Low-Magnitude High-Frequency Vibration Accelerates Callus Formation, Mineralization, and Fracture Healing in Rats

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ABSTRACT: Fracture healing is a biological regenerative process that follows a well-orchestrated sequence. Most healing is uneventful and enhancement of normal fracture healing is not commonly done, although it is clinically important in the recovery and regain of functions after fracture. This study investigated the osteogenic effect of low-magnitude high-frequency vibration (LMHFV, 35 Hz, 0.3 g) on the enhancement of fracture healing in rats with closed femoral shaft fracture by comparing with sham-treated control. Assessments with plain radiography, micro-CT as well as histomorphometry showed that the amount of callus was significantly larger (p = 0.001 for callus area, 2 weeks posttreatment); the remodeling of the callus into mature bone was significantly faster (p = 0.039, 4 weeks posttreatment) in the treatment group. The mechanical strength of the healed fracture in the treatment group at 4 weeks was significantly greater (p < 0.001). The results showed the acceleration of callus formation, mineralization, and fracture healing in the treatment group. It is concluded that LMHFV enhances healing in the closed femoral shaft fracture in rats. The potential clinical advantages shall be confirmed in the subsequent clinical trials. © Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: low-magnitude high-frequency vibration; fracture healing; callus; femur; remodeling

Fracture healing is a biological regenerative process that follows a well-orchestrated sequence. Most fracture healing is uneventful because it is a very mature biological process with multiple backup mechanisms and alternative pathways. However, complications do occur like delayed unions and nonunions (with an incidence up to 10%). Therefore, enhancement of fracture healing has been one of the major goals in modern fracture management because it is clinically important for the recovery and regain of functions after fracture. The biological environment for osteogenic potential, influenced by blood supply, bone-forming cells, growth factors, etc., as well as the mechanical environment are the key determinants for the enhancement of the fracture healing. Proper loading conditions have been proven crucial for bone repair and remodeling, but the optimal timing and amount of mechanical stimulation remain inconclusive. Low-magnitude high-frequency vibration (LMHFV) is a form of biophysical intervention that provides cyclic loadings. Its osteogenic potential has been proven in animal models and clinical trials, with positive effects shown on bone mineral density (BMD), blood circulation, muscle functions, and balance control. Although the detailed cellular mechanism and molecular pathways remain to be explored, this biophysical stimulation does provide noninvasive and systemic effects on musculoskeletal system and its osteogenic effect is promising.

Because fracture healing is a regenerative process of osseous tissue, the osteogenic property of LMHFV may also be effective to enhance the biological process.

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compartmented cages fixed on the vibration platform for 20 min/day, 5 days/week. The walls of the cages were opaque and painted in black to minimize the surrounding disturbance. The control group had sham treatment with the rats standing on the vibration platform without switching on the machine using the same regime. At 1, 2, and 4 weeks posttreatment, six rats from each group were euthanized using overdose of sodium pentobarbital and the femora were immediately harvested for the micro-CT and histomorphometric assessments. An additional eight rats from each group were euthanized for mechanical testing at 4 weeks posttreatment.

The transmission of the vibration from paws to thigh in rats was confirmed by using a uniaxial accelerometer (Model 3115A, Dytran Instruments, Chatsworth, CA) attached on the skin surface on the middle of the right thigh in our pilot study. The electrical signals were acquired by the data acquisition system (S/R 500 Hz, Model DI-220, USA). Constant vibration signals (35 Hz) were detected during vibration therapy. Also, the possible effects of sound, magnetic field, and heat produced by the customized vibration platform were assessed. During vibration, the noise produced by the platform was minimal. The intensity of the static magnetic field generated by the vibration, the noise produced by the platform was minimal. The control group had sham treatment with the rats standing on the platform without switching on the machine using the same regime. At 1, 2, and 4 weeks posttreatment, six rats from each group were euthanized using overdose of sodium pentobarbital and the femora were immediately harvested for the micro-CT and histomorphometric assessments. An additional eight rats from each group were euthanized for mechanical testing at 4 weeks posttreatment.

