference in the fracture healing scores between groups C and L ( $2.8 \pm 0.5$  vs  $3.8 \pm 0.5$ ,  $P = 1.000$  and  $5.3 \pm 0.5$  vs  $6.5 \pm 1.7$ ,  $P = 1.000$ , respectively). Significantly more cartilaginous tissue was formed in groups M ( $5.5 \pm 1.3$ ) and H than in group C ( $P = 0.018$  and  $P = 0.010$ , respectively) at 2 wk post-fracture (Figure 3). Moreover, significantly more immature bone was formed in groups M ( $8.8 \pm 1.9$ ) and H ( $8.5 \pm 1.3$ ) than in the group injected with normal saline ( $P = 0.032$  and  $P = 0.050$ , respectively) at 6 wk post-fracture (Figure 4).

### *In vitro studies*

**Comparison of protein expression levels of chemokines related to MSC migration and angiogenesis at 2 wk post-fracture:** The fold change in mRNA expression (all reported values are fold-changes relative to the comparator) of SDF-1 was significantly higher in groups L, M, and H than in group C ( $P < 0.001$  all). Compared to group L, groups M and H showed significantly higher SDF-1 expression ( $P = 0.031$ ,  $P = 0.014$ , respectively). There was no significant difference in SDF-1 expression between groups M and H ( $P = 0.974$ ). TGF- $\beta$ 1 expression was significantly higher in groups L, M and H than in group C ( $P = 0.003$ ,  $P < 0.001$ ,  $P < 0.001$ , respectively). Compared to group L, groups M and H also showed significantly higher TGF- $\beta$ 1 expression at 2 wk post-fracture ( $P < 0.001$ , all). There was no significant difference in TGF- $\beta$ 1 expression between groups M and H ( $P = 0.997$ ). VEGF expression was significantly higher in groups M and H than in group C ( $P < 0.001$ , all). In addition, VEGF expression was significantly higher in groups M and H than in group L ( $P = 0.025$ ,  $P < 0.001$ , respectively). There was no significant difference in VEGF expression between groups M and H ( $P = 0.239$ ) (Figures 5 and 6) (Supplementary Table 2).

**Comparison of the mRNA expression levels of osteogenesis-related factors and chemokine related to angiogenesis at 6 wk post-fracture:** Bone morphogenetic protein-2 (BMP-2) expression was significantly higher in groups L, M, and H than in group C ( $P < 0.001$ , all). Compared to group L, BMP-2 expression was significantly higher in groups M and H ( $P = 0.037$ ,  $P = 0.038$ , respectively). There was no significant difference in BMP-2 expression between groups M and H ( $P = 1.000$ ). TGF- $\beta$ 1 expression was significantly higher in groups L, M and H than in group C ( $P = 0.002$ ,  $P < 0.001$ ,  $P < 0.001$ , respectively). Compared to group L, TGF- $\beta$ 1 expression was significantly higher in groups H ( $P = 0.016$ ). There was no significant difference in TGF- $\beta$ 1 expression between groups M and H ( $P = 0.824$ ).

VEGF expression was significantly higher in groups L, M and H than in group C ( $P < 0.001$ , all). In addition, VEGF expression was significantly higher in groups M and H than in group L ( $P = 0.021$ ,  $P = 0.010$ , respectively). There was no significant difference in VEGF expression between groups M and H ( $P = 0.943$ ) (Figure 7) (Supplementary Table 3). The protein expression levels of BMP-2, TGF- $\beta$ 1 and VEGF are listed in Supplementary Table 4.

