

LOW-MAGNITUDE HIGH-FREQUENCY VIBRATION ENHANCED MESENCHYMAL STEM CELL RECRUITMENT IN OSTEOPOROTIC FRACTURE HEALING THROUGH THE SDF-1/CXCR4 PATHWAY

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Abstract

Low-magnitude high-frequency vibration (LMHFV) has been proven to promote osteoporotic fracture healing. Mechanical stimulation was reported to enhance SDF-1/CXCR4 signalling in mesenchymal stem cells (MSCs). We hypothesised that LMHFV promoted osteoporotic fracture healing by enhancing MSC migration through the SDF-1/CXCR4 pathway. 152 ovariectomised SD-rats received closed femoral fracture in groups of vibration+MSC (VMG) (20 min/d, 5 d/week), vibration+MSC+AMD3100 (VMAG; AMD, a CXCR4 inhibitor) (1 mg/kg/d, intraperitoneal), MSC (MG) (1×10^6 MSC, intracardiac) or control (CG) for a treatment duration of 2, 4 or 8 weeks. MSC migration was evaluated by *ex-vivo* green fluorescent protein signal in the callus; and fracture healing was examined by weekly radiographs, endpoint computed-tomography and mechanical test. At week-2 and week-4, *ex-vivo* callus GFP intensity of VMG was significantly higher than other groups ($p < 0.05$). From week-2 to week-3, both callus width and callus area in VMG were significantly larger; and from week-7 to week-8, smaller than other groups ($p < 0.05$). At week-8, high-density bone volume fraction, bone volume fraction, bone mineral density and stiffness in VMG were significantly higher than other 3 groups ($p < 0.05$). This study demonstrated that LMHFV promoted MSC migration and fracture healing in osteoporotic rats. This effect was attenuated by CXCR4 inhibitor, providing strong evidence that SDF-1-mediated MSC migration was one of the important mechanisms through which LMHFV enhanced fracture healing.

Keywords: Osteoporotic fracture, mechanical stimulation, vibration treatment, mesenchymal stem cell recruitment, stromal derived factor-1.

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Introduction

Osteoporotic fracture healing is a growing public health concern due to the ageing global population (Cummings and Melton, 2002). Millions of fractures still occur annually as a result of traumatic injuries. Although most fractures will successfully heal within a few months, a proportion of fracture cases still result in delayed healing or even non-union (Claes *et al.*, 2012) which may prolong treatment period and increase morbidity of the patients. Healing of fractures in osteoporotic bone is impaired (Giannoudis *et al.*, 2007) in many ways, including callus formation and remodelling capacities (Chow *et al.*, 2011; Giannoudis *et al.*, 2007; Shi *et al.*, 2010), gene expression (Cheung *et al.*, 2011; Chung *et al.*, 2014), angiogenesis (Sun *et al.*, 2012), and also systemic recruitment of reparative mesenchymal stem cells (MSCs) (Granero-Molto *et al.*, 2009; Marsell and Einhorn, 2011). MSCs are the musculoskeletal lineage of multipotent stromal cells capable of differentiating into bone, cartilage, tendon, adipose and muscle cells (Caplan, 1991; Minguell *et al.*, 2001; Short *et al.*, 2003) with high reparative potentials. In case of a fracture, MSCs are recruited to the site of fracture from various tissues and organs (da Silva Meirelles *et al.*, 2006) by the inflammatory cascade (Kolar *et al.*, 2011). Therefore, MSCs play an important role in the early phase of fracture healing.

Although the fracture healing process has been very well described, the molecular regulation of the event is not completely understood. Amongst the cytokines released that regulate the healing process and the systemic recruitment of MSCs, the stromal cell-derived factor-1 (SDF-1) and its target receptor of chemokine (C-X-C) receptor-4 (CXCR4) are suggested to be highly related (Houthuijzen *et al.*, 2012; Ma *et al.*, 2011; Ponomaryov *et al.*, 2000). Granero-Molto *et al.* (2009) found that implanted MSCs were recruited to the fracture site in an exclusively CXCR4-dependent manner; and Kitaori *et al.* (2009) showed that SDF-1 level was elevated in the periosteum of injured bone, which recruited MSCs homing to the graft bone at the fracture site and promoted endochondral bone formation. Moreover, SDF-1 was found to be highly expressed and involved in MSC trafficking in a novel induced membrane technique used to repair large bone defects (Cuthbert *et al.*, 2013).

Low-magnitude high-frequency vibration (LMHFV) treatment was reported to be a promising non-pharmaceutical

intervention with the potential to accelerate fracture healing in both normal and osteoporotic bones (Chow *et al.*, 2011; Chung *et al.*, 2014; Leung *et al.*, 2009; Shi *et al.*, 2010). Our research group has previously reported that LMHFV was able to enhance ovariectomy-induced osteoporotic fracture healing through enhancing the endochondral ossification process (Shi *et al.*, 2010), the callus remodelling process (Chow *et al.*, 2011), and also the increase of blood flow, neo-vasculature and the protein expression of vascular endothelial growth factor (VEGF) in fracture callus when compared to non-vibrated controls (Cheung *et al.*, 2012b). As all of these are important contributing factors and their enhancement are much related to the recruitment of highly reparative MSCs to the fracture site, we hypothesised that the enhancement effect of LMHFV on osteoporotic fracture healing was related to the enhanced systemic recruitment of MSCs, regulated by the SDF-1/CXCR4 signalling pathway. The objectives of the study were to investigate (i) the effect of LMHFV on the recruitment of MSCs to the fracture site; and (ii) the role of the SDF-1/CXCR4 signalling pathway during the recruitment process.

Materials and Methods

Experimental design

In this study, we employed the well-established closed femoral fracture model in ovariectomy-induced osteoporotic rats. Exogenous green-fluorescent protein (GFP)-labelled MSCs were used to demonstrate the treatment effect of LMHFV in terms of MSC recruitment that could be objectively quantified and detected by a fluorescence imaging system (Cheung *et al.*, 2013). A CXCR4 antagonist, AMD3100 (Chabannon *et al.*, 2011; Wei *et al.*, 2014), was used to block the SDF-1/CXCR4 pathway to reveal the role of this pathway in MSC recruitment during osteoporotic fracture healing augmented with LMHFV. Grouping of the osteoporotic rats is as stated in Table 1. Sample size for each time-point was $n = 10$ for green fluorescent protein (GFP) signal and μ CT assessments, and $n = 8$ for the endpoint mechanical test. A total of 152 rats were used for this study. Fracture healing related outcomes are assessed at time-points of 2, 4 and 8 weeks after fracture creation.

Osteoporotic closed femoral fracture model

All animal experimentation procedures were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong (Ref: CUHK 11/010/GRF-5). The osteoporotic fracture rat models were created according to our previously described protocol (Cheung *et al.*, 2011; Leung *et al.*, 2009; Wei *et al.*, 2014). In brief, each rat received bilateral ovariectomy under general anaesthesia at the age of 6 months and aged for 3 additional months for the development of osteoporosis. At the age of 9-months, an intramedullary K-wire (390152A, Stryker, Kalamazoo, MI, USA) was surgically inserted followed by closed femoral fracture creation by a standardised 3-point-bending apparatus. Post-operational X-ray (MX-20, Faxitron Bioptics LLC, Tucson, AZ, USA) was used to confirm a transverse and non-comminuted fracture for

Table 1. Animal grouping.

Group	AMD3100	Vibration	MSC injection
VAMG	☑	☑	☑
VMG		☑	☑
MG			☑
CG			

Sample size (n) was 10 *per group per time-point*.

Abbreviations: V = vibration, A = AMD3100 injection, M = MSC injection, G = group.

each successful case. All animal surgeries were performed by the same researcher to ensure consistency.

Intra-cardiac injection of GFP-labelled MSCs

The procedure was performed according to our previously established protocol (Wei *et al.*, 2014). On day 3 post-fracture (2 d before vibration treatment), 1.0×10^6 GFP-labelled MSCs (SD Rats, RASM-X-01101, Cyagen, Guangzhou, China) in 500 μ L phosphate-buffered saline (PBS) were transplanted by intracardiac injection as previously described (Cheung *et al.*, 2013; Furlani *et al.*, 2009). Briefly, the GFP-labelled MSCs were sub-cultured to the fifth passage, and then were trypsinised and diluted in 0.9 % normal saline. A 23G needle was inserted through the thoracic wall at a point left to the sternum on a line connecting the left axillary pivot with the caudal tip of the sternum. The syringe containing the MSC suspension was aspirated followed by the injection of the MSC suspension by gently pressing the piston of the syringe. The position of the needle was confirmed by an ultrasound system (Vevo 770, VisualSonics, Toronto, ON, Canada) under B-mode.