The wall of the cages was regularly used as the magnitude of the vibration (g)

\[ g = \frac{n}{6} \] for each group at each time point were scanned by micro-CT system (μCT-40, Scanco Medical, Brütisellen, Switzerland). The scan range covered 5 mm proximal and 5 mm distal to the fracture line with a resolution of 16 μm per voxel and 1024 × 1024 pixel image matrices. Contoured region of interest (ROI) was selected in two-dimensional (2D) CT images. Three-dimensional (3D) reconstruction of mineralized tissue was performed using a low-pass Gaussian filter (Sigma = 1.2, Support = 2). To differentiate newly formed mineralized callus from old cortices, the low- and high-density mineralized tissues were reconstructed using different thresholds (high attenuation = 325, low attenuation = 120) defined in 2D images using our recently established evaluation protocol. \[ 42,43 \] The high-density tissues represented old cortices and newly formed, highly mineralized callus, whereas the low-density tissues represented newly formed callus. \[ 42,43 \] Quantitative analysis was performed covering the mid 300 slices of the 2D images with the low- and high-density mineralized tissues evaluated separately. Morphometric parameters used for evaluation were total tissue volume (TV, mm³), volume of high-density bone (BVh, mm³), volume of low-density bone (BVl, mm³), total bone volume (BVv, mm³), i.e., equivalent to BVh + BVl or TV—interstitial space and normalized percentage of the tissue volumes including BVh/TV, BVl/TV, BVv/TV.

**Histomorphometric Analysis**

After micro-CT scanning, the femora were decalcified, cut into halves along the midsagittal plane, and embedded in paraffin. The specimens were then cut in 5 μm-thick sections longitudinally and stained with hematoxylin-eosin (H&E). \[ 41 \] Each specimen was analyzed with respect to the cellular components in periosteal and fracture areas in two central sections using light microscope system (Leica DMRB DAS, Leica, Heerbrugg, Switzerland). \[ 44 \] For quantitative analysis, the ROI was evaluated in H&E-stained slides covering 1.5 mm proximal and distal to the fracture line (total 3 mm). The external callus tissues within the ROI were quantified as total callus area (TCA) while the cartilaginous callus area (CCA) was manually defined using Metamorph Image Analysis System (Fig. 4). The percentage of CCA in callus area was expressed as CCA/TCA.

**Radiographic Analysis**

For the rats euthanized at 4 weeks posttreatment, fracture healing was monitored by weekly anteroposterior and lateral radiography according to established protocol. \[ 41 \] Two experienced orthopedic surgeons, who were blinded to the animal groupings and time points of the study, performed independent assessments of the fracture healing status of digitized anteroposterior and lateral images from each rat. The radiographic healing was defined as complete mineralized callus bridging of all four cortices on both anteroposterior and lateral radiographs. The external callus width (CW) and callus area (CA) were measured on digitized lateral view images with calibration using Metamorph Image Analysis System (Universal Imaging Corporation, Downingtown, PA).

**Micro-CT Analysis**

After removal of soft tissues and the intramedullary Kirschner wire, the harvested femora (n = 6 for each group at each time mechanical testing) were scanned by micro-CT system (μCT-40, Scanco Medical, Brütisellen, Switzerland). The scan range covered 5 mm proximal and 5 mm distal to the fracture line with a resolution of 16 μm per voxel and 1024 × 1024 pixel image matrices. Contoured region of interest (ROI) was selected in two-dimensional (2D) CT images. Three-dimensional (3D) reconstruction of mineralized tissue was performed using a low-pass Gaussian filter (Sigma = 1.2, Support = 2). To differentiate newly formed mineralized callus from old cortices, the low- and high-density mineralized tissues were reconstructed using different thresholds (high attenuation = 325, low attenuation = 120) defined in 2D images using our recently established evaluation protocol. \[ 42,43 \] The high-density tissues represented old cortices and newly formed, highly mineralized callus, whereas the low-density tissues represented newly formed callus. \[ 42,43 \] Quantitative analysis was performed covering the mid 300 slices of the 2D images with the low- and high-density mineralized tissues evaluated separately. Morphometric parameters used for evaluation were total tissue volume (TV, mm³), volume of high-density bone (BVh, mm³), volume of low-density bone (BVl, mm³), total bone volume (BVv, mm³), i.e., equivalent to BVh + BVl or TV—interstitial space and normalized percentage of the tissue volumes including BVh/TV, BVl/TV, BVv/TV.