## DISCUSSION

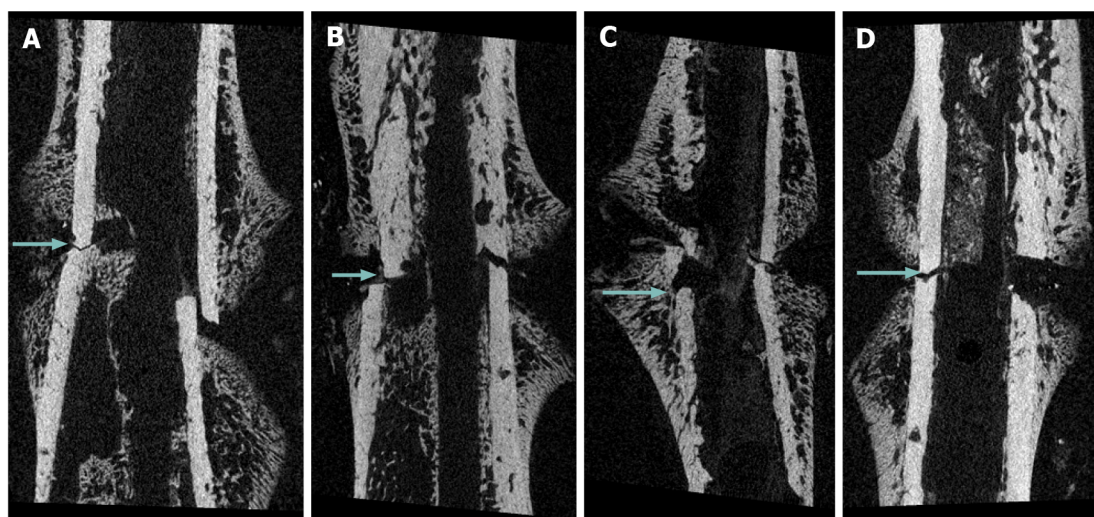
In this study, fracture healing was significantly improved in the groups injected with MSCs compared with that in the control group. In addition, after injection of MSCs at different concentrations, the mRNA and protein expression of genes related to MSC migration, angiogenesis, and osteogenesis were higher in groups injected with  $5.0 \times 10^6$  and  $10 \times 10^6$  MSCs than in the group injected with  $2.5 \times 10^6$  MSCs. This study is meaningful as it is the first animal study to confirm that an MSC concentration of



**Table 2 Radiologic evaluation of the fractures using micro-computed tomography analysis**

		Normal saline	$2.5 \times 10^6$	$5.0 \times 10^6$	$10.0 \times 10^6$
Two weeks post-fracture	BV ( $\mu\text{m}^3$ )	$34.9 \pm 3.1$	$37.3 \pm 4.5$	$45.3 \pm 2.9$	$45.7 \pm 4.9$
	Normal saline	-	0.878	0.048	0.040
	$2.5 \times 10^6$	0.878	-	0.137	0.112
	$5.0 \times 10^6$	0.048	0.137	-	0.999
	$10.0 \times 10^6$	0.040	0.112	0.999	-
	PBV (TV/BV, %)	$7.3 \pm 0.8$	$9.0 \pm 0.5$	$10.8 \pm 1.2$	$11.0 \pm 2.6$
	Normal saline	-	0.517	0.079	0.067
	$2.5 \times 10^6$	0.517	-	0.509	0.445
	$5.0 \times 10^6$	0.079	0.509	-	0.999
	$10.0 \times 10^6$	0.067	0.445	0.999	-
Six weeks post-fracture	BV ( $\mu\text{m}^3$ )	$71.3 \pm 8.0$	$78.7 \pm 8.8$	$101.4 \pm 14.0$	$107.2 \pm 6.9$
	Normal saline	-	0.798	0.023	0.009
	$2.5 \times 10^6$	0.798	-	0.083	0.030
	$5.0 \times 10^6$	0.023	0.083	-	0.887
	$10.0 \times 10^6$	0.009	0.030	0.887	-
	PBV (TV/BV, %)	$13.4 \pm 2.2$	$15.0 \pm 3.1$	$20.5 \pm 2.2$	$23.6 \pm 3.3$
	Normal saline	-	0.769	0.004	0.001
	$2.5 \times 10^6$	0.769	-	0.026	0.003
	$5.0 \times 10^6$	0.004	0.026	-	0.425
	$10.0 \times 10^6$	0.001	0.003	0.425	-

CT: Computed tomography; BV: Bone volume; PBV: Percentage bone volume; TV: Tissue volume.

