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Original Article

Intermittent low-magnitude high-frequency vibration enhances biological and radiological parameters during fracture healing in sheep

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Abstract: Background: Fracture is one of the main concerns for public health worldwide. Low-magnitude high-frequency vibration (LMHFV) accelerates facture healing by enhancing the callus formation, mineralization and remodeling. We aimed to investigate the effects of intermittent LMHFV on fracture healing in sheep models. Methods: Ninety 12-month-old female sheep were randomly divided into four groups: baseline control (BCL), sham osteotomized (Sham-O), osteotomized control (OSX-C) and osteotomized vibration groups (OSX-V). OSX-V group was further subdivided into six groups by different treatment intermittences. An open transverse osteotomy metatarsal model was created and the fractures were fixed. Whole-body vibrations were performed with 0.3 g peak-to-peak acceleration and a frequency of 35 Hz. Serum bone formation and resorption markers, and radiology results were analyzed. Results: Levels of serum BALP, BGP and TGFβ1 were markedly elevated and TRAP5b declined in all OSX-V groups compared to OSX-C at week 4. At week 8, significant increase of TGFβ1 and decrease of TRAP5b were still observed in OSX-V groups. Radiology study showed that the sheep treated with LMHFV had better bone callus, more blurred fracture lines, and higher callus width and callus area. Our study also suggested that one-day intermittent vibration group was better than other vibration groups in serum bone markers at 4 weeks and in the CW and CA at week 8. Conclusion: Our study confirmed the efficacy of LMHFV in fracture healing and it was the first to show that LMHFV with one day rest may have greater potential in clinical applications for fracture healing.

Keywords: Whole-body vibration, LMHFV, fracture healing, intermittent, TGF-β1

Introduction

Fracture is one of the main concerns for public health problems worldwide, leading to large numbers of co-morbidities and mortalities [1]. Most fracture healing is uneventful. However, complications do occur like delayed unions and nonunions with an incidence up to 10% [2, 3], resulting in the failure of internal fixation and joint stiffness. The fracture healing is largely dependent on the biological environment for osteogenic potential, which is influenced by growth factors, bone-forming cells [4, 5], blood supply [6], as well as the mechanical environment [7]. Treatment that potentially accelerates the healing process could help decrease the rate of this large problem and regain of functions after fracture.

Low-magnitude high-frequency vibration (LMHFV) is a form of mechanical intervention that provides cyclic loadings. LMHFV can enhance the muscle strength, reduce fatigue, improve the material metabolism, promote tissue nutrition and limb blood flow, and accelerate wound recovery [8, 9]. Its osteogenic potential has been suggested in animal models [10-12] and clinical trials [13, 14], as well as its positive effects on bone cell proliferation [15]. Although the detailed cellular mechanism and molecular pathways remain to be explored, LMHFV has engendered great interest because of its low cost, ease of handling, noninvasive nature and promising osteogenic effect [16]. In order to understand the mechanism of LMHFV on fracture healing, scientists have been investigating important parameters of LMHFV including intensity, frequency and duty cycle. The optimal values of these parameters have been selected based on scientific evidence support. The parameter considered in this work is the intermittent period. Our previous study proved that intermittent vibration may be superior to continuous vibration in a rat osteoporosis model [17]. Whereas other study reported inconsistent results [18, 19].

Therefore, the present study aimed to determine whether intermittent period LMHFV promote fracture healing in osteotomized metatarsal treated by plate and screws in sheep models. Sheep metatarsal fracture model can better imitate human physiological state of maximum weight bearing bone than other small animal models. We also adopted steel plate fixation, which is commonly used in clinical practice. The healing status was assessed using biochemical markers and radiography.

Materials and methods

Animals

Ninety 12-month-old skeletally mature female small-tail sheep weighted from 14 to 16 kg and housed at the research animal laboratory of Jilin University were used in this study. All sheep were allowed to roam freely cage movement. At the end of the experiment, the sheep were euthanized by an overdose of sodium pentobarbital. The Animal Experimentation Ethics Committee of the first Hospital of Jilin University has approved the care and experimental protocol of this study (Ref. no: 2010/010).