LMHFV treatment

Rats receiving the LMHFV treatment were placed in a custom-made bottomless rat holder directly on the custom-built vibration platform transmitting vertical vibrations at 35 Hz and a magnitude of $0.3 \times g$. Treatment was started on the fifth day after fracture creation and was given 20 min *per day*, 5 consecutive days (Mon-Fri) *per week* until the endpoint of the experiment based on our previously reported treatment scheme (Cheung *et al.*, 2012b; Chow *et al.*, 2011; Leung *et al.*, 2009; Shi *et al.*, 2010). Rats were euthanised with an overdose of 20 % pentobarbital at endpoints.

AMD3100 administration

According to previously published protocol, AMD3100 was dissolved in 0.9 % saline solution to a final concentration of 1 mg/mL for intraperitoneal injection at a dose of 1 mg/kg/d (De Clercq, 2003; Wei *et al.*, 2014) 30 min before LMHFV treatment to the VAMG group. All other groups received equivalent amounts of vehicle injection.

Weekly radiography

Two-dimensional digital radiographs (MX-20, Faxitron, Lincolnshire, IL, USA) were taken weekly post-fracture to confirm the quality and degree of fracture healing. The

quantified temporal changes of callus morphology were evaluated by using the Metamorph Image Analysis System (Universal Imaging Corporation, Downingtown, PA, USA) according to our previously established protocol (Shi *et al.*, 2010), where callus width (CW) was defined as the maximal outer diameter of the mineralised callus minus the outer diameter of the femur; and callus area (CA) was calculated as the sum of the areas of the external mineralised callus, assessed independently by three researchers.

Ex-vivo GFP intensity analysis

After euthanasia at the specified endpoints (2, 4 and 8 weeks post-fracture), fracture femora were harvested with soft tissue carefully removed. GFP signals were quantified and fluorescent images were acquired (IVIS 200, Xenogen, Alameda, CA, USA). The fluorescence analysis was calibrated to the standard as recommended by the IVIS imaging operation manual. The intensities (in counts) were measured using the Live Image 2.5 software at an exposure time of 5 s, binning of 8 and f/16 (Cheung *et al.*, 2013; Granero-Molto *et al.*, 2009). A standard circular region-of-interest (ROI) with a diameter of 1 cm was defined around the callus area for all measurements. After background noise reduction, the intensities within the ROI were used to evaluate the average intensities, based on our previously established protocol (Cheung *et al.*, 2013).

μ CT analysis

Ex vivo micro-computed tomography scans (μ CT40, Scanco Medical, Brüttisellen, Switzerland) were then performed after GFP intensity analysis, based on our established protocol (Cheung *et al.*, 2012a; Leung *et al.*, 2009). The femora were positioned vertically with normal saline-soaked gauze in the sample holder during scanning. The newly formed bone (low-density bone, threshold = 165-350) and highly mineralised bone (high-density bone, threshold = 350-1000) were reconstructed separately (Fung *et al.*, 2012; Leung *et al.*, 2009). The ratios of low-density bone volume over total tissue volume (BV_{l}/TV), high-density bone volume over total tissue volume (BV_{h}/TV), total bone volume fraction (BV_{t}/TV) and bone mineral density (BMD) were evaluated.

Immunohistochemistry (IHC) of GFP

After completing the μ CT scanning, the fractured femora were fixed, decalcified and paraffin embedded. Histological sections were cut at 5 μ m thick for observation. Protein expression of GFP was stained by immunohistochemistry using commercially available mouse monoclonal primary antibodies for GFP (ab38689 at dilution of 1:100, Abcam, Cambridge, UK) and rabbit specific colour developing kit (ab80437) with Harris haematoxylin counter-staining as previously reported (Chow *et al.*, 2014). Three random sites were selected at the bony callus for image capturing under a 10 \times objective (Leica DFC495, Leica Microsystems GmbH, Wetzlar, Germany). The stained area was segmented by colour thresholding normalised with negative control, and expressed as percentage (%) area fraction averaged from three separate images for comparison.

Mechanical test

A four-point bending test was performed at week 8 post-fracture. A separate batch of 32 rats ($n = 8$ per group) were euthanised at week 8 post-treatment with the whole femur dissected free from soft tissue. A material testing machine (H25KS Hounsfield Test Equipment Ltd, Redhill, UK) was used to test the femora to failure loaded in the anterior-posterior direction and a constant displacement of 5 mm/min according to established protocol (Cheung *et al.*, 2012a; Shi *et al.*, 2010). Ultimate load, stiffness and energy to failure were recorded for further analysis.

Statistical analysis

All quantitative data were expressed as mean \pm standard deviation (SD) and analysed with SPSS version 20.0 software (IBM, Armonk, NY, USA). One-way analysis of variance (ANOVA) with Tukey's *post-hoc* test were used for two-group or multiple-group comparisons, respectively. Statistical significance was set at $p < 0.05$.

Results

Clinical observations

Apparent bone mineral density (BMD) drop in the ovariectomised groups was confirmed in 5 random rats by μ CT to be 396.4 ± 57 to 250.6 ± 49 mgHA/ccm ($p = 0.0000287$). All fractured rats in all groups returned to normal locomotion within the first week of fracture creation. All rats in control and treatment groups progressed to complete healing with no case of non-union observed. Twelve rats (7.9 %) resulted in comminuted fractures after creation and were immediately euthanised, excluded from the study and later replaced. All rats receiving LMHFV treatment showed signs of relaxation by self-grooming behaviour, but no signs of depression or excessive weight loss.

Weekly radiography

Callus morphometry by X-ray (Fig. 1) expressed in width and area are summarised in Fig. 2 and Fig. 3. In general, callus width was shown to be significantly higher in the VMG at week 2 ($p = 0.001$ vs. VAMG group, and $p < 0.0005$ with both MG and CG groups) and week 3 ($p = 0.012$ vs. VAMG group, $p = 0.017$ vs. MG group, and $p = 0.019$ vs. CG group) as compared to all other groups. The VAMG group showed no significant difference with the CG and MG groups. Callus width decreased more rapidly in the VMG group compared to all other groups at week 7 ($p = 0.007$ vs. MG group, and $p = 0.005$ vs. CG group) and week 8 ($p = 0.002$ vs. MG group, and $p = 0.001$ vs. CG group) with significant difference. Similar results were observed for the callus area.

Ex-vivo GFP signal intensity

Qualitatively, the signal intensity was highest in the VMG at an early time point of 2 weeks compared to other groups, and the increased signal was not detected in the VAMG group. Blue circle with uniform diameter indicates the ROI