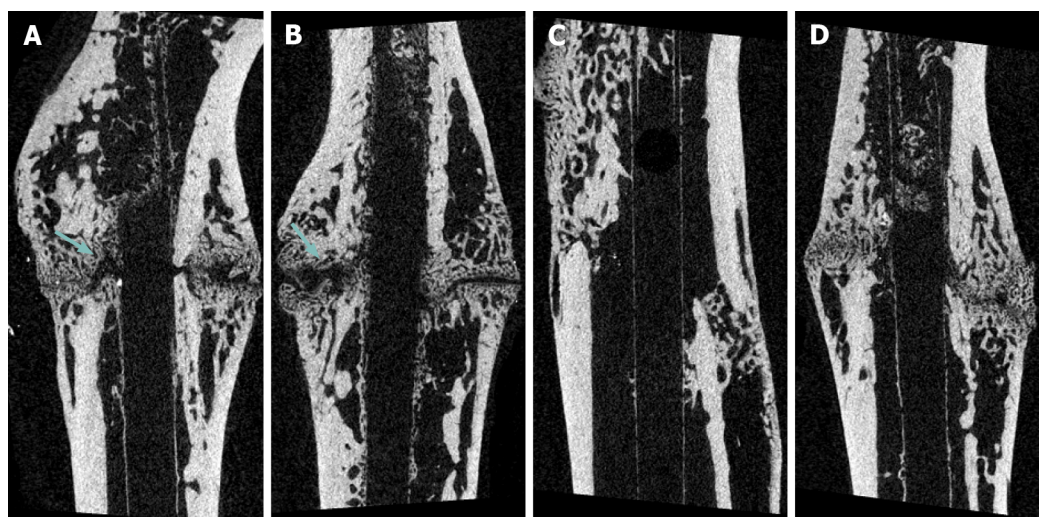


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**Figure 1 Micro-computed tomography imaging at 2 wk post-fracture.** A: Rats were injected with normal saline; B: Rats were injected with  $2.5 \times 10^6$  mesenchymal stem cells (MSCs); C: Rats were injected with  $5.0 \times 10^6$  MSCs; D: Rats were injected with  $10.0 \times 10^6$  MSCs. Callus formation was observed in all groups; however, fracture lines (arrows) were clearly observed, indicating that union had not yet occurred.

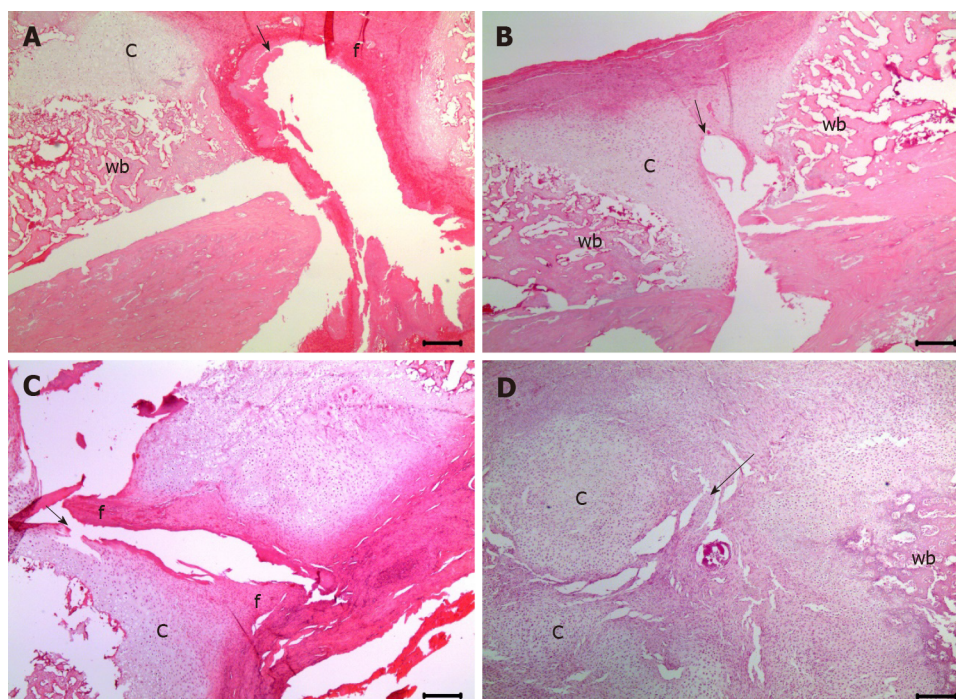
$5.0 \times 10^6$  cells, which has been used in several previous studies, maximizes fracture healing.