Study design

The sheep were randomly divided into four groups: (1) baseline control (BCL) (n = 10) without osteotomy or vibration treatment; (2) sham osteotomized (Sham-O) (n = 10) with only skin incision but no further operation; (3) osteotomized control (OSX-C) (n = 10) with osteotomy and fixed fracture without vibration; (4) osteotomized vibration (OSX-V) (n = 58). The OSX-V group was further subdivided into six groups with different treatment intervals (vibrational loading for X day followed by X day rest). Subgroups were divided as either 0, 1, 3, 5, 7 or 14 days of loading intervals in 6 groups, respectively (labeled as OVXR, X = either 0, 1, 3, 5, 7 or 14; n = 10 in OV0R, OV1R, OV7R and n = 9 in OV3R and OV5R subgroup, respectively). OV0R sheep was treated with daily vibration treatment without rest for 8 weeks. LMHFV therapy in the vibration group was started at day 7 postoperatively for all sheep in the vibration group. The control group (Sham-O and OSX-C) sheep without vibration treatment stood on the machine without switching it on for the same period of time.

Fracture model

After 1 week of acclimatization, open transverse right metatarsal osteotomy was performed following plate and screws fixation in the right metatarsal shaft according to our established protocol [17]. Anesthesia was achieved by intravenous injection of katemine (20-60 mg/kg) and xylazine (2.5 mg/kg). Temgesic (1 ml/kg) was also given intravenously 15 minutes before surgery and for three consecutive days after surgery to relieve pain.

During The low-energy osteotomy, the right metatarsal was firstly fixed by a steel plate and screws. A Gigli wire saw was used for cutting the right metatarsal in the middle of the steel plate to create an osteotomy of 3 mm. The fractures were then fixed by unilateral compression plate and cortical screws. Osteotomy and fixation were carried out by one experienced orthopedics doctor for consistency. Of the 80 operated sheep, two unexpected deaths occurred after fracture. Therefore, a total of 88 sheep were available for final analysis.

Vibration treatment

LMHFV treatment was started 7 days after surgery when the sheep recovered and regained full weight-bearing walking. OSX-V sheep received LMHFV treatment using a specially designed vibration platform providing vertical LMHFV at 35 Hz with a peak-to-peak acceleration of 0.3 g, according to our previous protocol [17]. The specific vibration signal (35 Hz, 0.3 g) used in this study was chosen according to the previous investigations reported by us [17] and another research group [16]. The animals were allowed to stand on their hind legs in the bottomless and compartmented cages fixed on the vibration platform for 20 min/day. Whereas sheep in BCL and OSX-C received the same regime with the vibration platform turned off for 20 min/day. The walls of the cages were opaque and painted in black to minimize the surrounding disturbance.

ELISA analysis on bone markers

At 4 and 8 weeks after the surgery, all sheep were fasted for 8 hours and refrained from any

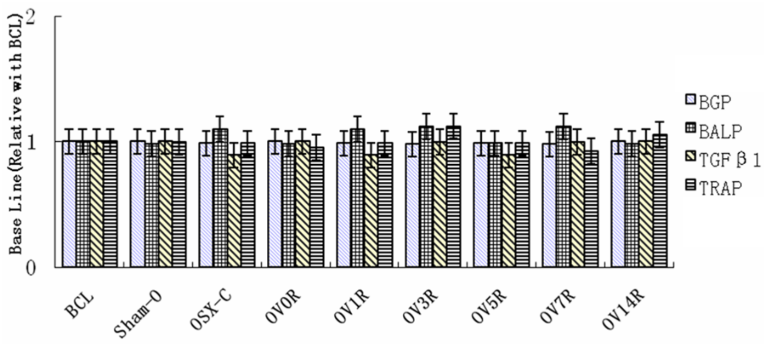

Figure 1. Bone maker analysis at baseline. BCL: baseline control; Sham-O: sham osteotomized; OSX-C: osteotomized control; OS0-V: continuous vibration, OV1R: vibrational loading for 1 day followed by 1 day rest; OV3R: vibrational loading for 3 days followed by 3-day rest; OV5R: vibrational loading for 5 days followed by 5-day rest; OV7R: vibrational loading for 7 days followed by 7-day rest; OV14R: vibrational loading for 14 days followed by 14-day rest. Data were reported as mean ± SD.