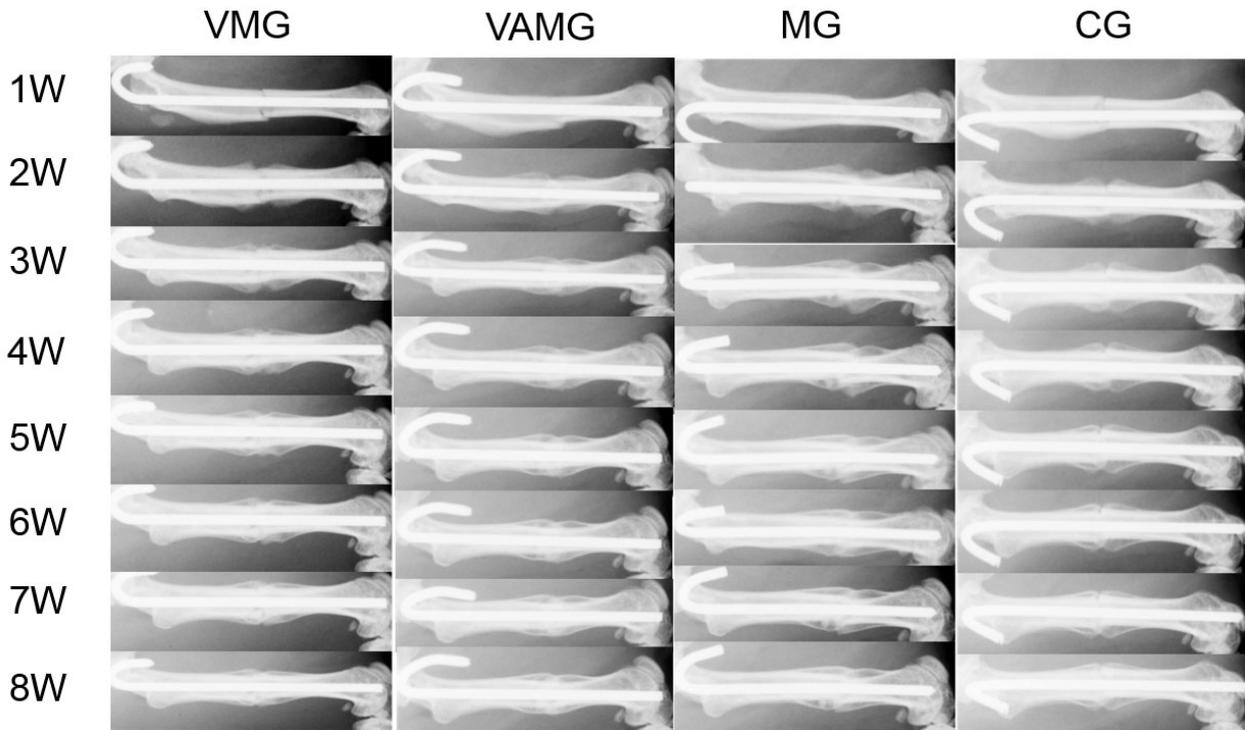


Fig. 1. Serial X-ray. Representative X-ray images showing the healing process in each group. In general, the VMG group showed earlier bridge at week 3 to week 4 with greater callus size detected at earlier time-points followed by a more rapid decrease in callus size at later time-points. VMG, MG and CG groups showed similar trends throughout the observed period of 8 weeks.

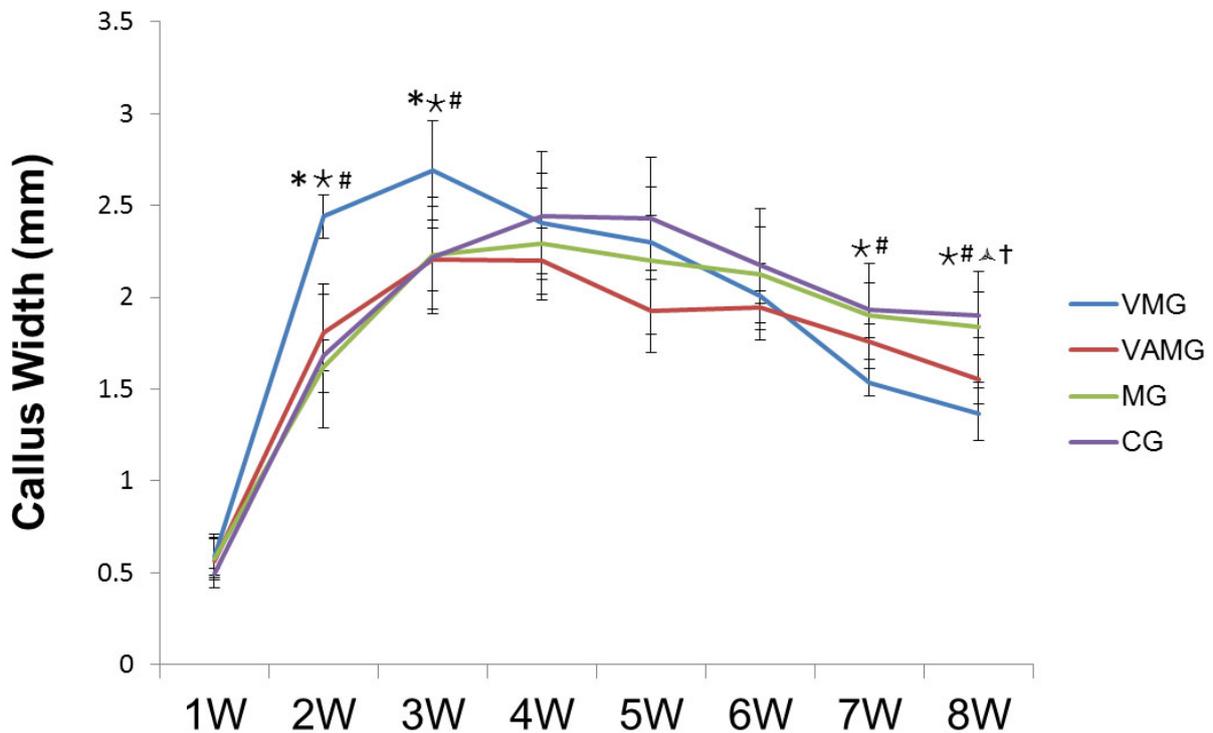


Fig. 2. Callus width. Callus width was found significantly enhanced by both LMHFV treatment along with exogenous MSC injection in the VMG group at week 2 to 3. The enhancement effect was suppressed by AMD3100 as shown in the VAMG group. Sample size of $n = 10$ per group per time-point with one-way ANOVA adjusted by Tukey's *post-hoc* test at $p < 0.05$ considered statistically significant. * $p < 0.05$ between VMG & VAMG; * $p < 0.05$ between VMG & MG; # $p < 0.05$ between VMG & CG; ^ $p < 0.05$ between VAMG & MG; † $p < 0.05$ between VAMG & CG.

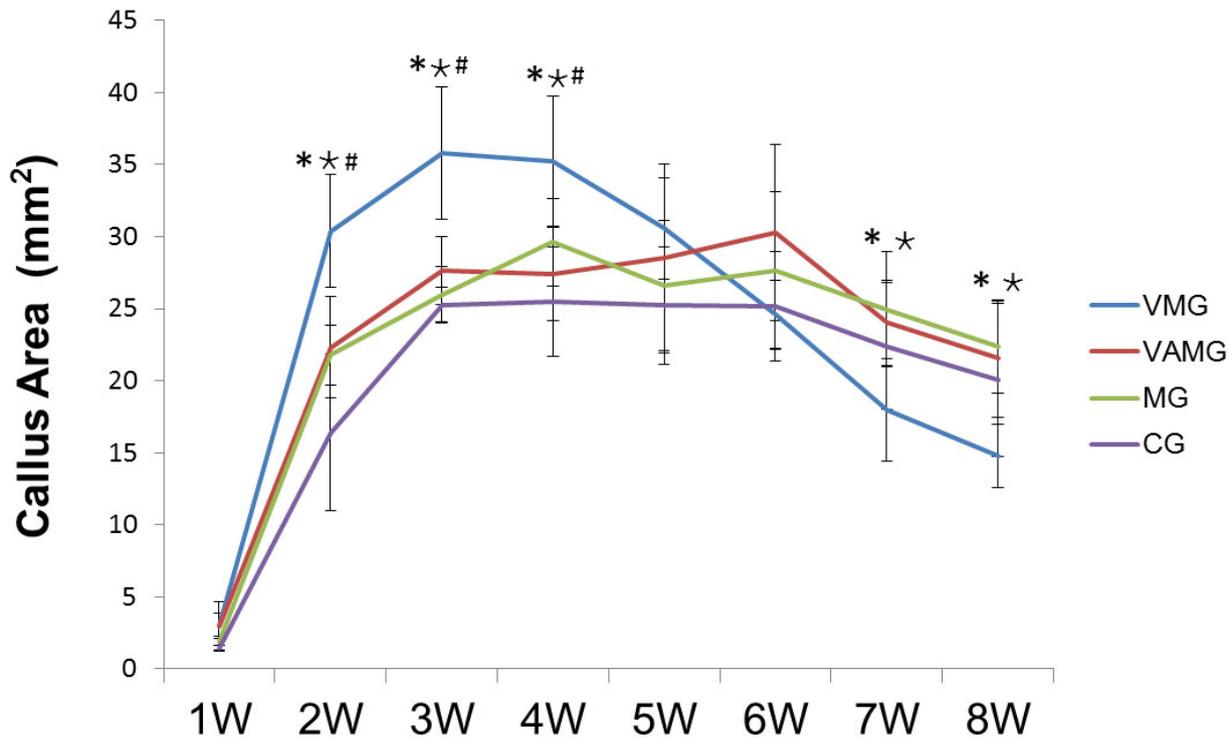


Fig. 3. Callus area. Callus area was also found significantly enhanced by both LMHFV treatment along with exogenous MSC injection in the VMG group at week 2 to 4. The enhancement effect was suppressed by AMD3100 as shown in the VAMG group. Sample size of $n = 10$ per group per time-point with one-way ANOVA adjusted by Tukey's *post-hoc* test at $p < 0.05$ considered statistically significant. * $p < 0.05$ between VMG & VAMG; * $p < 0.05$ between VMG & MG; # $p < 0.05$ between VMG & CG.

selected for signal quantification (Fig. 4). The *ex vivo* GFP intensity in the VMG was significantly higher than those in the VAMG ($p = 4.7 \times 10^{-5}$), MG ($p = 8.5 \times 10^{-5}$) and CG ($p < 5.0 \times 10^{-7}$) at week 2. No significant difference was detected between MG and CG at week 2 ($p = 0.062$). No significant difference was detected at week 4 to week 8. (Fig. 5).