The nonunion of long bone fractures results in a high socioeconomic burden and long treatment duration[7]. Studies aimed at enhancing fracture healing through bone regeneration[25,26] and those



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**Figure 2 Micro-computed tomography imaging at 6 wk post-fracture.** A: Rats were injected with normal saline; B: Rats were injected with  $2.5 \times 10^6$  adipose-derived-mesenchymal stem cells (AD-MSCs); C: Rats were injected with  $5.0 \times 10^6$  AD-MSCs; D: Rats were injected with  $10.0 \times 10^6$  AD-MSCs. In the group injected with normal saline and  $2.5 \times 10^6$  AD-MSCs, fracture lines (arrows) were clearly observed, indicating that union had not yet occurred.



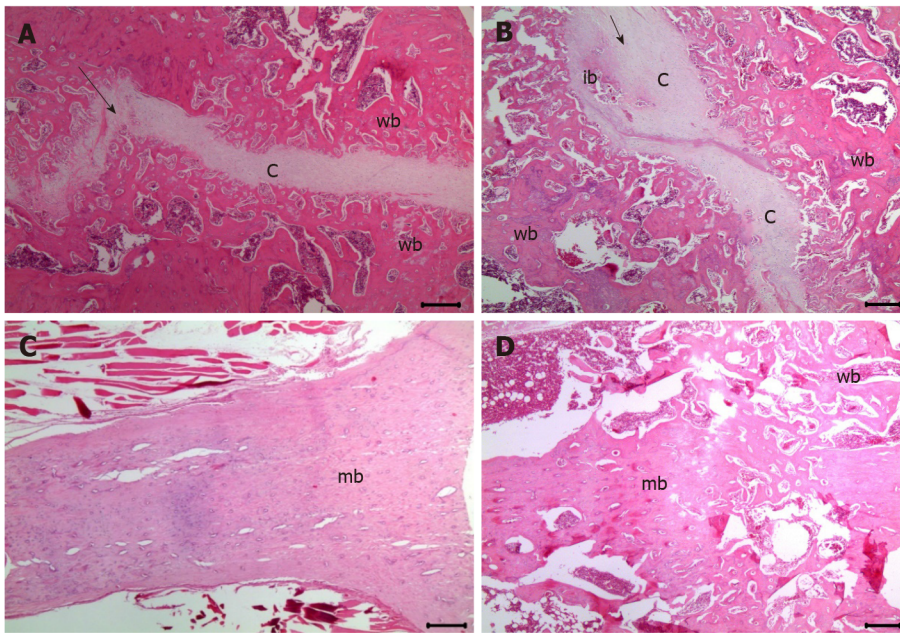
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**Figure 3 Assessment of histological scores for fracture healing at 2 wk post-fracture using hematoxylin and eosin staining (200 × magnification).** A: The histological score was 2 in the group injected with normal saline; B: The histological score was 3 in the group injected with  $2.5 \times 10^6$  mesenchymal stem cells (MSCs); C: The histological score was 4 in the groups injected with  $5.0 \times 10^6$  MSCs; D: The histological score was 4 in the groups injected with  $10.0 \times 10^6$  MSCs. The black arrows indicate the fracture lines. c: Cartilage in the fracture area; f: Fibrous tissue; wb: Woven bone.

that used MSCs as a cell therapy have been conducted[12,13,27]. Obermeyer *et al*[13] compared fracture healing after the injection of MSCs and saline in an animal model of impaired fracture healing. They reported that significantly more callus formation was observed in the group injected with MSCs and that MSCs migrated and homed to the fracture site, contributing to fracture healing. However, they evaluated only the migration of labeled MSCs to the fracture site *via* immunofluorescence staining; they did not quantitatively evaluate the levels of factors related to MSC homing.