exercise for 24 hours before blood collection. Five milliliters of blood were collected from the antecubital vein. Whole blood samples were then centrifuged at 3,500 rpm (2,000 g) for 15 min to separate the serum. The sample was aliquoted, and stored in -80°C until analysis. Commercially available ELISA kits were used to measure serum concentration of bone alkaline phosphatase (BALP), bone gia protein (BGP), transforming growth factor β1 (TGF-β1) and tartrate-resistant acid phosphatase 5b (TRAP-5b, a bone resorption marker) (Immunodiagnostic System Limited, Frankfort am Main, Germany) to analyze bone formation and resorption rates respectively [20]. Instructions from the manufactures were followed strictly.

Radiological analysis

At 8 weeks after surgery, the formerly osteotomized metatarsus and contralateral intact metatarsus were harvested from sheep. Frontal and lateral X-ray films of the developing callus were taken in order to monitor the fracture healing status (Faxitron X-ray system model 43855C, Wheeling, IL). Two experienced orthopaedic surgeons, blinded by the time points or study groups, were responsible for grading fracture healing state of each sheep's digital X ray. Fracture healing was assessed through observation the bridging of all four layers of cortical bone mineralization tissue by lateral and anterior and posterior X-ray films. CW, the maximum width of callus minus the width of the cortical bone and medullary cavity at the diaphysis, and CA, the size of radio-opaque area of callus, were measured on lateral X-rays using the Metamorph Image Analysis System from each radiograph (Universal Imaging Corporation, Downingtown, PA).

Statistics

All quantitative data were expressed as mean ± standard deviation (SD) and analyzed using SPSS for Windows (SPSS version 16.0, SPSS Inc, Chicago, IL). All measurements including the quantitative radiographic analysis and ELISA data among groups at different time points were compared by one-way analysis of variance (ANOVA) (a = 0.05) after Bonferroni post-hoc tests for multiple group comparisons. Data not normally distributed were compared using the Mann-Whitney nonparametric comparison of means. A two-tailed $P < 0.05$ was considered as statistically significant.

Results

Serum concentration of bone markers

Mean changes from baseline for markers of bone turnover, and bone regulation at 4 and 8 weeks were presented in **Figures 1-3**. There were no significant differences in bone turnover markers (BALP, BGP, TGFβ1 and TRAP5b) at baseline across different groups (**Figure 1**).

At week 4, levels of serum BALP, BGP (bone formation markers) and TGFβ1 were markedly elevated in all OSX-V groups (**Figure 2A-C**) whereas TRAP5b level was declined in all OSX-V groups compared to OSX-C group (**Figure 2D**). More importantly, OV1R had significantly higher levels of BALP, BGP and TGFβ1, and significantly lower level of TRAP5b compared to the OV0R group.

At week 8, the results were more complex. OV1R had significantly higher level of BGP however other vibration groups had similar levels compared to OSX-C group (**Figure 3A**). All groups had similar levels for BALP group (**Figure 3B**). TGFβ1 level were markedly elevated in the

