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Original Article

Mechanism of low-intensity pulse ultrasound combined with Rhodiola to promote bone formation in spinal fusion

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Abstract

This study aimed to explore the influence and mechanism of low-intensity pulsed ultrasound (LIPUS) combined with Rhodiola bone penetration on the formation of spinal fusion bone. Sixty clean-grade New Zealand white rabbits were selected for randomization and divided into combined group and Rhodiola group, with 30 rabbits in each group to construct a rabbit lumbar intervertebral fusion model, using Rhodiola intervention and Rhodiola combined with LIPUS intervention protocol, respectively. The axial strength, axial stiffness, maximum compressive load, vascular endothelial growth factor (VEGF), cycloxygenase-2 (COX-2), prostaglandin E2 (PGE2) and transforming growth factor-β (TGF-β) were compared after HE staining, immunohistochemistry and biomechanical detection. Spine fusion rate was 100.00%; the combined bone graft tissue had implanted bone cell degeneration, cell necrosis and cell hyperplasia, chondrocytes differentiated into trabecular bone and some hematopoietic cells, severe cell necrosis and fiber cell proliferation and late bone formation in the Rhodiola group, VEGF, COX-2, PGE2, TGF-β, axial strength, axial stiffness, and maximum compression load in the combined group significantly increased (P<0.05). Spinal fusion using LIPUS combined with Rhodiola can enhance biomechanical properties and promote the role of PGE2, COX-2, VEGF, TGF-β expression and bone formation, and this protocol is worthy of clinical application.

Keywords: Biomechanics, Bone formation, *Rhodiola rosea*, Low intensity pulsed ultrasonic, Rabbit interlumbar fusion model

1. Introduction

Spinal fusion is a good treatment for spinal reconstruction and spinal sequence, the spinal instability, trauma, tumor and infection in clinical disease is widely used with good results, the formation of false joint failure is spinal fusion, the fusion unsolved problems, will further aggravate the patient's pain and cause deformity, some patients need surgery again, Therefore, increasing the rate of bone graft fusion has become the key to spinal fusion [1]. With the development of medical technology, bone graft materials and bone morphogenetic protein can play a good role in assisting spinal fusion, but there are still some shortcomings such as high cost and poor safety. It is of great significance to explore new effective, safe and economical auxiliary programs to promote spinal fusion for the treatment of clinical spinal diseases [2]. Rhodiola (*Rhodiola rosea*) is a kind of herb growing in the polar alpine region, tenacious vitality, its roots have the role of replenishing qi and clearing lungs, anti-tumor, anti-cardiovascular diseases, widely used in sports medicine research, ultrasound therapy has the advantages of simple operation, economy, safety and so on. In recent years, it has achieved certain results in skeletal muscle soluble system diseases. LIPUS can mechanically stimulate body tissues and participate in biological reaction processes such as bone formation and can promote fracture healing [3]. At present, there are few literatures to analyze the influence mechanism of LIPUS combined with Rhodiola treatment on the formation of spinal fusion bone, so this study aims to further analyze this direction in this direction and provide medical basis for subsequent clinical research.

2. Data and Methods

2.1. Source of Materials

Sixty clean-grade New Zealand white rabbits were selected for randomization and divided into combined group, Rhodiola group, with 30 rabbits in each group, weighing 2-2.5 (2.24±0.15) kg. All animals were provided by the Experimental Animal Center of Southern Medical University, and fed and drinking water were not restricted.

2.2. Methods

2.2.1. Modeling method

(1) Preoperative preparation. Rabbits were artificially raised in a single cage for 1 week in advance and then fed with cage markers after adaptation to the feeding environment. Before operation, the values were weighed and recorded, and the water was forbidden for 1 day. (2) Surgical