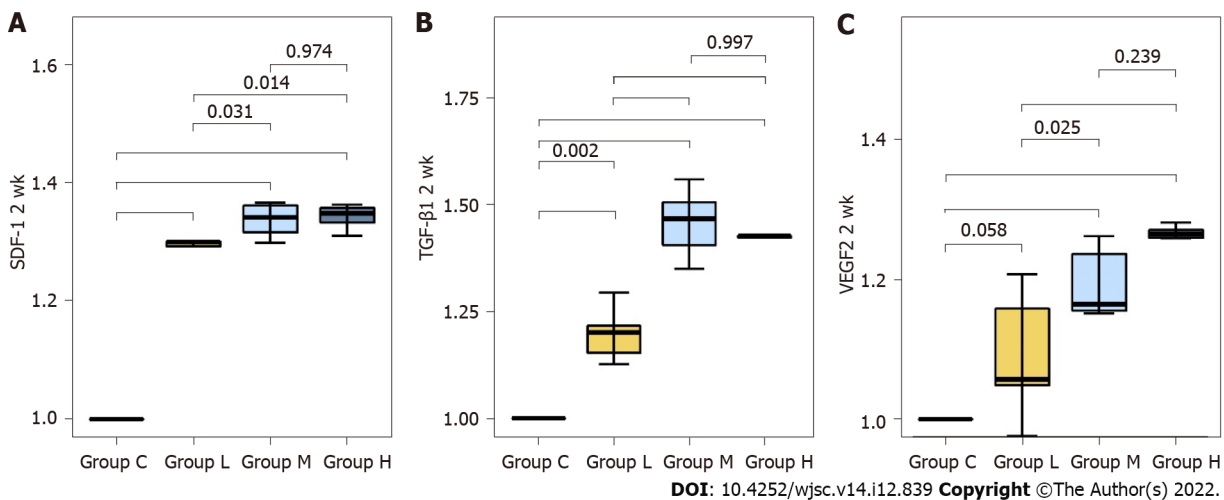
The homing of MSCs to the fracture site is a key mechanism during the early stages of fracture healing. After MSCs are recruited to a fracture site, they differentiate into osteogenic cells to enhance healing[28]. In addition, Caplan[29] reported that directly injected MSCs did not differentiate in the





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**Figure 4** Assessment of histological scores for fracture healing at 6 wk post-fracture using hematoxylin and eosin staining (200 × magnification). A: The histological score was 5 in the groups injected with normal saline; B: The histological score was 5 in the groups injected with  $2.5 \times 10^6$  mesenchymal stem cells (MSCs); C: The histological score was 9 in the group injected with  $5.0 \times 10^6$  MSCs; D: The histological score was 10 in the group injected with  $10.0 \times 10^6$  MSCs. The black arrows indicate the fracture lines. c: Cartilage in the fracture area; wb: Woven bone; ib: Immature bone; mb: Mature bone.



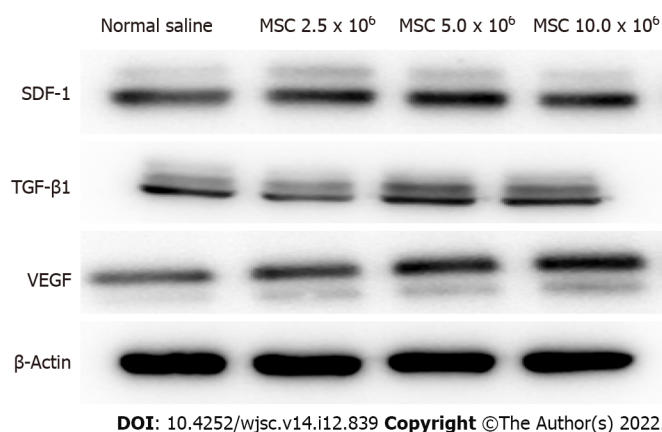
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**Figure 5** Relative protein expression levels of chemokines related to mesenchymal stem cell migration and angiogenesis at 2 wk post-fracture. SDF-1: Stromal cell-derived factor 1; TGF-β1: Transforming growth factor-beta 1; VEGF: Vascular endothelial growth factor.