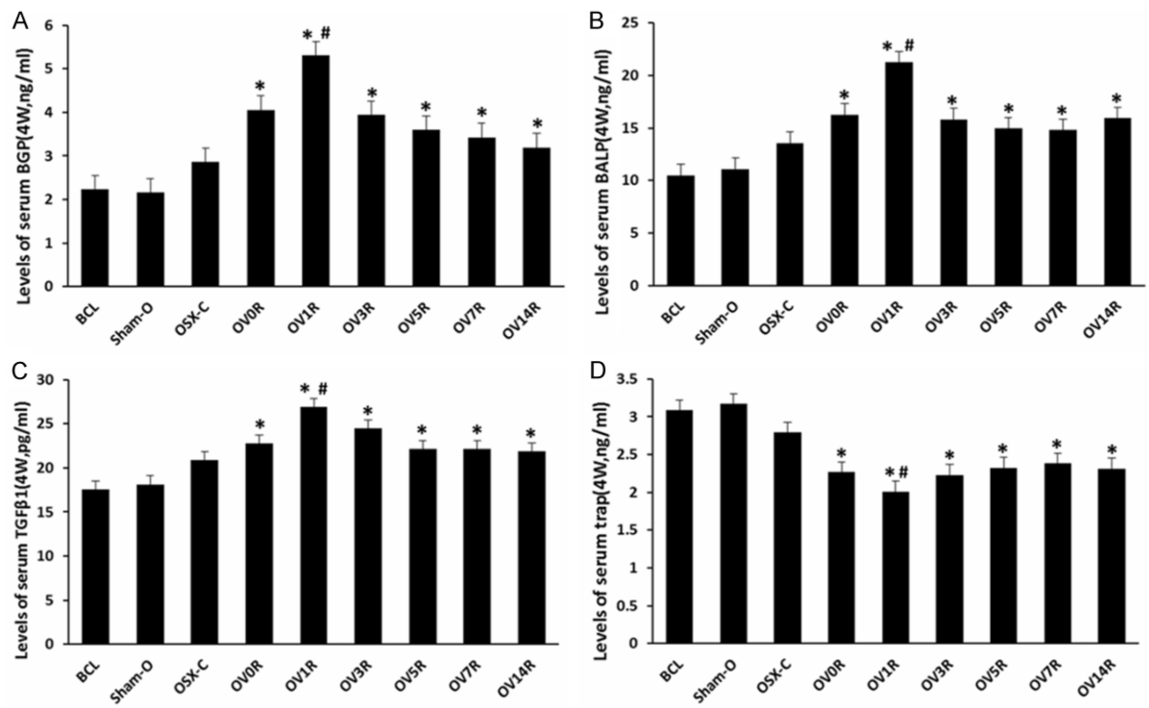

Figure 2. Results of bone makers analysis at 4 weeks after operation. A: BGP; B: BALP; C: TGF-β1; D: TRAP5b. Significant difference was found compared with OSX-C (*); and OV0R (#) (P < 0.05). Data were reported as mean ± SD. BCL: baseline control; Sham-O: sham osteotomized; OSX-C: osteotomized control; OS0-V: continuous vibration; OV1R: vibrational loading for 1 day followed by 1 day rest; OV3R: vibrational loading for 3 days followed by 3-day rest; OV5R: vibrational loading for 5 days followed by 5-day rest; OV7R: vibrational loading for 7 days followed by 7-day rest; OV14R: vibrational loading for 14 days followed by 14-day rest.

