Effects of Low-Intensity Pulsed Ultrasound on the Proliferation of Human Umbilical Cord Mesenchymal Stem Cells and Their Chondrogenic Differentiation via Bmp2 /Smad Signaling Pathway

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**Effects of low-intensity pulsed ultrasound on the proliferation of human umbilical
cord mesenchymal stem cells and their chondrogenic differentiation via BMP2
/smad signaling pathway**

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Abstract

Background: Although Low-intensity pulsed ultrasound (LIPUS) regiment has been applied to the treatment of cartilage injury clinically, the effect of LIPUS stress loading on the proliferation and differentiation of hUCMSCs is still controversial. Yet the specific mechanical mechanism of LIPUS stimulates cartilage differentiation of hUCMSCs has not been clarified.

Objective: To investigate the effects of low intensity pulsed ultrasound (LIPUS) on proliferation and chondrogenic differentiation of human umbilical cord mesenchymal stem cells (hUCMSCs) via BMP2 /smad signaling pathway in vitro.

Method: The hUCMSCs at P4 were randomly divided into control group(no LIPUS), L₃₀ group (30mW/cm²), L₅₀ group (50mW/cm²), L₈₀ group (80mW/cm²), each LIPUS group respectively loaded 5, 10, 20 min/d, the cell proliferation activity was detected by CCK-8 assay after continuous irradiation for 5 days. For chondrogenic differentiation, hUCMSCs were divided 4 groups as follows: control group, Noggin group, LIPUS group, Noggin+LIPUS group, LIPUS irradiation was given 50w/cm², 5min/d. Cartilage formation after 3 weeks was evaluated by Alician blue staining for GAG and COL II immunohistochemical staining. Furthermore, RT-PCR analysis of Sox-9, COL II Aggrecan, BMP2, Smad1, Smad5 and Smad9. **Result:** (1) The proliferation activity of L50 group(50w/cm² , 5min/d) was noticeably higher compared

with other groups, Alcian blue staining of GAG , COL II fluorescent staining in the LIPUS group were much stronger, quantitative RT-PCR assays showed that the expression of COL II, GAG, Sox-9, smad1, smad5 and smad9 in LIPUS group was significantly increased. **Conclusion:** The suitable LIPUS irradiation can promote cell proliferation, 50mW/cm² intensity at 5min/d is most significant. And it can promote chondrogenesis of hUCMSCs via upregulating MP2 /smads signaling pathway.

Key word: Low-intensity pulsed ultrasound; Umbilical cord mesenchymal stem cells; Bone morphogenetic protein; cell proliferation; chondrogenesis

1. Introduction

Stem cell based tissue engineering has emerged as a promising strategy for articular cartilage regeneration[1-4]. Human umbilical cord mesenchymal stem cells (hUCMSCs) derived from Wharton jelly tissues has the characteristics of stem cells and pre-chondrocytes, and naturally expresses Sox-9 and COL II positively, and it has the ability to differentiate and express multi-directional cells stably under specific conditions and stably express specific cell phenotypes. These characteristics make hUCMSCs an ideal seed cell for cartilage tissue engineering[5-8].

It is well known that stress loading is one of the most basic biological stimulation to cells in the process of biological evolution. MSCs can recruit, proliferate and

differentiate into chondroblast under stress stimulation, which is the cytological basis of new cartilage formation[9-13]. In recent years, it has been found that Low intensity pulsed ultrasound intensity ultrasound (LIPUS) integrates mechanical stimulation of compressive stress and fluid shear stress through high frequency and small amplitude pulsed pressure wave. LIPUS direct act on joints can improve the nutrition of articular cartilage, reduce the production of inflammatory mediators, promote the repair of articular cartilage defects and promote the proliferation, directional differentiation and homing of stem cells in vitro and in Vivo. It has been gradually applied to the repair and healing treatment of cartilage injury clinically[14-15].

However, the effect of LIPUS stress loading on the proliferation and differentiation of hUCMSCs is controversial[16-20].The suitable parameters of LIPUS including irradiation intensity and time need to be clarified. The specific mechanical mechanism of LIPUS stimulates cartilage differentiation and proliferation of hUCMSCs has not been fully elucidated.

Therefore, in our study, by evaluating the proliferation of hUCMSCs under different parameters of LIPUS loading conditions, suitable LIPUS loading conditions that could produce good chondrogenic differentiation effect on hUCMSCs in vitro were selected, and then the suitable parameter of LIPUS was loaded into hUCMSCs for chondrogenic differentiation and its effects through the BMP2 /Smad signaling

pathway was further evaluated.