μ CT analysis

From the 3D reconstruction images, the callus volume in the VMG group was found to be greatest at week 4 compared to the VAMG, MG and CG groups. It was also found that the decrease in callus size was fastest in the VMG group as compared to all other groups (Fig. 6). Mean tissue volume (TV) in the VMG was significantly larger than the VAMG ($p = 0.006$), MG ($p < 0.005$) and CG ($p < 0.005$) groups at week 2, and significantly larger than VAMG ($p = 0.023$) and CG ($p = 0.027$) at week 4 post-fracture (Fig. 7A). Mean bone volume (BV) in the VMG group was significantly larger than those in the MG ($p = 0.006$) and CG ($p = 0.031$) groups at week 2, and higher compared with the VAMG group ($p = 0.001$) at week 4 post-fracture (Fig. 7B). Mean high-density bone volume (BV_h) in VMG was significantly higher than MG ($p = 0.028$) and CG ($p = 0.015$) at week 2, and higher than MG ($p = 0.044$) at week 8 post-fracture (Fig. 7C). Mean BV_h/TV in VMG was significantly higher than VAMG ($p < 0.005$), MG ($p < 0.005$) and CG ($p < 0.005$) at week 8 post-fracture (Fig. 7D). Mean total bone volume fraction (BV/TV) in the VMG group was significantly

higher than the VAMG ($p < 0.005$), MG ($p < 0.005$) and CG ($p < 0.005$) groups at week 8 post-fracture (Fig. 7E). Mean bone mineral density (BMD) in the VMG group was significantly higher than in the VAMG ($p < 0.005$), MG ($p < 0.005$) and CG ($p < 0.005$) groups at week 8 post-fracture (Fig. 7F).

GFP immunohistochemistry

GFP was detected at the fracture callus in all groups, with the strongest signals at the intramedullary canal within the remodelling fracture callus and site where endochondral ossification occurred (Fig. 8A-C). Mean area fraction of GFP by IHC showed a significantly higher signal detected in the VMG group against all other groups at week 2 ($p < 0.005$), and no significant difference was detected between VAMG and MG groups ($p = 0.834$). Likewise, a slightly higher signal was detected in the VMG group at week 4 against all other groups ($p < 0.05$) and no significant difference was detected among all other groups. At week 8, no significant difference was detected between VMG and VAMG groups (Fig. 9).

Mechanical test

Four-point bending results at 8-week post-fracture indicated that the ultimate load, stiffness and energy to failure were generally higher in the VMG group when compared with other groups. Statistically significant higher stiffness was detected in the VMG group compared with VAMG, MG and CG groups ($p = 0.050$, $p < 0.0005$ and $p = 0.001$, respectively). No significant difference was

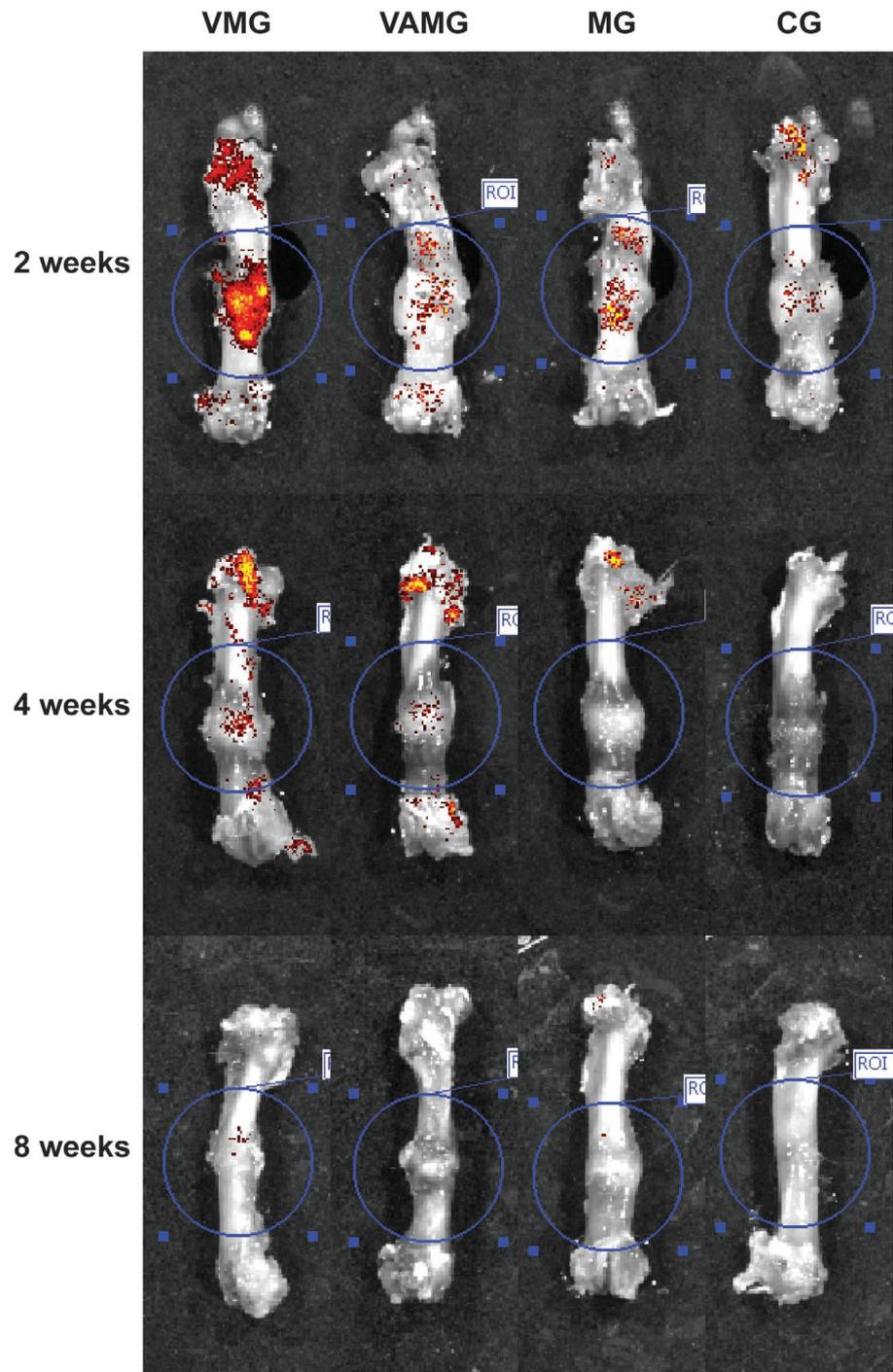


Fig. 4. Overview of *ex vivo* fluorescence imaging of GFP signal intensity. Representative images of whole femur specimens in all groups shown at all time-points. In general, the signal intensity was highest in the VMG group at an early time point of 2 weeks compared to other groups, and the increased signal was not detected in the VAMG group. Blue circle with uniform diameter indicates the ROI selected for signal quantification.

found between the VAMG, MG and CG groups in all of the mechanical properties tested (Table 2).

Discussion and Conclusion

This study aims to investigate the effect of LMHFV on MSC recruitment to the diaphyseal fracture site and the role of the SDF-1/CXCR4 signalling pathway, where diaphyseal fracture healing occurs predominately by the process of endochondral ossification. The results in this study reconfirmed the capacity of LMHFV, in combination with transplanted MSCs, to enhance fracture healing in osteoporotic bone (Chow *et al.*, 2011; Chung *et al.*, 2014; Shi *et al.*, 2010). In this study, MSC recruitment is the

Table 2. End-point mechanical test results in all groups.

