



## Effect of low-intensity pulsed ultrasound combined with *Rhodiola rosea* on proliferation and differentiation of osteoblasts

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### ABSTRACT

Osteoblasts (OB), as the mesenchymal progenitor cells differentiated from the inner and outer periosteum and the stroma in bone marrow, can specifically secrete a variety of bioactive substances, thereby regulating and influencing the process of bone formation and reconstruction. Therefore, promoting the proliferation and differentiation of OB plays an important role in promoting bone formation. Based on this, this study studied the proliferation and differentiation of OB by low-intensity pulsed ultrasound (LIPUS) combined with *Rhodiola rosea* to provide a reliable theoretical basis for bone repair. In this study, rat OB was used as the research material and divided into groups A, B, C and D according to different intervention methods after osteogenic culture. Joint *Salvia miltiorrhiza* LIPUS ever seen from the results of the study promotes the strongest OB proliferation, at the same time, effectively reduces the apoptosis rate of the OB and apoptosis-related proteins expression, and promotes the OCN and ALO protein expression, indicated by LIPUS *Salvia miltiorrhiza* can effectively promote the osteoblast proliferation, differentiation, in order to promote cartilage repair and bone strengthen provides an effective means.

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### Introduction

Bone enhancement inhibition in articular cartilage injury and osteoporosis is a difficult and hot spot in clinical research, but the continuous development of tissue engineering technology also brings hope for the treatment of patients with cartilage injury and osteoporosis (1). At present, the problem still exists in the treatment work is how to obtain a sufficient number of seed cells and further optimize the performance of tissue-engineered cartilage (2). Low-intensity pulsed ultrasound (LIPUS) has been proved by most animal studies to promote the repair of subchondral defects, and its mechanism is mainly through the compound stress stimulation to promote the production of mesenchymal stem cells and does not require excess growth factors or enzymatic digestion factors to supplement (3-4). At the same time, according to scholars Tabuchi Y(5), LIPUS can promote the formation and proliferation of mesenchymal progenitor cells or hematopoietic stem cell colonies in vitro. These studies suggest that LIPUS may promote the proliferation and differentiation of OB, thereby improving cartilage performance. As a traditional Chinese medicine, related pharmacological studies confirmed that *Rhodiola rosea* can not only promote blood circulation and remove blood stasis, but also improve local microcirculation and enhance OB function (7). However, there are few studies on the combination of LIPUS and *Rhodiola rosea* in OB proliferation and differentiation. To further provide theoretical reference for improving bone repair, rat OB was cultured in vitro and relevant experiments were conducted in this study.

### Materials and Methods

#### Material

Rat OBMC3T3-E1 was purchased from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.  $\alpha$ -MEM was used as the basic culture medium, and the growth medium was made of 10% fetal bovine serum, 1% penicillin and streptomycin. On the basis of the growth medium, 50  $\mu$ g/ml vitamin C and 10 mM  $\beta$  sodium glycerophosphate were added to make the induced differentiation medium. Mouse OBMC3T3-E1 was subcultured in the presence of a growth medium, and the subcultured cells were placed in a carbon dioxide incubator for routine culture.

#### Methods

##### Cell treatment and grouping

After trypsin digestion, the cells were blown repeatedly to make single-cell suspension and counted. When the concentration reached  $1 \times 10^4$ /L, the cells could be seeded into the culture plate and divided into four groups: A, B, C and D. The cells in group A were used as the control group and were treated with normal saline. Group B cells were treated with LIPUS alone. Cells in group C were treated with 20  $\mu$ g/ml *Rhodiola rosea* alone. Group D cells were treated with LIPUS combined with 20  $\mu$ g/ml *Rhodiola rosea*.

In addition, SonaCell ultrasound equipment was used for LIPUS stimulation, and the equipment parameters were set as follows: pulse ultrasonic frequency 1.5 MHz, pulse repetition rate 1 kHz, pulse cycle -20%, and average ultrasonic intensity 100 mW/cm<sup>2</sup>. During stimulation, a

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sleeve should be closely combined with a coupling agent at the bottom of the culture plate for stimulation.