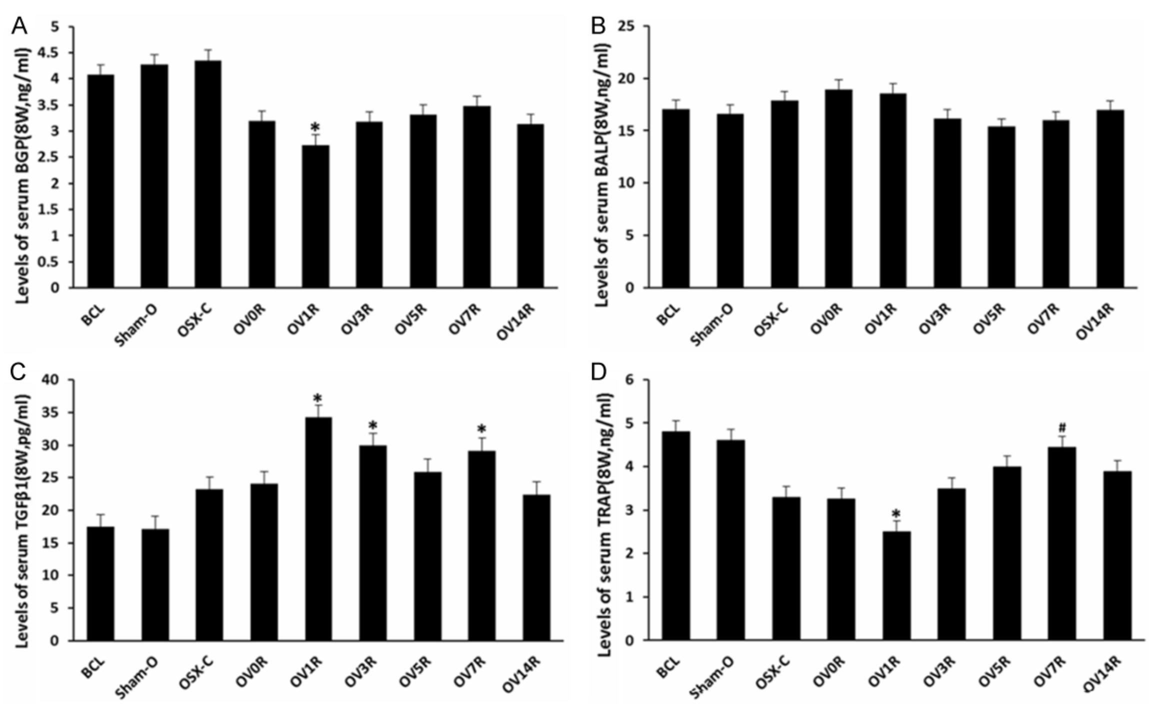

Figure 3. Bone markers analysis at 8 weeks after operation. A: BGP; B: BALP; C: TGF-β1; D: TRAP5b. Significant difference was found compared with OSX-C (*); and OV0R (#) (P < 0.05). Data were reported as mean ± SD. BCL: baseline control; Sham-O: sham osteotomized; OSX-C: osteotomized control; OS0-V: continuous vibration; OV1R:

vibrational loading for 1 day followed by 1 day rest; OV3R: vibrational loading for 3 days followed by 3-day rest; OV5R: vibrational loading for 5 days followed by 5-day rest; OV7R: vibrational loading for 7 days followed by 7-day rest; OV14R: vibrational loading for 14 days followed by 14-day rest.

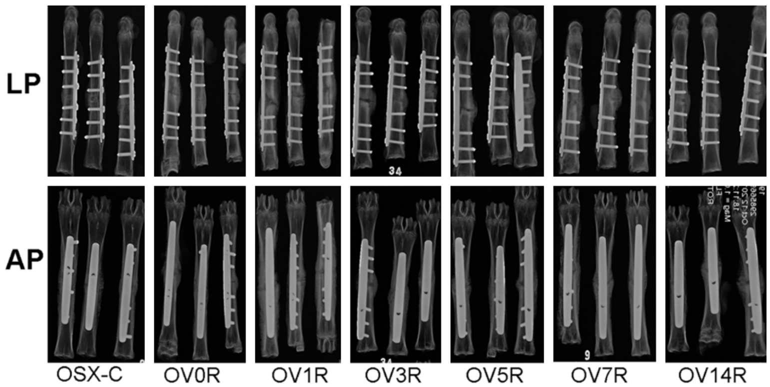

Figure 4. Three representative longitudinal radiographic images of mid-diaphyseal metatarsal fractures in each group at week 8. LP: Lateral Position; AP: Anterior-posterior Position; OSX-C: osteotomized control; OS0-V: continuous vibration; OV1R: vibrational loading for 1 day followed by 1 day rest; OV3R: vibrational loading for 3 days followed by 3-day rest; OV5R: vibrational loading for 5 days followed by 5-day rest; OV7R: vibrational loading for 7 days followed by 7-day rest; OV14R: vibrational loading for 14 days followed by 14-day rest.

OV1R, OV3R and OV7R groups compared to OSX-C group (**Figure 3C**). Whereas TRAP was significantly declined in OV0R and elevated in OV7R groups compared to OSX-C group (**Figure 3D**).

Radiographic analysis

Figure 4 showed representative longitudinal radiographic images of bilateral mid-diaphyseal metatarsal fractures at 8 weeks after treatment. For all the groups, Callus formation was detected in all groups. In OSX-C group, the fracture line still clear in 50% of the specimens whereas the fracture line on the original bone disappeared in 91.3% of the specimens in the vibration groups.