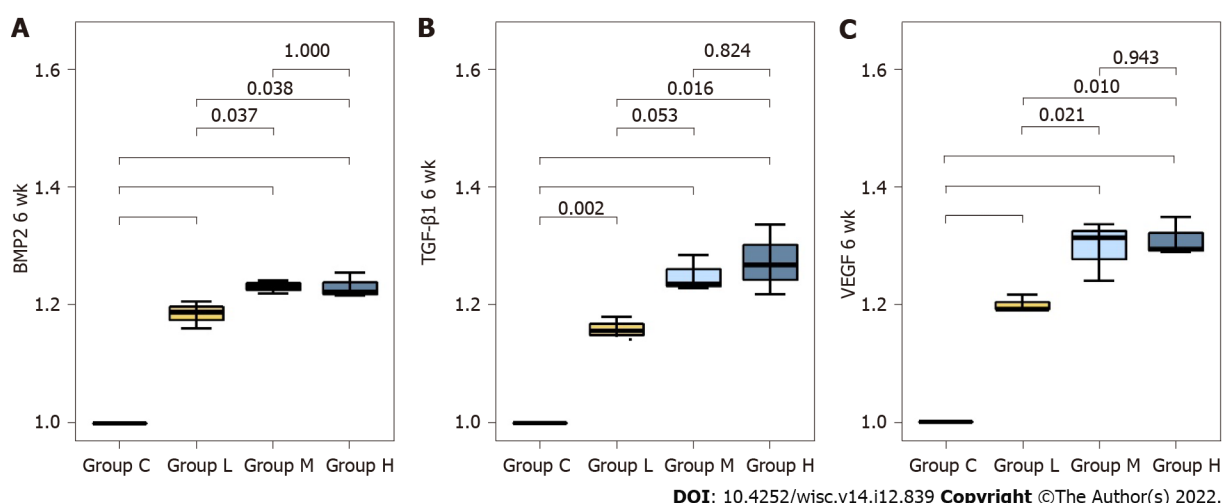
injured tissue, but homed to the injury site to secrete bioactive factors, thereby resulting in therapeutic effects. Therefore, in this study, we evaluated the homing effect by quantitatively analyzing the expression of SDF-1, which is known to be an important chemokine for the recruitment of MSCs to fracture sites[20], and TGF-β1, which is known to enhance MSC proliferation[30], using western blotting at 2 wk post-fracture. The expression of factors related to MSC homing to the fracture site was higher in the groups injected with  $5.0 \times 10^6$  and  $10.0 \times 10^6$  MSCs than in the control group injected with normal saline.

Known methods of injecting MSCs for fracture healing include systemic intravenous and direct injections[20,31]. Systemic MSC injection is convenient and minimally invasive[15], and repeated administration is possible[20]. Therefore, it has been used in many animal studies. Ra *et al*[15] evaluated the safety of systemic MSC injection in animals and humans and reported no serious side effects in any animal or patient. However, other than this study, no studies have analyzed the stability, toxicity, and possible adverse effects of systemic MSC injection. On the other hand, Galindo *et al*[32] suggested that systemic injection of MSCs may be associated with a high risk of side effects, and the number of cells





**Figure 6 Western blot analysis of factors related to mesenchymal stem cell homing and angiogenesis.** At 2 wk post-fracture, the protein expressions of stromal cell-derived factor 1 (SDF-1), transforming growth factor-beta 1 (TGF-β1) and vascular endothelial growth factor (VEGF) were significantly higher in the groups injected with  $5.0 \times 10^6$  and  $10.0 \times 10^6$  mesenchymal stem cells (MSCs) compared to the group injected with normal saline. No significant difference in SDF-1, TGF-β1 and VEGF protein expression was found in the groups injected with  $5.0 \times 10^6$  and  $10.0 \times 10^6$  MSCs. MSCs: Mesenchymal stem cells; SDF-1: Stromal cell-derived factor 1; TGF-β1: Transforming growth factor-beta 1; VEGF: Vascular endothelial growth factor.



**Figure 7 Relative mRNA expression levels of osteogenesis-related factors and chemokines related to angiogenesis at 6 wk post-fracture.** BMP: Bone morphogenetic protein; TGF-β1: Transforming growth factor-beta 1; VEGF: Vascular endothelial growth factor.

reaching the target area may be small. The treatment of fractures often requires opening of the fracture site to fix the injured area. Therefore, in this study, the above-mentioned disadvantage of systemic injection was reflected, and direct injection of MSCs into the fracture site was performed instead.