2. Materials and methods

2.1 The LIPUS exposure sonacell ultrasound device

The LIPUS Stimulation SonaCell Ultrasound Equipment (Intelligent-Nano, Canada) is based on biological and clinical research. Its equipment parameters are: Pulse Ultrasound Frequency 1.5 MHz, Pulse Repetition Frequency 1 KHz, Pulse Cycle 20%. The average intensity of ultrasound can be adjusted to 0-100 mW/cm². The instrument probe is irradiated against the bottom of the culture plate through the coupling agent.

2.2 Cell culture and morphological observation

The P2 generation hUCMSCs (Saiye Biotechnology, Guangzhou, China) were adherent cultured in Dulbecco's modified Eagle's medium (DMEM; Saiye Biotechnology, Guangzhou, China) with 10% fetal bovine serum (Saiye Biotechnology, Guangzhou, China), 100mg/mL streptomycin, and 100U/mL penicillin at 37°C in an incubator with 95% air and 5% CO₂. After 24h, the non-adherent cells were discarded by fluid exchange, and the fluid was changed every 2-3 days. When the cell fusion reached 80%, the cells were digested by trypsin and passaged at the ratio of 1:2.

The cells were characterized morphologically under an inverted microscope (Olympus, Tokyo, Japan). Phenotype of hUCMSCs including CD29, CD34, CD44,

CD45, CD73 and CD105 was identified by flow cytometry, CD34 and CD45 are markers of haematopoietic stem cell.

2.3 CCK-8 assay of cell viability

P4 of hUCMSCs growing on 96-well plates at a density of $100 \mu\text{L}$ with $1.5 \times 10^4/\text{ml}$ were randomly divided into four groups: control group, L30 group ($30 \text{ mW}/\text{cm}^2$), L50 group ($50 \text{ mW}/\text{cm}^2$), L80 group ($80 \text{ mW}/\text{cm}^2$). The LIPUS irradiation groups exposed the cell culture plate to the probe element of the SonaCell device through the coupling agent and placed it in the cell incubator for LIPUS stimulation at 5min/d, 10min/d or 20 min/d respectively for 5 consecutive days, the control group was treated without LIPUS. And the culture medium was replaced after 24h. Another blank hole was taken and added into the culture medium as blank group. $10 \mu\text{L}$ of CCK-8 solution was added 24h, 48h, 72h, 96h and 120h later. Cells were cultured for an additional 4 hours and the absorbance at 450nm was measured using a microplate reader. The proliferation inhibition rate = $(\text{OD of experimental group} - \text{OD of blank group}) / (\text{OD of control group} - \text{OD of blank group}) \times 100\%$. Four groups of repeated experiments were performed. The data were statistically analyzed.

2.4 Effect of LIPUS on Chondrogenic differentiation of hUCMSCs

2.4.1 Experimental grouping

Adjust the cell density of P4 generation hUCMSCs to $1 \times 10^5/\text{ml}$, inoculate in a

6-well culture plate, and perform LIPUS stress loading (50mW/cm² , 5 min/d.).The hUCMSCs cultured with chondrogenic medium were divided into 4 groups as follow:

(A)control group: hUCMSCs were cultured in chondrogenic medium for 3 weeks;

(B)LIPUS group: hUCMSCs were given for LIPUS (50mW/cm², 5min/d) stress loading for 3 weeks; (C)Noggin group: hUCMSCs were added with BMPs antagonist Noggin 50ng/ml treatment and then cultured for 3 weeks; (D)LIPUS+ Noggin group: hUCMSCs were added BMPs antagonist Noggin 50ng/ml and LIPUS (50w/cm², 5min/d)were continuously loaded for 3 weeks.

2.4.2 Alcian blue staining

After the hUCMSCs were cultured in chondrogenic medium for 3 weeks, the cells of each group were washed with PBS three times, fixed with 4% paraformaldehyde at room temperature for 30min, PBS washed 3 times, 0.1M HCl solution was adde for 5 minutes, pH value drops to 1.0; 1% alein blue staining for 30 minutes; PBS washed thrice.Then the cells were observed under an inverted microscope, and took pictures with Image-ProPlus software.

2.4.3 Immunofluorescence staining

After the hUCMSCs were cultured in chondrogenic medium for 3 weeks, and the cells were washed thrice with PBS, fixed with 4% paraformaldehyde at room temperature for 15 min, and then washed with PBS three tims, treated with 0.5%

Triton×-100 transmembrane for 30min. PBS washed thrice, 0.5% bovine serum albumin (BSA) wet box at room temperature for 30min, anti-human primary antibodies against collagen II (dilution 1:100; Abcam UK) were added and incubated at 4°C overnight. After washing with PBS thrice, the fluorochrome-conjugated secondary antibody (1:500; Abcam UK) were added and incubated at 37°C in the dark for 1h. Followed by washing thrice with PBS, counterstained with 1ml diamidine phenylindole (DAPI; Molecular Probes, Waltham, MA, USA) at 37°C for 30min. The cells were observed and counted under an inverted phase contrast fluorescence microscope (Olympus, Tokyo, Japan). Applying IPP 5.0 software to analyze the average value of Col-2 absorbance of each group of cells (average absorbance = absorbance value/field of view).