	Ultimate load (N)	Stiffness (N/mm)	Energy to fail (N·mm)
	Mean ± SD	Mean ± SD	Mean ± SD
VMG	144.83 ± 59.54	182.28 ± 61.97 ^(2,3,4)	0.0519 ± 0.0274
VAMG	118.98 ± 75.61	107.62 ± 40.53 ⁽¹⁾	0.0365 ± 0.0213
MG	91.03 ± 20.57	41.32 ± 34.99 ⁽¹⁾	0.0354 ± 0.0174
CG	81.38 ± 37.72	53.02 ± 33.00 ⁽¹⁾	0.0302 ± 0.0231

Superscript of ^(1, 2, 3, 4) represent Bonferroni *post-hoc* test with significant difference ($p < 0.05$) compared with VMG, VAMG, MG and CG, respectively

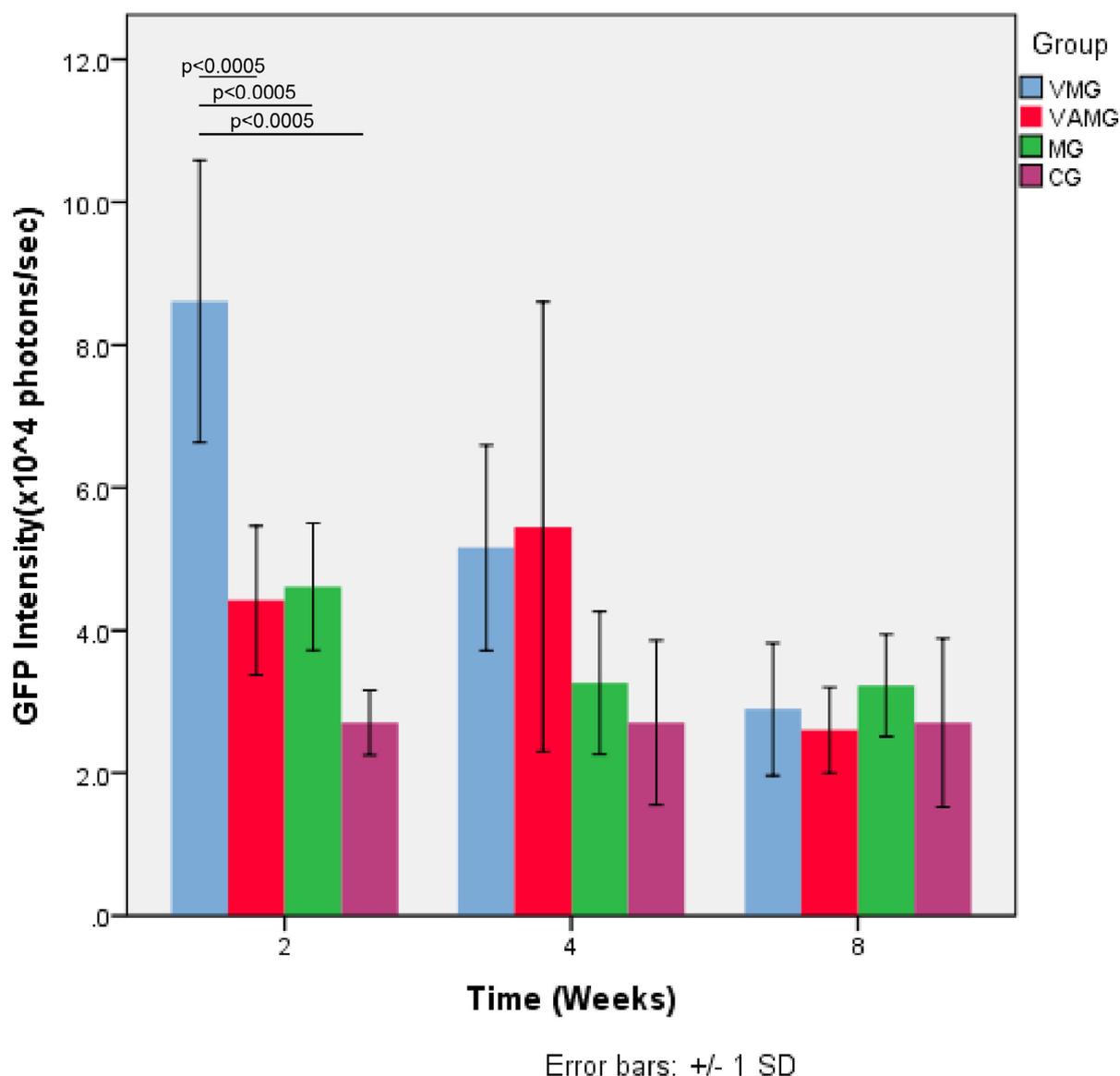


Fig. 5. Quantification of GFP signal intensity. MSC recruitment was found to be highest by GFP signal at week 2 to week 4 in the VMG group; and significantly smaller in the VAMG group with no statistical significant difference from the MG group.

focus and is demonstrated to be enhanced in response to the treatment of LMHFV through the SDF-1/CXCR4 signalling pathway. No fracture non-union has been observed and all fracture models progressed to union at end-point of the study in all groups.

In the process of fracture healing, SDF-1 was reported to modulate the homing and engraftment of circulating MSCs by binding to its receptor CXCR4, thus expanding the pool of osteogenic precursor cells in the fracture site (Wei *et al.*, 2014). In this study, GFP-labelled MSCs at the fracture site were shown to be enhanced at week 2 post fracture in the VMG group when compared to the MG and CG groups, which confirmed the effectiveness of LMHFV treatment in recruiting MSCs to the fracture site in osteoporotic bone. The VMG, VAMG and MG groups showed a 3.18 \times , 1.63 \times and 1.70 \times increase in *ex vivo* GFP signals, respectively, when compared to the CG group. Upon the addition of the CXCR4 antagonist (AMD3100), the enhancement effect of LMHFV on the VAMG group

immediately dropped from 3.18 \times to 1.63 \times ; which is also confirmed by immunohistochemistry of GFP at the fracture site to show a consistent trend. This observation indicates the importance that SDF-1/CXCR4 played in recruitment of MSCs to the fracture site in response to LMHFV. LMHFV might have achieved this by increasing the expression and secretion of SDF-1 at the fracture site, as SDF-1 is expressed and secreted by several cell types including osteoblasts, fibroblasts and endothelial cells (Jacobs *et al.*, 2010). Moreover, a similar *in vivo* study in mice by Leucht *et al.* (2013) demonstrated that application of strain would enhance SDF-1 expression in osteoblasts and osteocytes and the expression of CXCR4 in marrow MSCs, and thus concluded the role played by MSCs in mechanoadaptation of bone. Also, the current finding is consistent with our previous report regarding the *in vitro* up-regulation of SDF-1 and CXCR4 mRNA expression, as well as the release of SDF-1 in the culture medium after treating MSCs with another form of mechanical