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modeling. The rabbits were anesthetized by intravenous injection of 2% sodium pentobarbital (30 mg/kg) through the ear margin. After the skin was prepared, the rabbits were placed on the operating table in a lateral decubitus position. The operative area was disinfected with iodine and alcohol and then covered with a towel. A 6-8cm longitudinal incision was made along the left 12th costal end down to the line of the iliac spine, the skin was cut, subcutaneous, and the iliac bone was removed. The skin was pulled to the iliac spine and the aponeurosis was cut. The periosteum of the iliac spine was dissected to completely expose it. Bone surface was smeared with bone wax and sutured layer by layer without obvious bleeding. Lumbar building, along with lumbar-sacral spine muscle outside part of the muscle and fascia clearance exposed to L4 ~ waist 5 transverse process and vertebral body side, remove the corresponding transverse process make the waist intervertebral disc 4/5 completely exposed, intervertebral disc and endplate excision of L4/5 use curet processing, until the bone surface ooze blood, fill the ilium after flushing, additional steel screw side is fixed, no active bleeding after flushing and wound suture, The model is finished. For 3 consecutive days after operation, 100 ml auricular vein rehydration and 50,000 U penicillin were injected intramuscularly. 24 h after operation, rabbits were fed and watered. Fusion was assessed by two orthopedic surgeons (non-experimenters) in a double-blind setting.

2.2.2. Evaluation of bone graft fusion

The specimens were removed and examined by hand touch method. After removing the soft tissues around the lumbar spine, the posterior junction complex, and fixing the plate, the motion of fusion was detected by torsion. No motion was considered as successful fusion, and any movement was defined as non-fusion.

2.2.3. Intervention plan and specimen collection

The Rhodiola group intervened with the 100 mg/L Rhodiola group, and the combined group administered LIPUS on its basis as follows: LIPUS stimulation was given, and the frequency, intensity, pulse width and repetition rate were set to 1.5 MHz, 30 m W/cm^2, 200μs and 1 k Hz, respectively. The LIPUS working head was applied with coupling agent and the probe was kept stable, 20 min/time, once a day. Rabbits were sacrificed before intervention, 3 weeks after intervention and 6 weeks after intervention by air embolization of ear vein, and their tissues were taken as experimental samples, 10 rabbits each time.

2.2.4. HE dyeing

The sections were routinely dewaxed with xylene, treated for 10 min, and the operation was repeated once. Then the sections were hydrated with alcohol according to the concentration gradient of 100%, 100%, 95%, 90%, and 85%, each concentration lasted for 1 min, and then rinsed repeatedly with tap water. Hematoxylin staining solution was used for 15 min, and then rinsed with tap water for 1 min. Cell differentiation was carried out by adding 1% hydrochloric acid and alcohol solution. After 20 s of continuous reaction, the cells were rinsed under tap water for 1 min. Apply 1% dilute ammonia to reverse blue treatment, for 30 s and then rinse under tap water for 1 min; The cytoplasm was stained with 0.5% eosin aqueous solution. After 3 min, dehydration was carried out according to the concentration gradient of 85%, 90%, 95% and 100%. The dehydration time of 100% concentration was 2 min, and the dehydration time of the remaining concentration was 20 s, 30 s, and 1 min, respectively. After transparent treatment, xylene was used for treatment, lasting for 2 min. This operation was repeated for 3 times, and then the slices were sealed with neutral gum and placed under the microscope for detection and observation.

2.2.5. Immunohistochemical detection

Vascular endothelial growth factor (VEGF), cyclooxygenase 2 (COX-2) and prostaglandin E2 (PGE2) were detected by immunohistochemistry. PGE2 and transforming growth factor-β (TGF-β). Tissue samples were sectioned and dewaxed into water, and the antigen was repaired by microwave for 10 min. After repeated washing with phosphate-buffered saline (PBS) 3 times and incubation with 3% hydrogen peroxide for 20 min, goat blood was added and cleared and blocked at 37℃ for 30 min. After washing, rabbit anti-human VEGF antibody (1:200), rabbit anti-human COX-2 antibody (1:200), rabbit anti-human PGE2 antibody (1:200), rabbit anti-human TGF-β antibody (1:200) were added, and the samples were washed at 4℃ overnight. After washing with PBS, the secondary antibody working solution was added separately and incubated in a warm chamber for 30 min. After adding the horseradish peroxidase-labeled chain enzyme ovalbumin, the reaction was incubated for another 30 min. DAB was used to develop the color and terminate the reaction. The known positive VEGF, COX-2, PGE2 and TGF-β were used as positive control, and PBS was used as negative control. Two experienced pathologists independently read and interpreted the film, randomly selected 5 high-power microscopic fields (×400), VEGF, COX-2, PGE2 and TGF-β were considered as positive with brown-yellow particles in cytoplasm, and the percentage of positive cells was calculated, 0 scores: the percentage of positive cells < 25%; 1 score: 26% ~ 50%; 2 points: 51% ~ 75%; Three points: & gt; 75%; Dyeing intensity score, 0: no color; 1: light brown yellow; 2 points: brown yellow; 3 points: tan. The scores of the two items were summed, and ≥3 was considered as positive and < 3 as negative. The pathologist who read the film selected typical staining sites, excluded background staining and non-specific staining, and then took microphotography photos. Image Pro-PLUS 6.0 software was used for qualitative analysis of the photos, and the average optical density value of the samples with satisfactory histochemical staining was detected.

