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Low-intensity pulsed ultrasound promotes spinal fusion and enhances migration and proliferation of MG63s through sonic hedgehog signaling pathway

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ABSTRACT

Low-intensity pulsed ultrasound (LIPUS) has been found to accelerate the healing process of spinal fusion via a process closely related to osteoblast differentiation and migration. Sonic hedgehog (Shh) signaling plays an important role in development and homeostasis, including a critical function in bone formation. However, its role in spinal fusion during LIPUS treatment is still unknown. This study showed that LIPUS treatment after spinal fusion surgery increased bone formation. The increased bone mass under LIPUS treatment appeared to result from the increased migration and proliferation of osteoblasts, resulting from upregulation of the Shh signaling pathway. In contrast, inhibition of Shh reduced the migratory and proliferative ability of osteoblast-like MG63 cells and blocked the efficacy of LIPUS treatment.

1. Introduction

Spinal fusion is commonly used to rebuild the spine and enhance stability, and a successful spinal fusion is regarded as the gold standard for treating an unstable spine. Spinal fusion has also been widely performed to treat spinal degeneration, trauma, tumor, and infection. However, aggregated data have revealed that approximately 3%–12% of Swedish spinal fusion patients [1], 9.98% of spinal fusion patients in Germany [2], 8.89% of spinal fusion patients in the USA [3], and 9.4% patients in Spain [4] require subsequent surgeries to treat various complications. Thus, a new clinical treatment that can promote spinal fusion and accelerate the process of healing is urgently needed.

Low-intensity pulsed ultrasound (LIPUS) has been found to enhance spinal fusion in various studies. It has been shown to provide micromechanical stress and promote mineralization by directly regulating osteogenic cells, thereby, accelerating the process of bone fracture healing [5]. The first study investigated the effect of LIPUS on spinal fusion by Cook et al. reported that LIPUS promoted spinal fusion mainly through endochondral ossification [6]. Lu et al. [7] demonstrated enhanced spinal fusion and a greater amount of trabecular bone mass in the fusion area following LIPUS compared to the control group in a rabbit spinal fusion model.

Clinically, LIPUS has been approved for the treatment of fresh bone fractures and non-unions by the US FDA [8]. It can significantly shorten the time for fracture healing and can be used to treat non-unions [9–11]. Compared with other the interventions used for bone repair, LIPUS represents one of the safest and least invasive techniques [12].

Sonic hedgehog (Shh) is a morphogen that regulates the patterning and growth of limb buds at an early stage and is a promising signaling pathway in bone repair and bone formation [13]. It can be activated in adults undergoing bone repair [14]. Shh has a direct effect on the differentiation of mesenchymal stem cells into an osteogenic lineage [14]. Researchers have demonstrated the pivotal role of Shh signaling pathway in mediating the enhanced effect on MG63 proliferation and differentiation [15]. Another novel effect of Shh is its influence on the balance between osteogenesis and adipogenesis in adipose-derived stromal cells. Significantly, Shh increased in vitro osteogenic differentiation and abrogated adipogenic differentiation by activating markers such as Runx2, Col1, and ALP [16]. Hedgehog signaling pathway can be activated by cyclic mechanical stress and promote osteoblastogenesis of bone marrow

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stromal cells [17]. Nevertheless, the underlying mechanism by which LIPUS accelerates spinal fusion and whether the Shh pathway is involved in bone regeneration remains unclear.

The hypothesis tested in the present study was that LIPUS can promote the proliferation and migration of osteoblasts and, thus, accelerate the process of spinal fusion via activation of the Shh pathway.

2. Methods and materials

2.1. Animals

All animals were bred and all the experimental procedures were performed according to the animal experimental ethics guidelines of the Laboratory Animal Center of the Second Military Medical University. Male Sprague Dawley rats (n = 10), each weighing 300–350 g, were provided by Shanghai Super–B&K Laboratory Animal Corp. Ltd. The 10 rats were randomly allocated into two groups: five comprised the control group and five comprised the LIPUS group. The demineralized freeze-dried bone graft material was provided by Aorui Biological Material Co., Ltd., Shanxi, China (License: SHIYAOJIANXIEZI 2012-3460481).

2.2. Surgical procedures

Each anesthetized rat was placed in the prone position. Anesthesia was maintained by administration of isoflurane (0.5%–2%) and oxygen via a coaxial breath cone. A midline incision was made over the lumbar spine, and the surgeon sequentially incised the skin, subcutaneous tissue, and fascia. The transverse process was exposed after the longissimus lumborum muscle was bluntly split. The transverse process was decorticated with a rongeur and bur, and the decorticated fusion bed was implanted with the bone graft. Finally, fascia, subcutaneous tissue, and skin were closed with interrupted suture. Antibiotics were intramuscularly injected (at 0.1 mL/per 100 g) for 3 consecutive days after surgery. Rats (five in the sham LIPUS control group and five in the LIPUS group) were sacrificed 4 weeks after surgery.