**Mechanical Testing**

At 4 weeks posttreatment, eight rats from each group were euthanized and the entire femora were dissected free from soft tissues and preserved in gauze with saline and stored at −20 °C. In performing the mechanical testing, the femora were thawed overnight at room temperature. The Kirschner wire was removed and the specimen was prepared for four-point bending test. The femora were loaded in the anterior–posterior direction with the posterior side in tension. \[ 45 \] The inner and outer span of the blades was 8 and 20 mm, respectively, with the long axis of the femora oriented perpendicular to the blades during the test. A constant displacement rate of 5 mm/min was used to test the femora to failure using a material test machine (H25KS Hounsfield Test Equipment Ltd. Redhill, Surrey, UK). The ultimate load (UL) and stiffness were recorded and analyzed using built-in software (QMAT Professional Material testing software. Hounsfield Test Equipment Ltd. Redhill, Surrey, UK).
Statistical Analysis
All quantitative data were expressed as mean ± standard error (SE) and analyzed with SPSS version 13.0 software (SPSS Inc, Chicago, IL). Independent t-test was used to compare the differences between treatment and control groups at corresponding time points. Significant difference was set at a probability level of 95% (p < 0.05).

RESULTS
All rats resumed partial weight bearing within a few hours after surgery. All rats recovered full weight-bearing walking before the start of vibration or sham treatment (day 5 postoperatively). During treatments, the rats stood calmly with both hind paws on the vibration platform without panic behaviors. No significant difference in body weight was observed between groups in the study period. Of the 55 operated rats, one died and two were excluded due to nontransverse fractures created; thus, 52 rats were included in the study.

Radiographic Analysis
Obvious radio-opaque external callus around the fracture site was observed in both groups from 1 week posttreatment and throughout the study period. Compared with the control, the vibration treatment group showed a faster bridging of callus gaps in the serial lateral radiographies (Fig. 1). For the healing status, at 3 weeks posttreatment, 50% (7/14) of the rats in the treatment group and 21.4% (3/14) in the control showed radiographic healing. At 4 weeks posttreatment, 85.7% (12/14) in the treatment group and 57.1% (8/14) in the control showed radiographic healing. For quantitative analysis, at 1 week posttreatment, the treatment group presented 22.5% larger CW (p = 0.003) and 29.7% larger CA (p = 0.004) compared with the control (Fig. 2). At 2 weeks posttreatment, CW and CA increased in both groups with the treatment group showing 21.1% larger CW and 25.1% larger CA than the control (p < 0.001 and p = 0.001, respectively). At 3 weeks posttreatment, CW and CA decreased in both groups. Difference in CW between groups was no longer significant, while larger CA (p = 0.004) still presented in the treatment group (Fig. 2). By 4 weeks posttreatment, the treatment group showed smaller CW, larger CA, and flatter shape of the external calluses compared with the control. No significant difference was found between groups at this time point (Fig. 2).

Micro-CT Analysis
At different time points, reconstructed mineralized calluses showed different morphologic characteristics between groups in the 3D micro-CT images (Fig. 3). At 1 week posttreatment, newly formed callus (low-density bone) appeared in the perifracture region, together with a larger callus gap in the treatment group compared with the control (Fig. 3). The quantitative measurements of TV and BV/ showed higher values in the
treatment group, but the differences were not statistically significant (Table 1). At 2 weeks posttreatment, remarkable callus formation was observed in both groups while the treatment group showed more regular, symmetrical, and complete appearance with a smaller callus gap at fracture site (Fig. 3). Quantitatively, 15.3% larger BVt and 21.4% larger BVl were observed in the treatment group at this time point ($p = 0.025$ and 0.004, respectively), while TV demonstrated slightly higher value yet not statistically significant (Table 1). At 4 weeks posttreatment, the callus gaps were completely bridged by mineralized callus (low-density bone) in the treatment group, compared with the persistent callus gaps left in the control (Fig. 3). The treatment group presented 32.8% larger BVh and 24.8% higher BVh/TV than the control with $p = 0.026$ and 0.017, respectively (Table 1).