2.2.6. Biomechanical test

The specimens were thawed at room temperature and the spinal muscles were removed, leaving intact spinal ligaments, bones and joints. After the upper and lower segments of the lumbar spine were embedded, the strain sensor was used to load the animal specimens in stages. The parameters were set as 0, 50, 100, 150, and 200 N, and the load rate was set as 1.4 mm/min. The loading was applied to the mechanical center of gravity.

2.3. Statistical Methods

SPSS 24.0 software was used to process the data in the study. The count data were expressed as % by χ^2 test, the measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$) by t-test, and the multi-group data were

analyzed by F test, $P < 0.05$, the difference was statistically significant.

3. Results

3.1. Effect of bone grafting and fusion

The manual touch examination determined that all specimens were osteofused, and the fusion rate was 100.00%. No complete prolapse of the implanted bone block was seen. In four specimens, the anterior edge of the vertebral body was displaced, and the displaced area did not exceed 20% of the material, which did not affect the overall fusion effect of the patients. Attached is Fig. 1.

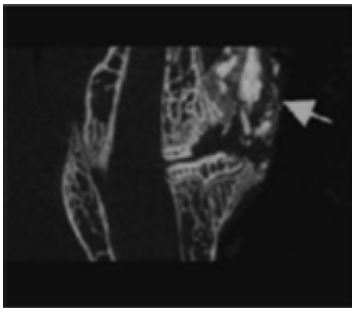

Fig. 1. Bone graft displacement. The arrow is for displaced bone grafting.

3.2. Histological results of the HE staining

In the combined group, some bone graft tissue underwent implanted osteocyte degeneration, cell necrosis, fibrocell proliferation and chondrocyte hyperplasia, chondrocytes differentiated into trabecular bone, hematopoietic cells appeared between some trabecular bone, the Rhodiola group, there were severe necrosis and fibrocell hyperplasia and late trabecular bone formation. Attached is Fig. 2.

Fig. 2. HE staining chart of bone tissue at rabbit bone graft fusion. (A) Trabecular bone marrow, (B) necrotic bone tissue, (C) fibroplasia. (200×).

3.3. Immunohistochemistry test results

The expression levels of VEGF, COX-2, PGE2, and TGF-β were all higher in the combined group than in the Red scene day group, and the data were statistically different (P <0.05). Attached Table 1-2, Fig. 3.

3.4. Biomechanical test results

The level of axial strength, axial stiffness and maximum compression load were higher in the joint group than in the Red scene day group, and the data were statistically different (P <0.05). Table 3-4.

4. Discussion

Osteoblast and cartilage cell proliferation and differentiation, can play a promoting bone matrix and bone trabecular structure formation and enhance the effect of osteoclast remodeling activity period and is the main pathological basis of spinal bone fusion, blood vessels and nerve fibers in osteogenesis area growth plays an important role in the process of bone growth, and LIPUS can promote the role of bone cell proliferation, differentiation, Then stimu-

Table 1. Results of immunohistochemistry of VEGF and TGF-β ($\bar{x}\pm s$).

Group	Time point	VEGF	TGF-β
Joint group	T1	0.58±0.05	0.63±0.12
	T2	0.73±0.03	0.78±0.11
	T3	0.93±0.03	0.98±0.12
Red scene day group	T1	0.58±0.04	0.63±0.13
	T2	0.68±0.02	0.73±0.12
	T3	0.82±0.02	0.88±0.12
$F_{\text{Observation time point}}$		34.512	325.733
$P_{\text{Observation time point}}$		<0.001	<0.001
$F_{\text{Groups observation time points}}$		543.236	522.165
$P_{\text{Groups observation time points}}$		<0.001	<0.001

Table 2. The immunohistochemical test results of COX-2 and PGE2 ($\bar{x}\pm s$).