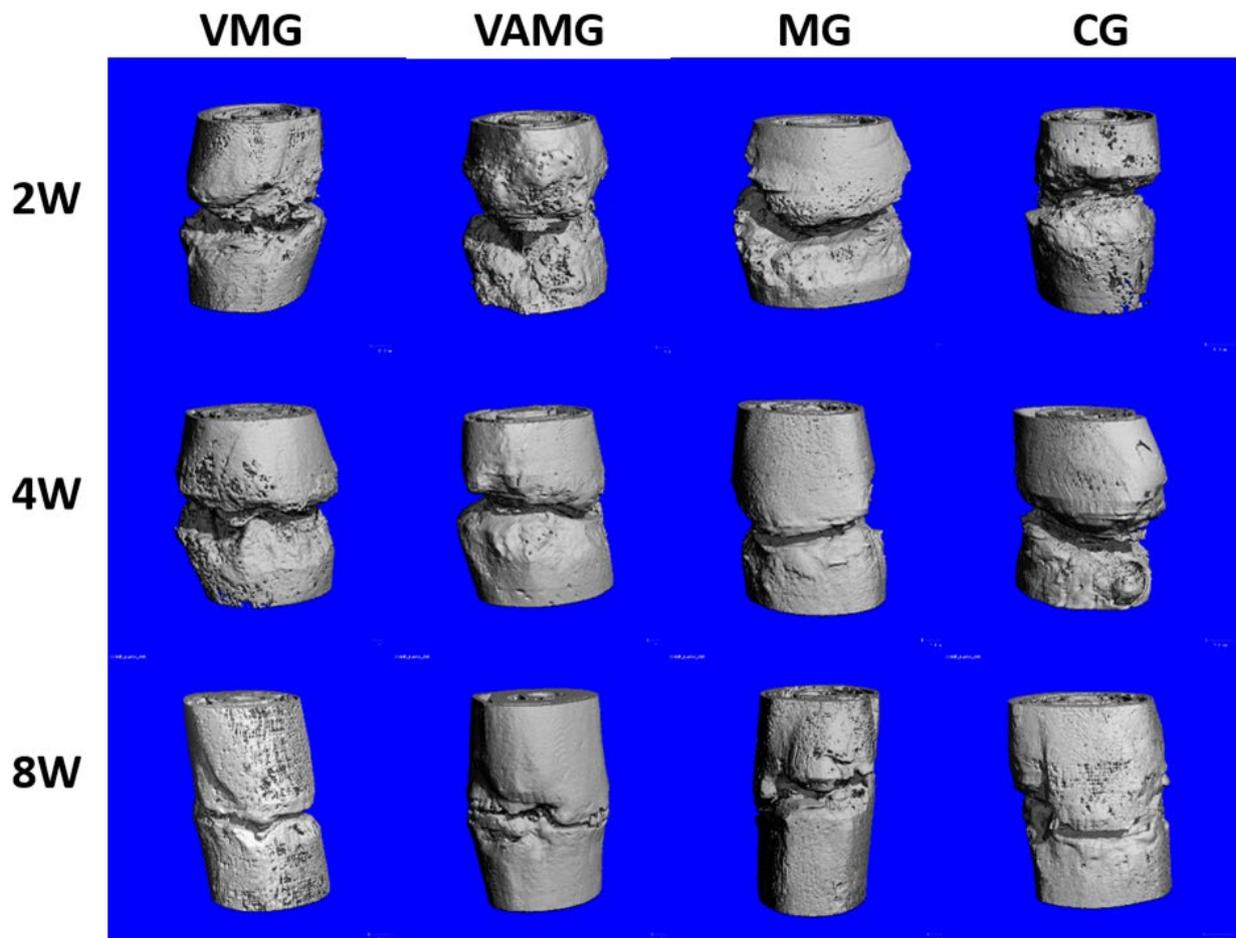


Fig. 6. μ CT reconstruction. Representative 3-dimensional reconstructed images of the fracture callus. In general, the callus volume in the VMG group was found to be greatest at 4 weeks compared to the VAMG, MG and CG groups. It was also found that the decrease in callus size was fastest in the VMG group and compared to all other groups.

stimulation, low intensity pulsed ultrasound (LIPUS) (Wei *et al.*, 2014). Moreover, recent reports have demonstrated similar findings that transplantation with or without overexpression of SDF-1 would both improve fracture healing (Ho *et al.*, 2015; Rapp *et al.*, 2015). Therefore, much like other forms of mechanical stimulation, LMHFV was shown to be capable of enhancing MSC recruitment to the fracture site; and the SDF-1/CXCR4 signalling pathway was essential during the process.

LMHFV treatment was also found to be capable of improving blood circulation and neo-angiogenesis (Cheung *et al.*, 2012b). In this study, exogenous MSCs were injected directly into the circulation, and treatment of LMHFV was proven capable of increasing blood circulation. Thus, it is not surprising that augmentation in blood circulation and angiogenesis may have played an important role in providing the required vessels for the successful recruitment of circulating MSCs. These two mechanisms may work synergistically during the augmentation by LMHFV, as it was reported by Kasper *et al.* (2007) that the *in vitro* mechanical loading of MSC would result in a paracrine stimulation of angiogenesis, possibly through the fibroblast growth factor receptor 1 (FGFR1) signalling pathway or the vascular endothelial growth factor receptor (VEGFR) signalling cascade. In this study, enhanced MSC recruitment was observed at week

2 to week 4 in the LMHFV-treated group, as compared to the observed enhancement in angiogenesis at week 2 to week 8 (Cheung *et al.*, 2012b) at the fracture site. This suggests a relationship between the two mechanisms that may work either independently or synergistically to achieve the enhancement effects, which requires further investigation to answer.

Subsequently, the enhancement effect of MSC recruitment by LMHFV in osteoporotic fracture healing was reflected in callus formation and remodelling parameters. As compared to classical stages of fracture healing (Einhorn, 1998; Schindeler *et al.*, 2008), after LMHFV treatment was given to osteoporotic fractured bone, callus size generally showed more rapid increase in the soft callus formation stage at early time-points and a more rapid remodelling stage at later time-points. As shown by the callus size and μ CT data, the VMG group had significant increase in callus width and area at early time points, increased high-density bone volume fraction at later time points (Fig. 7, week 8) and subsequently accelerating the remodelling process leading to faster healing as shown by the faster decrease in callus volume (Fig. 7). Ultimately, the enhancement in callus formation and remodelling in the VMG group was manifested by the end-point mechanical properties in stiffness (resistance to deformation) *versus* all other groups (Table 2). Since mechanical stimulation

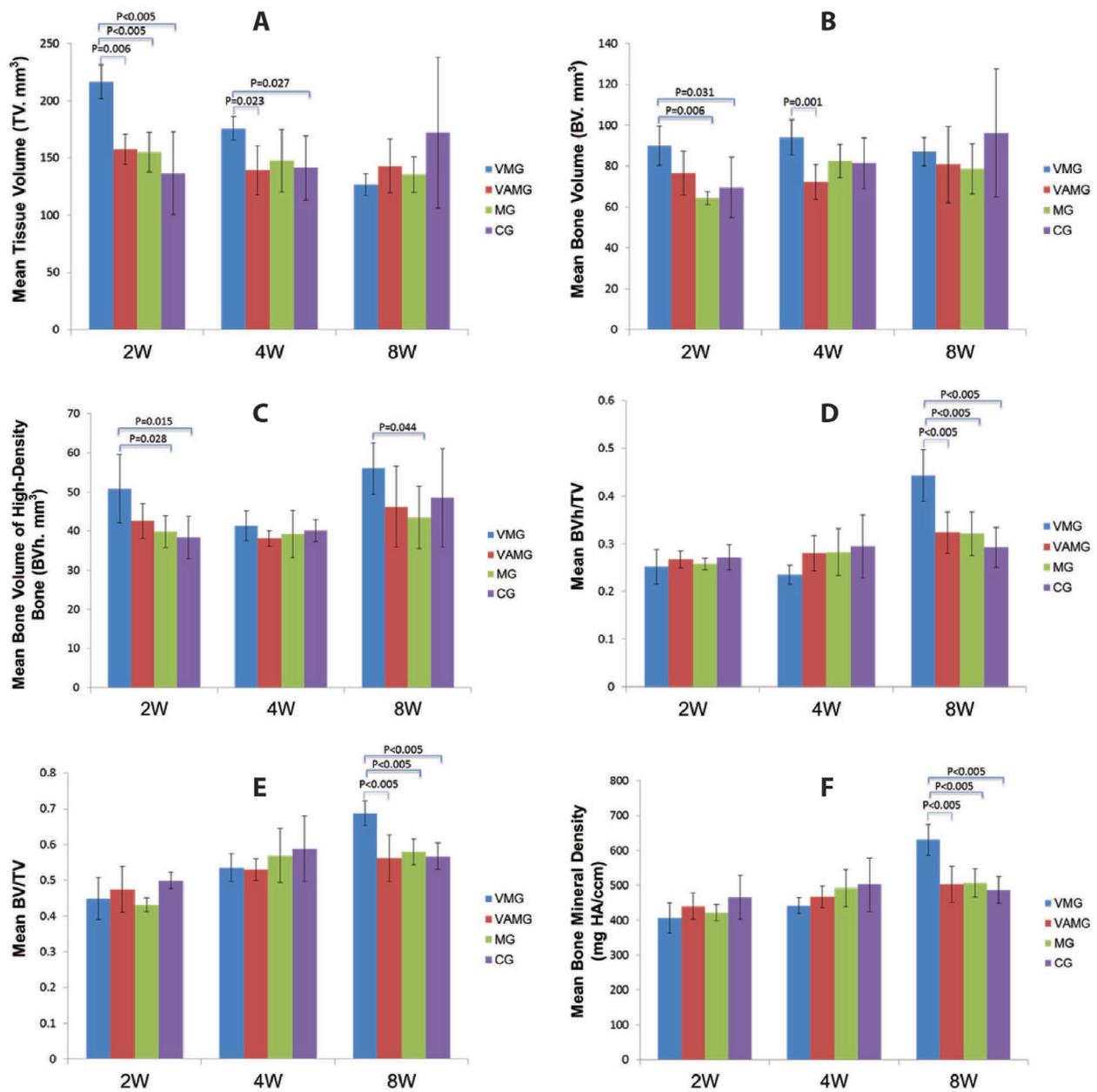


Fig. 7. (A) Mean tissue volume (TV). TV was the highest in the VMG group at week 2 to week 4 with statistical significance. No difference was detected in the VAMG group against MG or CG group. (B) Mean bone volume (BV). BV was the highest in the VMG group at week 2 to week 4 with statistical significance. No difference was detected in the VAMG group against MG or CG group. (C) Mean high density bone volume (BV_h). BV_h was the highest in the VMG group at week 2 against MG and CG groups, and at week 8 against the MG group with statistical significance. No difference was detected in the VAMG group against MG or CG group. (D) Mean high density bone volume fraction (BV_h/TV). BV_h/TV was the highest in the VMG group at week 8 against all groups with statistical significance. (E) Mean total bone volume fraction (BV/TV). BV/TV was the highest in the VMG group at week 8 against all groups with statistical significance. (F) Mean bone mineral density was found to be higher in the VMG group compared to the other groups with significant difference. Sample size of $n = 10$ per group per time-point with one-way ANOVA adjusted by Tukey's *post-hoc* test at $p < 0.05$ considered statistically significant.

may promote MSCs to differentiate into different cell types, including adipocytes, osteoblasts and chondrocytes, the results of this study suggests that LMHFV treatment might be capable of promoting differentiation of MSCs at the fracture site to osteogenic cell types (Qi *et al.*, 2008) that contribute to the enhanced process of callus formation, callus remodelling and ultimately improved mechanical properties.