**Histomorphometric Analysis**

All samples demonstrated secondary fracture healing with callus formation and endochondral ossification. In both groups, newly formed woven bone, blood vessels, and enlarged osteoblasts were observed beneath the periosteum adjacent to the fracture site, while the control showed persistent callus gaps left in the control (black arrows). At 1 week posttreatment, newly formed callus (low-density bone) was observed in both groups, while the vibration group showed a larger callus gap at fracture site than the control (black arrows). At 2 weeks posttreatment, remarkable callus formation was observed in both groups, while the treatment group showed more regular, symmetrical, and complete appearance with a smaller callus gap completely bridged by mineralized callus (low-density bone) in the treatment group, compared with the callus gaps left in the control (black arrows).

**Mechanical Testing**

Typical load–displacement curve of long bone was confirmed in all the individual bending tests performed on the fractured femora showing an initial short nonlinear response (preloading or engaging) followed by an upward-sloping linear component (elastic phase) and then a failure response at the break point. The UL of the treatment group was 50.1% higher compared with the control.
control group (p = 0.023) and 67.3% higher stiffness was observed in the treatment group (p = 0.001) (Table 2).

**DISCUSSION**

LMHFV is a form of biophysical stimulation delivering noninvasive, systemic, and cyclic mechanical stimuli. Its osteogenic effect on intact bone and stimulative effect on limb blood flow have been documented in animal and human studies, which are considered to be essential in fracture healing. The present study investigated the effects of LMHFV on fracture healing in a closed rat femoral shaft fracture model with the findings indicating a significant enhancement in fracture healing. The healing process in the treatment group was accelerated with enhanced callus formation and faster callus mineralization when compared with the control. This is the first evidence showing fracture healing enhancement with the application of LMHFV.

Mechanical stimulation is known to have osteogenic effects. Although a variety of methods and clinical facilities in mechanical or biophysical forms have been developed to enhance fracture healing, gap remains in the search of a practical enhancement possessing that could be noninvasive and effective with well-defined specifications and regimes. Comparing with other forms of enhancements, the application of LMHFV may have special indication in enhancement of fracture healing because of its noninvasiveness with systemic and osteogenic characteristics.

In this study, the animals were treated with the standard regime of LMHFV and showed positive effects on fracture healing. The animals were subjected to vibration stimulation, which was confirmed by the detection of the signal transmission at the midthigh level by an accelerometer. The enhancement of fracture healing in animals treated with LMHFV is shown by the increased callus formation after 1 eek of treatment in which both the callus width and the area were significantly increased as compared with the control. The callus width decreased much faster in the later phase of the treatment. This is explained by the faster mineralization and remodeling of the callus in the treatment group. The radiographic data were further confirmed by the micro-CT analysis where the enhancement of fracture healing in the treatment group was demonstrated at 1 week posttreatment despite the difference not reaching statistically significant level. At 2 weeks posttreatment, while the volumes of the high-density bone (the original bone ends) (BVh) were similar in both groups, the low-density bone (BVl) and the total bone volume (BVt) around the fracture site increased significantly in the treatment group, indicating that the callus formation was much stimulated following LMHFV treatment. At 4 weeks posttreatment, while the normal fracture healing was progressing into callus remodeling, the treatment group showed significant increase in the volume of high-density bone (BVh) and the ratio of volume of high-density bone to the total tissue volume (BVh/TV). The total tissue volume (TV) and total bone volume (BVt) in both groups also increased with the treatment group remaining higher, although the differences were not statistically significant. This indicated that the callus formed in the treatment group mineralized and matured faster than the control at the later phase (133.6% faster at 3 weeks and 50.1% more complete callus bridging at 4 weeks posttreatment).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Vibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultimate load (N)</td>
<td>96.4 ± 12.9</td>
<td>144.7 ± 13.9°</td>
</tr>
<tr>
<td>Stiffness (N/mm)</td>
<td>177.8 ± 22.9</td>
<td>288.5 ± 9.8°</td>
</tr>
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*°p < 0.05 (Independent samples t-test).
Histomorphometrically, the area of cartilaginous callus (CCA) was larger in the treatment group at 1 week posttreatment. The TCA and the CCA were found significantly larger in the treatment group, indicating a positive stimulation on callus formation from the very beginning of the fracture healing. At 2 weeks posttreatment, while the TCA was significantly larger; the CCA, however, did not show difference from the control group. This echoed with the increased BVh and BVl in micro-CT analysis. At week 4, cartilaginous callus was almost not found in the treatment group, which explained the significantly higher BVh detected in micro-CT analysis, indicating that the massive callus mineralization induced by the LMHFV was much faster than the control.