Hou *et al*[18] reported that MSCs at a concentration higher than  $3.0 \times 10^6$  should be administered in mice. Dreger *et al*[27] evaluated bone regeneration in a mouse femur shaft fracture model after the injection of  $2.0 \times 10^6$  MSCs, which was less than that reported by Hou *et al*[27]. Dreger *et al*[27] reported that injected MSCs significantly accumulate at the fracture site and enhance bone regeneration. However, there is still no consensus on the optimal number of MSCs to be injected to enhance fracture healing. Janko *et al*[19] injected MSCs using scaffolds in a large-sized bone defect rat model and reported that a range of  $(2.0-10.0) \times 10^6$  MSCs was an effective dose window for fracture healing. However, they evaluated only healing at 8 wk post-fracture and did not analyze the effect of MSC concentration on fracture healing in the early phase. In addition, they analyzed only callus formation *via* histological analysis, not *via* radiologic evaluation, such as micro-CT. Furthermore, the expression of factors related to osteogenesis and angiogenesis, which are important in evaluating fracture healing, was not analyzed. In this study, fracture healing was analyzed at 2 and 6 wk post-fracture, that is, in both the early and late phases. Histological analysis, micro-CT, and the expression of factors related to MSC migration, osteogenesis, and angiogenesis were analyzed. Our results showed that fracture healing was enhanced in the groups injected with  $5.0 \times 10^6$  and  $10 \times 10^6$  MSCs compared to the groups injected with normal saline and  $2.5 \times 10^6$  MSCs. There was no significant difference between the groups injected with  $5.0 \times 10^6$  and  $10 \times 10^6$  MSCs.

This study has several limitations. First, although previous reports have shown that human MSCs are safe when injected into animals[15], MSCs cultured in rats were not used in this study. However, because conventionally prepared MSCs are used, purity can be guaranteed. Second, in this study, fracture healing was compared by administering MSCs at different concentrations of  $2.5 \times 10^6$ ,  $5.0 \times 10^6$ , and  $10 \times 10^6$  cells; however, the criterion for determining the concentrations was ambiguous. Third, in cases of direct injection of MSCs mixed with normal saline, it may be difficult to retain MSCs at the fracture site for the long time. Additionally, we did not evaluate retention of the implanted MSC at the fracture site using fluorescence imaging analysis. Despite these limitations, this study is meaningful because it is the first animal study to analyze the optimal concentration of MSCs that maximizes the effect on fracture healing. In addition, this study could help to set the standard concentration of MSCs for evaluating fracture healing in an rat model of long bone fracture.

## CONCLUSION

Direct injection of various concentrations of MSCs enhances fracture healing in a rat model of long bone fractures. Among the various concentrations used,  $5.0 \times 10^6$  MSCs was optimal to promote fracture healing. Therefore, in order to evaluate the therapeutic effect on fracture healing of MSCs in a rat model of fractures, administration of at least  $5.0 \times 10^6$  MSCs is suggested.

## ARTICLE HIGHLIGHTS

### Research background

Previous studies have reported that injection of mesenchymal stem cells (MSCs) improves fracture healing. However, no studies have specifically reported the most effective concentration of MSC.

### Research motivation

There is no consensus on which concentration of MSCs to use for promoting fracture healing in a rat model of long bone fracture.

### Research objectives

The present study aimed to assess the optimal concentration of MSCs for promoting fracture healing in a rat model.

### Research methods

Wistar rats were divided into four groups according to MSC concentrations: Normal saline (C),  $2.5 \times 10^6$  (L),  $5.0 \times 10^6$  (M), and  $10.0 \times 10^6$  (H) groups. New bone formation was evaluated using micro-computed tomography (micro-CT). Histological analysis was performed to evaluate fracture healing score. The protein expression of factors related to MSC migration and angiogenesis was evaluated using western blot analysis. The expression of cytokines associated with osteogenesis was evaluated using real-time polymerase chain reaction.

### Research results

Micro-CT showed that new bone formation was significantly increased in groups M and H compared to that in group C at 6 wk post-fracture. Significantly more cartilaginous tissue and immature bone were formed in groups M and H than in group C at 2 and 6 wk post-fracture. At 2 post-fracture, the protein expression levels of factors related to MSC migration and angiogenesis were significantly higher in groups M and H than in group L. The mRNA levels of cytokines associated with osteogenesis and angiogenesis were significantly higher in groups M and H than in group C at 6 wk post-fracture. There were no significant differences between M and H groups.

### Research conclusions

Among the various concentrations used,  $5.0 \times 10^6$  MSCs was the optimal concentration that promoted healing of long bone shaft fractures.

### Research perspectives

This study could help to set the standard concentration of MSCs for evaluating fracture healing in an animal model of fracture.

## FOOTNOTES

**Author contributions:** Kim MS collected and assembled the data and performed data analysis and interpretation, manuscript writing; Chung HJ analyzed the data and performed interpretation; Kim KI provided the study material and designed the research study and performed final approval of manuscript; and all authors have read and approve the final manuscript.

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