The radiological results also indicated that vibration with different intermittence period resulted in inconsistent callus size. Quantitative analysis of CW showed that all vibration treated group had significantly higher CW values than OSX-C group (P = 0.034, < 0.0005, 0.025, 0.0029, 0.009 and 0.032 in the OV0R, OV1R, OV3R, OV5R, OV7R and OV14R, respectively). The intermittent groups also showed significantly higher CW than OV0R group (P = 0.027, 0.076, 0.084, 0.036 and 0.088 in the OV1R, OV3R, OV5R, OV7R and OV14R, respectively). Similarly, all vibration treatment groups were significantly higher than OSX-C group in CA measurement (P = 0.024, < 0.0005, 0.026, 0.0031, 0.012 and 0.037 in the OV0R, OV1R, OV3R, OV5R, OV7R and OV14R, respectively). The intermittent groups were also found significantly higher in CA than the continuous group (P = 0.037, 0.079, 0.089, 0.047 and 0.095 in the OV1R, OV3R, OV5R, OV7R and OV14R, respectively). The one-day-intermittent vibration group (OV1R) showed significantly higher CA than that of any other vibration groups (**Figure 5**).

Discussion

This study used sheep fracture model to demonstrate that low-magnitude high frequency vibration treatment after fracture fixation surgery was beneficial for fracture healing. LMHFV treatment up-regulated the expressions of BALP, BGP, TGFβ1, down-regulated of TRAP5b, increased mean callus width and mean callus area. More importantly, vibration treatment with one-day intermittence showed greater potential in accelerating bone healing. To our knowledge, this is the first study demonstrated the usefulness of intermittent vibration treatment of fracture in large animals

Fracture healing is affected by multiple factors. Some of these are patient related factors such as ageing, chronic disease, and medications whereas some are related to the severity of the injury (such as high energy damage) and the treatment type [21]. Some treatments including internal fixation devices may inadvertently compromise the healing progression resulted from the destructed blood supply and load sharing between the bone and the device. Under the physiological condition, the fracture surfaces are also under high frequency and low amplitude vibration due to muscle contraction.

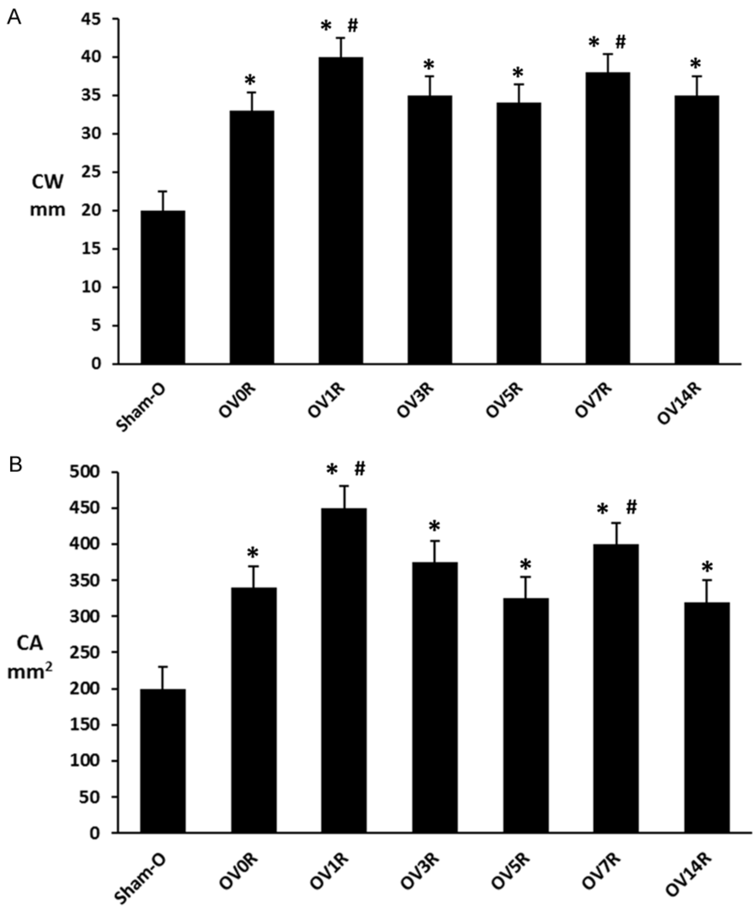

Figure 5. Radiographic analysis of the right metatarsal fracture. A: Mean callus width; B: Mean callus area. Significant difference was found compared with OSX-C (*); and OV0R (#) ($P < 0.05$). CW: callus width; MCA: mean callus area; Sham-O: sham osteotomized; OSX-C: osteotomized control; OS0-V: continuous vibration; OV1R: vibrational loading for 1 day followed by 1 day rest; OV3R: vibrational loading for 3 days followed by 3-day rest; OV5R: vibrational loading for 5 days followed by 5-day rest; OV7R: vibrational loading for 7 days followed by 7-day rest; OV14R: vibrational loading for 14 days followed by 14-day rest.