### Cell proliferation detected by CCK-8 (8)

Follow the relevant operations in the literature.

### Detection of apoptosis by flow cytometry

Cells were collected and then washed with PBS, and then resuspended with binding buffer. Annexin V-FITC (10  $\mu$ L) and PI (5  $\mu$ L) were added according to the instructions in the manual, and the cells were stained with dark light according to the temperature requirements in the manual. After 15min, the apoptosis rate was calculated by flow cytometry.

### WB was used to detect protein expression

Firstly, an appropriate amount of RIPA lysate was taken and slowly added into MC3T3-E1 cells. After adding MC3T3-E1, the total protein could be extracted accordingly. The BCA method was used here to accurately measure and calculate the concentration of relevant protein. Then the electrophoresis operation was continued, using SDS-PAGE, the protein gel was separated first, and then a PVDF membrane was transferred into the separated protein gel, which was blocked with 5% skim milk. The primary antibody was incubated overnight and the secondary antibody was Quantity One for subsequent protein quantification. Here we detected proteins including Bax, Bcl-2, OCN, ALP, and BMP-2.

### OB identification

Rat OB was further identified by ALP staining and alizarin red staining.

### Statistical analysis

Statistic Package for Social Science (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical analysis. Univariate ANOVA test was used to compare multiple groups, the Dunnett method was used for pairwise comparison with the control group as the reference, and the LSD method was used for pairwise comparison between groups.  $P < 0.05$  indicates a significant difference.

## Results

### Identification of rat OB

It could be seen from the staining results that the number of cells stained was small on the 3rd day of isolation and culture, and increased significantly on the 15th day as time went by. The cells formed calcium junctions on the 28th day of culture, and the results of alizarin red stain-

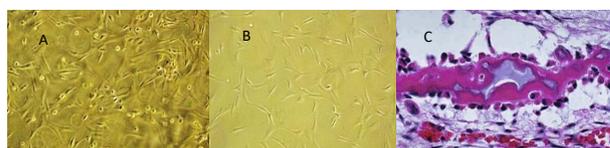
ing showed that the cells had been cultured as OB, which could be used for subsequent experiments. See Figure 1 for details.

### Comparison of cell proliferation in each group

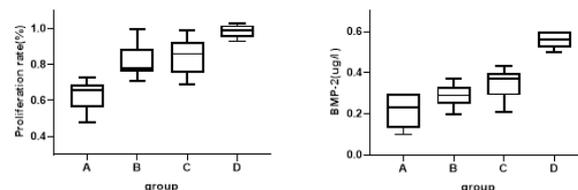
CCK-8 detection of cell proliferation and proliferation-related protein expression in each group showed that there were significant differences in OB proliferation rate and BMP-2 expression among the four groups, as shown in Table 1. The OB proliferation rate and BMP-2 expression in group D were higher than those in groups A, B and C, respectively, as shown in Figures 2 and 3.

### Comparison of apoptosis in each group

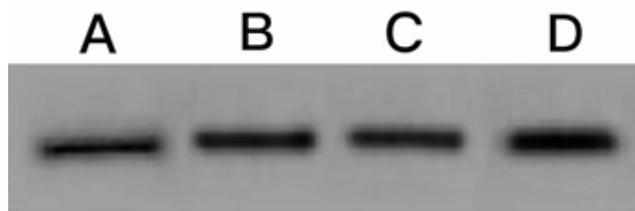
The apoptosis rate of group D was the lowest ( $0.34 \pm 0.02$ ), followed by group B ( $0.26 \pm 0.03$ ) and group C ( $0.26 \pm 0.03$ ), and there was no significant difference between the two groups. In addition, the apoptosis rate of group A ( $0.16 \pm 0.03$ ) was the highest, and the apoptosis rate of the four groups was significantly different ( $F=67.556$ ,  $P < 0.001$ ), see Figures 4 and 5 for details.