Although it was observed that the GFP signal intensity at the fracture site was apparently higher in the injection-only MG group at week 2 compared to the control CG group without significant difference, there were no significant enhancement in callus morphometry compared to the CG group in other assessed parameters. This indicates that the injection of exogenous MSCs alone did not provide an additional enhancement effect in callus formation in

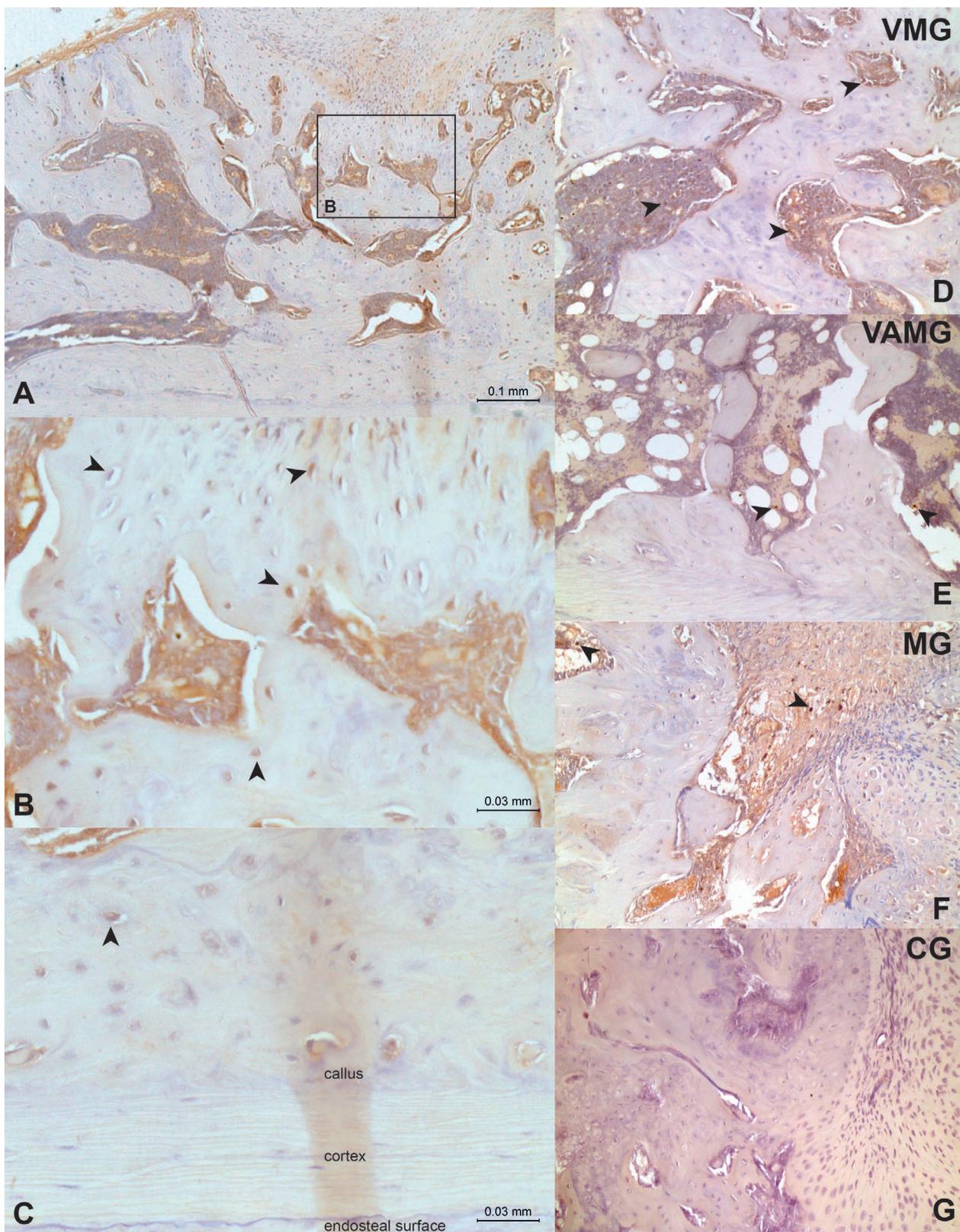


Fig. 8. GFP immunohistochemistry (representative images at 4 weeks post-fracture). (A) Showing positively stained GFP signal detected at the fracture callus counter-stained with haematoxylin captured under a 10× objective. (B) Enlarged area (at 40×) within mineralised callus showing positive signals detected at osteocyte-like cells from within their lacunae (with arrows), and osteoblast-like cells on mineralised surfaces. (C) Positively stained osteocyte-like cells in contrast with unstained osteocytes in the original cortex and the endosteal surface near the fracture site. (D-G) Positively stained cells were apparently found in the VMG group > VAMG and MG groups > CG group, captured at 20× at the callus undergoing mineralisation.

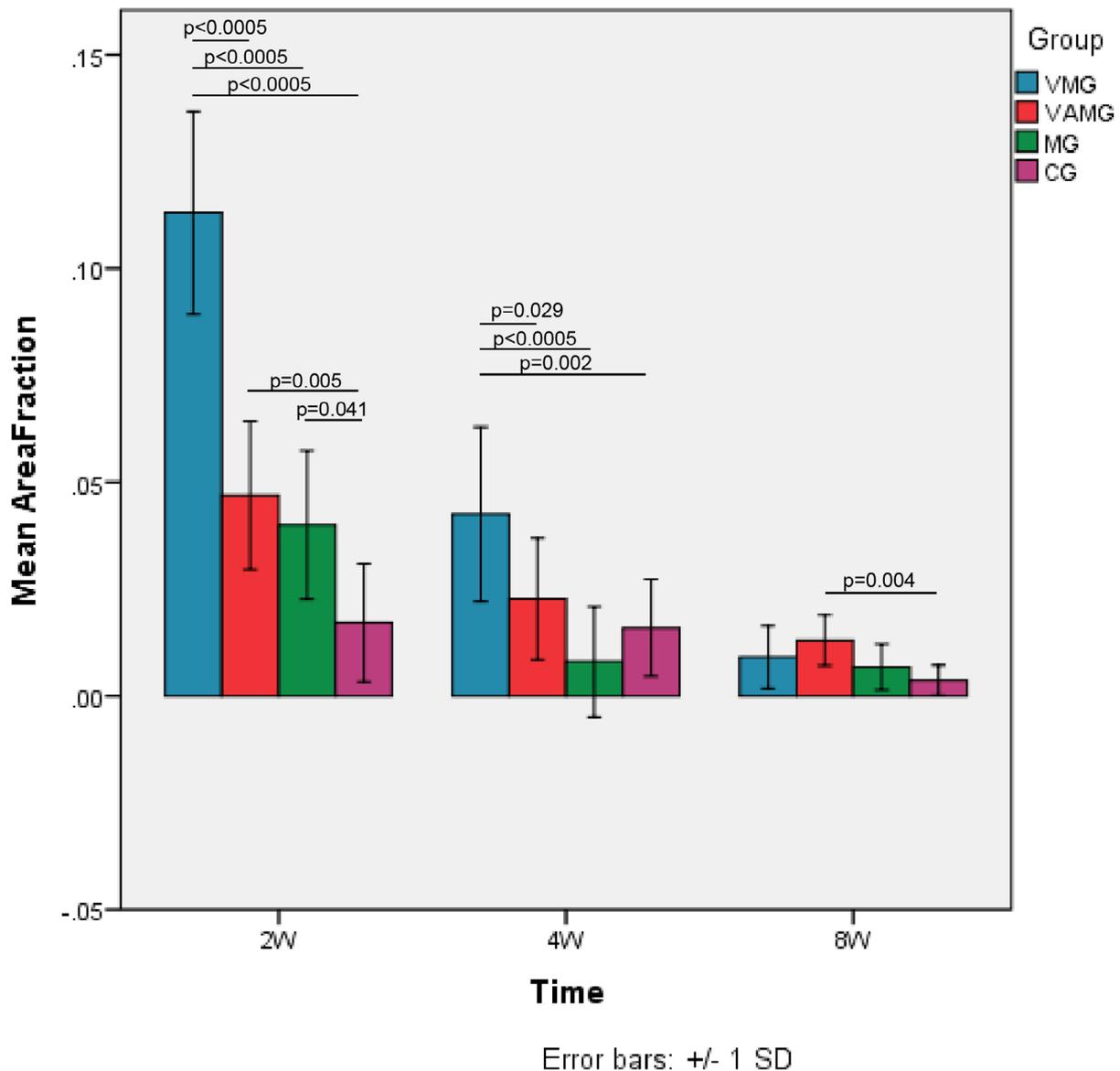


Fig. 9. Quantitative immunohistochemistry of GFP at the fracture site. GFP-labelled MSCs were found to be higher in concentration at the fracture site in the VMG group *versus* the SDF-1/CXCR4 blocked VAMG group. The result is demonstrated by higher positive signals detected in the VMG group at week 2 and 4 *versus* all groups ($p < 0.0005$, $p = 0.029$, respectively) and no difference was detected between VMG and MG groups at week 2 and week 4 ($p = 0.834$, $p = 0.149$, respectively).