2.3. LIPUS treatment for animals

A LIPUS machine (Exogen Ultrasound Bone Healing System, Bioventus LLC, Durham, NC, USA) was used in the experiment with an ultrasound frequency of 1.5 MHz ± 5%, with a 200 μs ± 10% signal burst width, and 1.0 kHz ± 10% repetition rate. The temporal average power was 117 mW ± 30%, and the power of the device was 30 mW/cm^2 ± 30% spatial average and temporal average incident intensity. The LIPUS device was set to operate 20 min at a time.

At day 3 following the operation, transcutaneous LIPUS treatment for the rats was started (20 min/day, 5 days/week for 4 weeks). The rats were anesthetized with isoflurane during LIPUS treatment. The LIPUS probe was carefully placed on the skin dorsal to the fusion site. Rats in the control group received a sham LIPUS treatment (probe placed on the skin with the LIPUS device turned off).

2.4. Micro-CT scanning

After sacrifice on week 4, specimens from the L1 vertebral body to the pelvis were removed and fixed in 4% paraformaldehyde. The specimen was scanned in air by a CT scanner (eXplore Locus, GE Healthcare BioScience Corp., Piscataway, NJ, USA) with parameters of 13 μm voxel size, 55KVp, 145 mA, 400 ms exposure per view and 360° angular range. Relevant data such as bone and total volume, trabecular thickness, trabecular number, and trabecular separation were analyzed by Micro View (GE Healthcare BioScience Corp., Piscataway, NJ, USA). The threshold was 0.25 g HA/cm^3.Volumes of interest (VOIs) were demarcated from the top of the fourth lumbar transverse process to the bottom of the fifth lumbar where LIPUS treatment occurred and bone allograft was implanted. Only the fusion mass outside the vertebral bodies was examined in the study.

2.5. Histologic staining

The L3–L5 were placed in 4% paraformaldehyde and fixed for 24 h. After 72 h of decalcification by 5% nitric acid, the samples were embedded in paraffin after being washed in distilled water. Then, sections (5 μm each) were taken along the midline of the fusion area near the transverse process. Hematoxylin and eosin (H&E) was used for staining, and all the sections were observed under light microscopy (40× and 400×).

All the images were evaluated using Image Pro Plus 5.02 (Media Cybernetics, Washington, USA). Five random fields of view of the fusion area were selected in each section for analysis. Bone tissue area/total image area (BA/TA) was calculated as: bone tissue area / total image area × 100%. Bone area (BA) was determined by 40× magnification under light microscopy for five randomly selected fields (TA) in each ROI, and the average was used for subsequent calculations.

2.6. Immunohistochemical staining

Immunohistochemical analysis was performed on 4% paraformaldehyde-fixed paraffin-embedded spine tissue after 4 weeks of LIPUS stimulation. Regions around the transverse process and graft were defined as the areas of interest. Sections were incubated with diluted primary antibody to Osterix (sc-393325), Shh (sc-1194), and Gli (sc-20687) from Santa Cruz Biotechnology, CA, USA. The osteoblasts-containing area was calculated by ImagePro Plus 5.02 (Media Cybernetics) under microscopy with 400× magnification.

2.7. MG63s culture and GDC0449 treatment

MG63 cells were chosen because of their own advantages in the present study. First, the LIPUS device works on human being, thus it is better to used human derived cells rather than mouse derived cells (MC3T3). Secondly, MG63 cells are easy to access, passage and survive compared with human primary bone derived cells. Third, MG63 cells have a higher level of proliferative and migratory ability at the baseline. So the changes would be more significant after treatment.

MG63 cells were obtained from Cell Bank of the Chinese Academy of Science Shanghai, China. Cell lines were cultured as monolayers in T-75 flask in DMEM (HyClone, #SH30243.01, Utah, USA) supplemented with 10% FBS (Gibco, #16000-044, Waltham, MA USA), 0.1 mg/mL streptomycin (Bio-light, #BL-CC-0033, Shanghai, China) and 0.1 mg/mL penicillin (Bio-light, #BL-CC-0033, Shanghai, China) at a final concentration of 15 μM in an incubator with humidified air plus 5% CO_2 at 37 °C. The cells were subcultured every 72 h at a density of 1×10^5 cells/cm^2 or when cell grew to approximately 80% confluence. Cells were detached form culture flasks by trypsinization using 0.025% trypsin-EDTA, then transfer the medium to the new culture flask. MG63s from passages 4–6 were used in the present experiment. The hedgehog antagonist (GDC0449, Vismodegib; Selleck, #S1082, Shanghai, China) was dissolved in DMSO (Bio-light, #BL-BR-0141, Shanghai, China) to a stock concentration of 10 mM and stored in 1.5-mL aliquots at 4 °C. The cells treated with GDC0449 in a concentration of 15 μM were cultured for 48 h before LIPUS treatment. The culture medium just described (but without GDC0449) was used as a control. All the cell experiments below were replicated for 3 times.

2.8. LIPUS treatment for cells

LIPUS treatment for MG63s was performed 20/40/60 min under separate wells on the same plate in the transwell migration assay. The LIPUS device was set to run 20 min at a time. Thus it need to be performed twice for 40 min and three times for 60 min treatment. The LIPUS probe was fixed under the board of each well, approximately 2–