**Figure 1.** Identification of rat osteoblasts. Note: "A" refers to rat osteoblasts cultured for 3d; "B" is when rat osteoblasts were cultured for 15 days; "C" is when osteoblast culture is completed.



**Figure 2.** Cell diagram of osteoblast proliferation rate and BMP-2 expression in each group.



**Figure 3.** Blotting of BMP-2 in each group.

**Table 1.** Osteoblast proliferation in each group (n=9).

Group	Proliferation rate (%)	BMP-2 ( $\mu$ g/l)
A	$0.63 \pm 0.08$	$0.22 \pm 0.08$
B	$0.82 \pm 0.09^*$	$0.29 \pm 0.05^*$
C	$0.84 \pm 0.10^*$	$0.35 \pm 0.07^*$
D	$0.99 \pm 0.04^{*}\#\&$	$0.56 \pm 0.04^{*}\#\&$
F	28.700	47.837
P	$< 0.001$	$< 0.001$

Note: "\*", # and "&" respectively denote  $P < 0.05$  compared with "A, B and C", as shown in the following table.

**Comparison of apoptotic protein expression in each component**

WB experiment showed that the expressions of Bax and Bcl-2 were significantly different among the four groups. From the results, it could be seen that the expression of Bax was in the order of group A > Group B > group C > group D from high to low, but there was no significant difference between groups B and C. However, the expression of Bcl-2 was in the order of Group D > Group B > Group C > Group D from high to low. Similarly, there was no significant difference in Bcl-2 expression between groups B and C, as shown in Table 2 and Figures 6 and 7.

**OCN and ALP protein expression in each group**

The protein expressions of OCN and ALP were significantly different among the four groups, with the lowest expression levels in group A, followed by groups B and C, and the highest in group D (see Table 3 and Figures 8 and 9 for details).

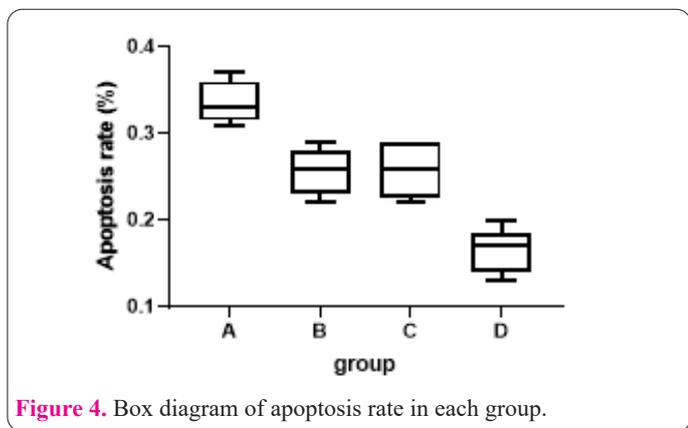


Figure 4. Box diagram of apoptosis rate in each group.

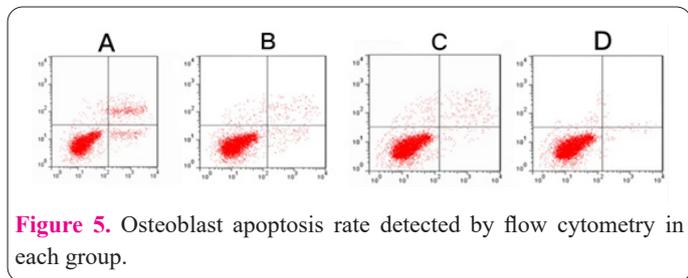


Figure 5. Osteoblast apoptosis rate detected by flow cytometry in each group.