osteoporotic fracture by simply increasing the amount of circulating MSCs. This observation is not surprising as our previous empirical results have indicated that enhancement in fracture healing would only occur when injected MSC number reached 4.0×10^6 in the same rat fracture model (Cheung *et al.*, 2013). Moreover, it has been previously reported by Furlani *et al.* (2009) that the injection of 3.0×10^6 exogenous MSCs in mice would not enhance cardiac functions. Our experimental design was intended to introduce a small reporter signal and thus injected only a relatively smaller amount of 1.0×10^6 isolated MSCs in the rat models (Cheung *et al.*, 2013; Nagaya *et al.*, 2005; Wei *et al.*, 2014), thus confirming that MSC recruitment was enhanced by LMHFV.

Mechanical stimulation in the form of LMHFV and LIPUS are both promising in providing adequate

stimulatory signals to achieve enhancements in fracture healing. In contrast to the systemic whole body vibration provided by LMHFV, LIPUS belongs to the type of local mechanical stimulation treated directly superficial to the fracture site. In our previous study on rat fracture healing augmented with LIPUS treatment, the *ex vivo* GFP signal intensity in the LIPUS treatment group also showed significant increase ($2.66\times$) in signal strength compared to the control group (Wei *et al.*, 2014). The enhancement in MSC recruitment was translated to comparable enhancement effects in callus formation capacity, at around 26.8 % to 35 % increase in callus size during week 1 to 3 in 8-week old rat fractures. Moreover, upon the addition of the AMD3100 in the modulation group, the enhancement effect of LIPUS showed similar abolishment as shown in this study, indicating that both types of mechanical

stimulation achieved the enhancement effects in bone healing through the SDF-1/CXCR4 pathway. Although the effect of AMD3100 on fracture healing might depend on the administration scheme of various doses, time and duration (Toupadakis *et al.*, 2013; Toupadakis *et al.*, 2012), the present findings are in line with these reports indicating that a sustained blockade of CXCR4 reduced callus formation and maturation. These *in vivo* studies together convey strong evidence to support the important role of the SDF-1/CXCR4 pathway in the recruitment of MSCs to the fracture site augmented by mechanical stimulation. Moreover, LIPUS also showed an *in vitro* positive effect on enhancing expression of SDF-1 and CXCR4 in MSCs, suggesting that a similar effect can be achieved by LMHFV. As it was also shown by another study that the application of low magnitude vibration treatment at 90 Hz would increase the MSC cell population in long bone marrow (Luu *et al.*, 2009), the effect of LMHFV on the expression of SDF-1 and CXCR4 in MSCs and various cell types needs further investigation to be confirmed.

It is demonstrated that exogenously injected MSCs are recruited to the fracture site through the SDF-1/CXCR4 pathway and the blockade of this pathway would suppress the enhancement effect brought about by mechanical stimulation. However, circulating MSCs only represent a small population of reparative MSCs which would normally reside as a majority in bone marrow (da Silva Meirelles *et al.*, 2006; Margulies *et al.*, 2015; Young *et al.*, 1995). Thus, our model was only able to demonstrate the recruitment of circulating MSCs to the fracture site and assumed that bone marrow MSCs were also recruited to the injury site in close proximity. Another limitation is the lack of an AMD3100-only modulation group in this study design, as this agent was only used as a tool to investigate the role of the SDF-1/CXCR4 signalling pathway in the mechanically stimulated fracture group, and its sole effects on similar models without mechanical stimulation were previously well reported (Leucht *et al.*, 2013; Toupadakis *et al.*, 2013; Toupadakis *et al.*, 2012). Also, future experiments on the expression of SDF-1/CXCR4 at the fracture site might provide additional insight to reveal the effect of LMHFV on resident MSC recruitment.

In conclusion, all rats in all groups progressed to complete healing. It was found that LMHFV enhanced exogenous injected MSC recruitment in osteoporotic diaphyseal fracture healing that led to enhancement in fracture healing in terms of callus formation, remodelling and improved mechanical properties. It was also shown that SDF-1/CXCR4 was essential for the enhancement effect in MSC recruitment provided by LMHFV. Our results suggest the possible clinical application LMHFV in combination with MSC treatments for osteoporotic fracture healing.

Acknowledgements

This project was supported by General Research Fund (GRF) of the University Grant Council, Government of The Hong Kong Special Administrative Region (Reference: 462811) and partially by the National Natural Science Foundation of China (NSFC) (Reference: A.03.15.02401).

Study design (Cheung, Leung, Li), data collection (Wei, Chow, Qin, Guo, Yu), data analysis (Wei, Chow, Yu, Cheung), writing (Wei, Chow, Cheung), supervision (Cheung, Leung). All authors have read and approved the final submitted manuscript. We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Discussion with Reviewers

Reviewer I: Can the authors speculate about a homing of the injected cells in other organs? Would intra-cardiac injection of MSCs also be the way for clinical application?

Authors: Systemic stem cell homing to treat a variety of diseases and damaged areas has been well reported in a number of original and review articles before (*e.g.* Chen *et al.*, 2010; Khaldoyanidi, 2008). Stem cells are mostly keen to migrate to defect sites, rather than other non-injured organs. Our study did not look into the migration of injected cells to other organs, because our colleagues had already reported this before (Huang *et al.*, 2015). In the study, they used a mouse femoral fracture model administered systemically with labelled MSCs through intra-cardiac injection and traced the fate of MSCs by *in vivo* bioluminescent imaging (a very similar approach to ours). Their findings confirm that the injected MSCs mainly went to the fracture site to promote the healing but rarely migrated to other organs. Therefore, we are confident that injected stem cells were specifically going to the fracture site (augmented by mechanical stimulation) but not the other organs. Intra-cardiac injection of MSCs was chosen in this study instead of a tail vein approach, because our preliminary evaluation indicated that the cells injected through the tail vein were more trapped in the lungs, as compared with the cardiac injection approach. However, we do not recommend the intra-cardiac approach for clinical practice, as this is relatively risky. In the rat model, we have not much choice (either tail vein or cardiac) as the animal is small; in the human, there are many more alternatives because human veins or arteries are big enough for injection, depending on the fracture site. For example, if the fracture site is at the femur, we recommend the MSC

injection at the femoral artery and most MSCs should go to the fracture site.

Reviewer III: It would have been interesting to see comparison between the VMG (LMHVF + MSCs) compared to LMHVF alone. The study design did, however, not contain the latter group. Do the authors suspect that the additional application of the MSCs does have a positive impact on fracture healing in combination with LMHVF?

Authors: We admit that injected MSCs may have positive effect on fracture healing. Our previous study (Cheung *et al.*, 2013) confirmed this conclusion, but please note that the dose of injection was 4×10^6 MSCs in order to give a positive effect. Therefore, we are careful in this study to use 1×10^6 MSCs only to minimise the fracture enhancement effect but emphasise the cell recruitment only. In this study, although we did not have a LMHFV alone group, we can compare MG and CG instead to look into the effect of MSCs on fracture healing (should be equivalent to LMHFV+MSCs vs. LMHFV only). All the related results (X-ray, CT and mechanical test) did not show any significant difference between MG and CG. Therefore, we believe that the MSC injection dose is good enough to demonstrate recruitment effect but not fracture enhancement effect in this study. In summary, we make use of injected MSCs to mainly look into the MSC recruitment effect of LMHFV to fracture site in this study. However, we cannot exclude the possibility of a combined enhancement potential of LMHFV and MSCs, provided a sufficiently high dose of MSCs is used, as proven in our previous study.

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Editor's Note: Scientific Editor in charge of the paper: Juerg Gasser.