Group	Time point	COX-2	PGE2
Joint group	T1	0.65±0.13	0.63±0.13
	T2	0.80±0.12	0.78±0.12
	T3	1.00±0.13	0.98±0.13
Red scene day group	T1	0.65±0.13	0.63±0.14
	T2	0.75±0.12	0.73±0.12
	T3	0.89±0.13	0.87±0.13
$F_{\text{Observation time point}}$		409.534	417.745
$P_{\text{Observation time point}}$		<0.001	<0.001
$F_{\text{Groups observation time points}}$		501.163	589.122
$P_{\text{Groups observation time points}}$		<0.001	<0.001

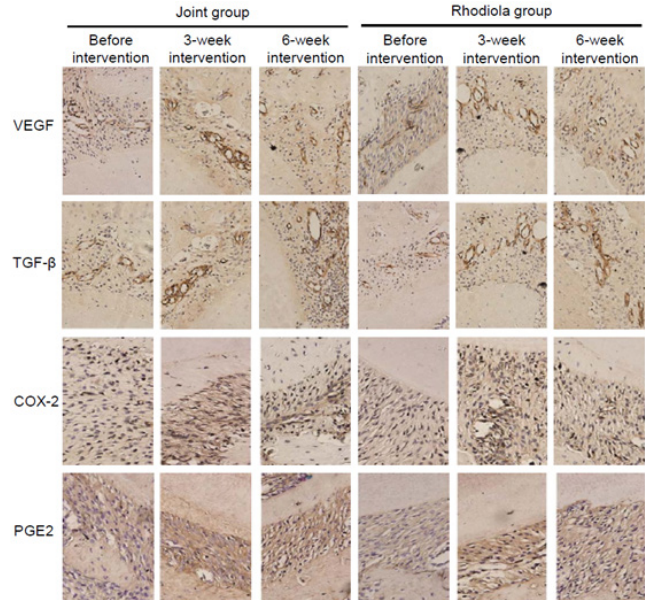

Fig. 3. The expressions of VEGF, COX-2, PGE2 and TGF-β were detected by immunohistochemistry.

late the secretion of bone matrix and osteogenesis, so as to promote bone formation [4].

The results of HE staining in this study showed that in the combined group, some bone graft tissues had degeneration of implanted osteocytes, cell necrosis, fibrocyte proliferation, chondrocyte proliferation, chondrocyte differentiation into bone trabeculae, and hematopoietic cells appeared in some bone trabeculae. In the Red Scene day group, the process of cell repair was relatively slow, and the chondrogenesis was retarded, cell necrosis was severe, and the formation of bone trabeculae was late. It is further demonstrated that the application of low-intensity ultrasound stimulation can accelerate the spinal fusion of rabbits by promoting chondrocyte differentiation [5].

PGE2 is an important messenger molecule produced by osteoblasts and plays an important role in the process of bone remodeling triggered by mechanical stimulation. COX-2 is an important enzyme protein for arachidonic acid demarcation and PGE2 synthesis and is closely related to tumor growth and inflammatory response. Previous studies have confirmed that COX-2 is an inducible expression gene. COX-2 plays an important role in bone remodeling and osteoblast proliferation and other biological processes. Under the action of mechanical stimulation, PGE2 and COX-2 in osteoblasts show abnormally high expression and regulate bone metabolism and bone remodeling [6, 7]. Angiogenesis is an important factor in bone fusion, which can provide necessary oxygen, nutrients and active factors for cells in the fracture area. VEGF is a cytokine that specifically promotes vascular growth and participates in the process of angiogenesis and bone tissue remodeling [8]. In this study, the levels of PGE2, COX-2, VEGF and TGF-β in the combined group were significantly increased after intervention, suggesting that LIPUS treatment could further enhance the promotion effect on bone formation. The mechanism of action is analyzed as follows: Type I Collagen (type 1 collagen, Col-1) is the main organic component of bone matrix and is a specific collagen secreted by osteoblasts. The realization of the mineralization function of osteoblasts depends on the network structure formed by Col-1, and Col-1 reflects the ability of osteogenic bone formation. Osteoblasts form mineralized nodules through a period of proliferation, Suggest that osteoblasts

Table 3. Biomechanical axial strength and stiffness changes ($\bar{x}\pm s$).