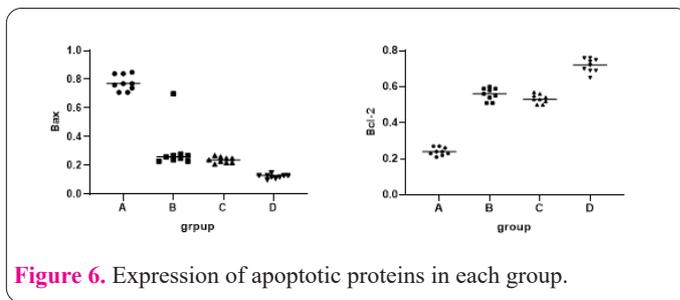


Figure 6. Expression of apoptotic proteins in each group.

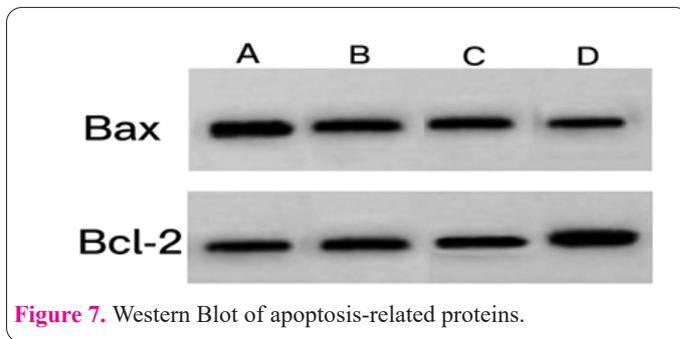


Figure 7. Western Blot of apoptosis-related proteins.

**Discussion**

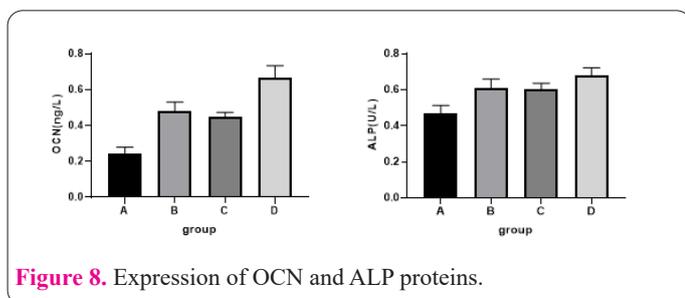
Ultrasonic wave is a high-frequency sound wave beyond the threshold range of human hearing, and it is also a form of mechanical energy, which transmits energy through high-frequency (1-12 MHz) pressure waves (8). LIPUS generally refers to intensity less than 100 mW/cm<sup>2</sup> (9). Duarte (10) first reported that LIPUS with an intensity of 30 mW/cm<sup>2</sup> could accelerate fracture healing in animals without any side effects, and proved that the stimulation mechanism may be related to the piezoelectric effect rather than the thermal effect. From 1994 to 2015, the Food and Drug Administration of the United States approved the clinical application of LIPUS for the treatment of delayed fracture union, nonunion, fresh fracture, etc. (11,12). In recent years, the promoting effect of LIPUS on bone defect healing has been widely applied in the medical field. A large number of in vivo and in vitro experiments have shown that LIPUS can promote the bone induction effect, accelerate the process of bone tissue repair, enhance

Table 2. Protein expression of apoptotic cells in each group (n=9).

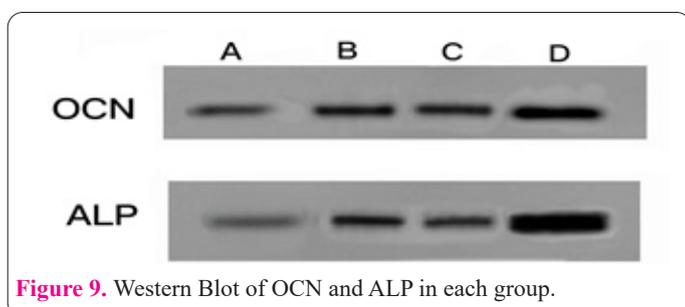
Group	Bax	Bcl-2
A	0.78±0.05	0.24±0.02
B	0.30±0.15*	0.56±0.03*
C	0.24±0.02*	0.53±0.02*
D	0.12±0.01*##	0.72±0.04*##
F	113.708	387.414
P	<0.001	<0.001

Table 3. Expression of OCN and ALP proteins (n=9).