Internal fixation may shield this vibration and slow down the healing time [22].

LMHFV is a form of biophysical stimulation delivering noninvasive, systemic, and cyclic mechanical stimuli. The effect of LMHFV on fracture healing has been investigated in previous studies showing that LMHFV was effective for fracture healing in mice [23]. No previous study has investigated the effect of intermittent LMHFV on fracture healing of large animals, treated with open reduction and plate fixation, which is a frequently used in clinical practice.

The present study investigated the effects of LMHFV on fracture healing in a sheep's metatarsal shaft osteotomy model fixed with plate and screws. Our results support previous studies [23, 24] and confirmed that intermittent and continued LMHFV increased serum expression related to osteogenesis and bone remodeling while down-regulated resorption related factors, leading to better healing responses.

BALP, BGP and TRAP are the specific experimental diagnostic indicators for bone remodeling evaluation [25]. Another chosen marker was TGF-β1, which has been implicated as a regulator of endochondral ossification during skeleton formation, but also during fracture healing [26]. In this study, compared with the osteotomized control group, TGF-β1 was significantly increased at the fourth and eighth week in all the vibration group, indicating accelerated endochondral ossification occurred by vibration treatment. Compared with the osteotomized control, BALP and BGP were significantly increased at week 4, whereas TRAP was decreased in all the vibration groups except for intermittent 5 and 7 days groups, indicating that early vibration resulted in enhanced bone formation. At week 8, the change of BGP and BALP was not as significant whereas TRAP level in the OV1R group was significantly lower, indicating that the main effects of vibration on bone mass was inhibiting bone resorption in reparative phase of fracture healing at later stage.

Radiology study confirmed that the sheep treated with LMHFV has better bone callus, more blurred facture line, and higher CW and CA values, indicating that LMHFV enhanced fracture healing. This was consistent with previous studies [15, 23, 24] except for the Wolf's study [27]. Wolf et al. reported that the effect of vibration was insignificant for healing under

0.02 mm of inter-fragmentary movements at 20 Hz of vibration in a metatarsal open osteotomy model. The reasons for the difference were probably due to the different setups of treatment specifications and regimes. In the current study, the fracture was fixed by plate and screws, which was different from external circular fixator. The disadvantageous effect of the high number of pins from external circular fixator might result in reduced bone vitality. The frequencies and amplitudes used and the interval period of vibration was also different.

In this study, the fracture healing process was promoted by LMHFV in both intermittent and continent groups. However, the OV1R group was more sensitive towards the mechanical stimulation of LMHFV than OV0R. An early increase in BALP, BGP, TGFβ1 and decrease in TRAP in OV1R group was observed at week 4. The OV1R group showed significantly higher CW and CA than OV0R and any other group at week 8. These results were different from Gao et al. which observed the most significant effect of LMHFV with 7-day-intermittent vibration in a rat model [18]. There are four possible reasons: In the current study: 1. The positive effects of LMHFV were testified in a fracture model of sheep, which had different bone structures and bear much higher load than small animals; 2. The fracture was fixed by plate and screws, which was different from the intra-medullar nail. Steel plate screw fixation is a rigid eccentric fixation, with strong stress shielding effect. Whereas the intramedullary pin is elastic center fixation with weak strong stress shielding effect; 3. Different fracture model: single side open metatarsal fracture versus bilateral close tibia fractures; 4. Different experimental designs. GAO et al. used vibrational loading for 7 day with or without 7-day rest. In summary, we used an open metatarsal fracture model in sheep to confirm the efficacy of one-day intermittent LMHFV in promoting fracture healing.

Conclusion

This is the first report to confirm the efficacy of intermittent low-magnitude high-frequency vibration treatment on fracture healing. These findings also suggested that LMHFV with one day rest may have greater potential in clinical applications for fracture healing. Studies in other animal models or clinical studies are warrant for further elucidation of the role of LMHFV in bone healing.

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Disclosure of conflict of interest

None.

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