Group	Time point	axial strength (N)	axial rigidity (N/mm^{-1})
Joint group	T1	84.18±2.82	35.00±2.76
	T2	127.32±3.88	51.31±2.04
	T3	149.90±2.84	77.86±3.83
Red scene day group	T1	84.67±2.23	35.04±3.06
	T2	120.22±2.94	43.05±1.96
	T3	132.01±3.26	64.69±2.48
$F_{\text{Observation time point}}$		354.224	409.255
$P_{\text{Observation time point}}$		<0.001	<0.001
$F_{\text{Groups observation time points}}$		461.253	508.046
$P_{\text{Groups observation time points}}$		<0.001	<0.001

Table 4. Maximum compression load change in biomechanics ($\bar{x}\pm s$).

Group	Time point	Maximum compression load (N)
Joint group	T1	326.75±17.47
	T2	423.40±15.61
	T3	496.47±12.20
Red scene day group	T1	323.91±15.95
	T2	382.07±3.90
	T3	438.99±17.52
$F_{\text{Observation time point}}$		409.514
$P_{\text{Observation time point}}$		<0.001
$F_{\text{Groups observation time points}}$		541.083
$P_{\text{Groups observation time points}}$		<0.001

are differentiated and mature and osteogenic, Osteogenic-specific transcription factors (runt-related transcription factor2, Runx 2) is a member of the Runx transcription factor family, As an important transcription factor for osteoblast differentiation, Play an important role in early proliferation and differentiation maturation of osteoblasts, Important regulation of the maturation and stabilization of osteoblasts, Rhodiola can achieve a role in promoting osteoblast differentiation by upregulating the expression of Runx 2 with Col-1, This can protect osteoblasts and promote bone formation [9]. Molecules on the cell surface can sense local fluid flow and generate downstream signals. Integrin antibodies can reduce the sensitivity of osteoblasts to LIPUS. LIPUS can promote the expression of integrin on the cell surface, and then promote COX-2 synthesis by changing local fluid shear stress, thus affecting bone remodeling. Furthermore, G protein-coupled receptors are activated to promote osteoblast proliferation and differentiation, thereby promoting bone remodeling [10, 11]. LIPUS can activate the NO and hypoxia-inducible factor HIF-1α signaling pathway and promote the secretion of VEGF by osteoblasts, thus leading to the high expression of VEGF, suggesting that a large number of neovascularization occurs in the body, and the increase of blood flow promotes the rapid transition of fibrous bone to skeletal callus. LIPUS can promote angiogenesis in bone remodeling regions by upregulating VEGF expression. Enhancing endochondral ossification and bone formation [12, 13]; TGF-β can promote the differentiation of bone marrow mesenchymal stem cells into chondrocytes. Therefore, LIPUS may promote the secretion of TGF-β by osteoblasts and chondrocytes, and then promote the differentiation of mesenchymal stem cells into chondrocytes [14]. In combination with the biomechanics of the present study results, the posterolateral lumbar transverse process between autogenous iliac fusion has a good supporting role, but there is autogenous iliac implant fusion implant prone to drawbacks, such as shift, loose, hernia or collapse so take advantage of low intensity ultrasound to accelerate the bone fusion and improving the quality of healing, to improve the biomechanical performance.

5. Conclusion

In conclusion, LIPUS combined with Rhodiola can enhance the biomechanical properties of spinal fusion, while promoting bone formation by stimulating the high expression of PGE2, COX-2, VEGF, and TGF-β, which can be used as a reliable reference protocol for subsequent clinical treatment.

Conflict of Interests

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

We have received approval from the Animal Ethics Committee of Zhejiang Hospital.

Informed Consent

Not applicable.

Availability of data and material

The simulation experiment data used to support the findings of this study are available from the corresponding author upon request.

Authors' contributions

PH contributed to the study conception and design. Experimental operation, data collection and analysis were performed by ZL, LY, ZY, TY and YH. The first draft of the manuscript was written by ZL. All authors commented on previous versions of the manuscript.

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