The biomechanical property of the healed fracture was tested with a four-point bending test. The results again confirmed that the fracture healed under LMHFV was mechanically 50.1% (p < 0.05) stronger than the control at 4 weeks posttreatment, indicating the acceleration of fracture healing in the treatment group that resulted in a stronger structure.

This study confirmed the positive effect of LMHFV on fracture healing in the closed femoral shaft fracture in rats. Although we did not investigate the molecular biology and the mechanism of the stimulation in the present study, the mechanical stimulation provided by the LMHFV may be involved in stimulation of blood flow in the muscles which surrounded the fracture site, thus providing a better biological environment for fracture healing. The mechanical stimuli to the fracture ends may also induce more endochondral ossification as depicted by a larger cartilaginous callus in early phase of the healing. The increased rate of callus mineralization may reflect the positive effect on cell differentiation as shown in our previous study with low-intensity pulsed ultrasound (LIPUS) on periosteal cells. Further studies on gene expression are undergoing in our institute to understand the different responses of the osseous tissues to LMHFV during fracture healing.

The rate of fracture healing induced by LMHFV was improved by 25–30%, which could have tremendous potential for clinical application. This effect is comparable to that from LIPUS stimulation, which has the limitation of local application on fractures with thin soft tissues envelope like tibia or distal radius. The application of LMHFV may have wider application because its effect is more systemic. Faster healing will facilitate earlier rehabilitation and regain of functions after fracture. The stimulatory effects may decrease the incidence of delayed union or nonunion, which is common in fractures resulted from high energy trauma like open fractures. A faster fracture healing rate will also lead to a decrease in the implant failure rate. All these will help to minimize the cost of treating fractures, which are still the commonest cause of loss of working force in the society where younger generation is prime victims.

Beneficial effect of vibration was also reported in Goodship’s study in which axial interfragmentary movement of 0.02 mm at 32 Hz (30,000 loading cycles) was confirmed to enhance endochondral bone repair with an ovine tibial open osteotomy model. However, Wolf et al. only found slight but insignificant difference of metatarsal osteotomy healing under 0.02 mm of interfragmentary movements at 20 Hz (6000 loading cycles). Disagreement in the results of these studies, as explained by Wolf et al., were probably due to the different setups of treatment specifications and regimes. In the current study, positive effects of LMHFV were testified in a closed fracture model, which was different from the open osteotomy models because the fracture hematoma was undisturbed and the soft tissue damage was minimal in the closed fracture model.

This study has a few limitations. First, we managed to detect the transmission of vibration signal at midthigh level of the rats, but neither real-time nor spot check of interfragmentary strain at fracture site on conscious rats in movements was done due to limited technology. Second, only the effects of LMHFV on diaphyseal fractures were addressed in this study. The stimulatory effect of LMHFV might not be able to apply directly to metaphyseal fractures. Third, investigation on the effect of LMHFV during the initiation of fracture healing was not addressed in this study because LMHFV treatment was not started immediately after surgery due to the fact that it took around 5 days for the rats to recover full weight-bearing standing and walking on the fractured limb. This time point also echoed the stabilization of hematoma and early angiogenesis.

In conclusion, the healing of the closed femoral shaft fractures in the rat model was significantly enhanced with low-magnitude high-frequency vibration. The enhancement was illustrated with positive osteogenic effect by formation of larger amount of callus, accelerated callus remodeling, and finally a complete restoration of stronger osseous tissue around the fracture site. These findings would imply that the anabolic effect of vibration treatment on fracture healing might have great potential in clinical applications.

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