Group	OCN(ng/L)	ALP(U/L)
A	24.33±0.04	0.47±0.04
B	0.48±0.05*	0.61±0.05*
C	0.45±0.03*	0.60±0.04*
D	0.66±0.07*##	0.68±0.04*##
F	117.454	37.333
P	<0.001	<0.001



**Figure 8.** Expression of OCN and ALP proteins.



**Figure 9.** Western Blot of OCN and ALP in each group.

the degree of bone bending resistance, and play an important role in all stages of fracture healing (13). Studies have shown that LIPUS affects the related cells that mainly take part in the bone healing between osteoblasts and bone marrow mesenchymal stem cells, osteoclasts, and cartilage cells, LIPUS to regulating cell proliferation, promotes its late early differentiation, the secretion of extracellular matrix, cell aggregation and migration, the synthesis of collagen and collagen, affect the secretion of cytokines, Thus, the healing of bone defects can be promoted through different pathways (14).

*Rhodiola rosea* is activating blood stasis, detumescence stops bleeding, step-down sedative meditation, and antibacterial effect of traditional Chinese medicine, modern pharmacology study shows that it has antioxidant, improve microcirculation and the effect of preventing thrombosis (15), in recent years, many studies showed that *Rhodiola rosea* monomer, combination drugs or the water-soluble and fat-soluble composition play an important role in contributing to bone and bone resorption, as the fat-soluble The sex component tanshinone II can promote the differentiation of osteoblasts and enhance cell activity (16). However, the effect of LIPUS combined with *Rhodiola rosea* on the proliferation and differentiation of osteoblasts has not been elucidated.

MC3T3-E1 cells are pre-osteoblastic cell lines with osteoblastic characteristics constructed from neonatal mouse skull osteoblasts, which can differentiate into mature osteoblasts under the effect of osteogenic agents. Therefore, MC3T3-E1 cells can be used as a cell model to study the differentiation process of osteoblasts (17). This study showed that compared with the cells in group A, the proliferation rate of cells in groups B, C and D was significantly increased, while the apoptosis rate was significantly decreased. LIPUS mainly transformed the mechanical effects of microcirculation caused by radiation, shear wave and liquid flow into biological effects for cells. They produce biological transmission mechanism is more complicated, however, are now known to include the MAPK signal transduction pathway and others of the kinase pathway, cell gap junction pathways, collection of integrin and raised, through this pathway role in tissue or cell, a change of a series of biological effects, such as cell

proliferation, differentiation, transfection, It regulates the secretion of growth factors and the expression of related genes (18). As a water-soluble active component of *Salvia miltiorrhiza*, also has significant effects on the proliferation, differentiation and anti-apoptosis of MC3T3-E1 cells. Xu X (19) explained its possible mechanism in his study, indicating that the osteogenic effect of water-soluble active components of *Rhodiola rosea* may be related to the activation of the Nrf2 signaling pathway.

Although the application of *Rhodiola rosea* and LIPUS alone can promote the proliferation and differentiation of OB to some extent, the combined application of *Salvia miltiorrhiza* and LIPUS showed that the effect of promoting the proliferation and differentiation of OB was more obvious, indicating that the combined effect of the two can promote the repair of bone tissue to a great extent. The results may provide a powerful theoretical basis for promoting cartilage repair and treating osteoporosis in the future. There are many reports (20-34) about the effect of medicinal plants in the treatment of various diseases.

In conclusion, LIPUS combined with *Rhodiola rosea* can effectively promote the proliferation and differentiation of OB. However, this study is still in the theoretical stage, and no relevant clinical studies have been conducted. Its clinical application value in patients with osteoporosis or cartilage injury needs to be further proved by clinical trials with large sample sizes.

#### Data Availability

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

#### Conflicts of Interest

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

#### Acknowledgments

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