2.4.4 Quantitative real-time (qRT)-PCR

Total RNA was extracted using qRT-PCR kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. RNA was converted into complementary DNA using an AMV RTase kit ((Tiangen Biotech, Beijing, China). PCR reaction body and reaction conditions are strictly according to the kit instructions, respectively detect Aggrecan, COL II, SOX-9, BMP2, smad1, smad5, smad9 gene expression. And according to the detected Ct value, calculate the mRNA expression level of each group of genes by $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = \Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}}$). Primer pairs

((Tiangen Biotech, Beijing, China) for target gene amplification are listed in Table 1.

Table I Fluorescence quantitative PCR primers.

Genes	Forward primer (5'3')	Reverse primer (5'3')
Sox-9	AAAGACCACCCCGATTA CAAG	TGAAGATGGCGTTAGG AGAGA
Aggrecan	ACCACAAATGGAGCAGT TCACC	GGTCTGGTTGGGGTAG AGGTA
COL II	GCTCCCAGAACATCACCC TACCAC	TGGACAGTAGACGGAG GAAAG
BMP2	CGTGAGGATTAGCAGGT CTTTG	TCGCTTGACGCTTTTC TCTTC
Smad1	CCAGTTTGTTCTCCTTC ATGA	CGCCCTTCTTCTTCTTC AGTT
Smad5	GGGTTTGAGGCAGTGTA TGAG	GGAGCCCATCTGAGTA AGGAC
Smad9	GCCCCCTGCACTGGTGCT CTG	GCGTACACCTCGCCCC CAC
GAPDH	AGAGCACCAGAGGAGGA CGA	TGGGATGGAAACTGTG AAGAGG

2.5 Statistical analysis

All data were expressed as mean $\pm$ standard deviation (SD) of three independent experiments and analyzed using SPSS 22.0 software (IBM Corp, Armonk, NY, USA). Multiple groups were compared by single-factor analysis of variance (ANOVA) and two groups by pair-wise Student's t test. P values < 0.05 were considered statistically significant.

3. RESULTS

3.1 General observation and characterization of hUCMSCs after LIPUS irradiation

The hUCMSCs were incubated under LIPUS stimulus for 5 days, the cells shaped as fusiform and grew in vortex, more cell colonies are observed in the L50 group (50w/cm², 5min/d) than in the control group. As increased with the pulse intensity and the irradiation time, the cell colonies was decreased. Flow cytometry assay showed that the hUCMSCs were positive for CD29 ($86.9 \pm 3.73\%$), CD44 ($99.91 \pm 7.33\%$), CD73 ($95.84.91 \pm 7.33\%$) and CD105 ($87.99 \pm 7.33\%$), and negative for CD34 ($4.54 \pm 0.83\%$) and CD45 ($1.19 \pm 0.88\%$).

3.2 The effect of LIPUS irradiation on cell proliferation

The results of hUCMSCs proliferation of each group after irradiating with different LIPUS intensities and duration time are shown(Fig. 1). At 5min/d, the cell proliferation rate of the L50 group was the highest ($P<0.05$). But the cell proliferation ability decreased with the increase of irradiation intensity and the pulse duration instead, cell proliferation is weakest at L80 group (80w/cm², 20min/d) ($P<0.05$).

Fig.1 After culture for 24, 48, 72, 96, and 120 hours, the effect of LIPUS on hUCMSCs proliferation capacity was tested by CCK-8 assay at (A) 5min/d, (B) 10min/d, (C) 20min/d. Note: * $P < 0.05$.

3.3 Effects of LIPUS irradiation on chondrogenic differentiation of hUCMSCs

3.3.1 Immunohistochemical staining results of cartilage-specific proteins GAG, COL II and DAPI

After 3 weeks of chondrogenic induction, Alcian blue staining of GAG in the LIPUS group was much stronger than that in the control, Noggin, LIPUS+ Noggin groups (see Fig.2:A3-D3). The staining in Noggin group was weaker than that in the control group(Fig.2:C3). Immunofluorescence staining for COL II and DAPI results showed that COL II green-stained fluorescence distribution and nucleus blue were seen around and between the nuclei of each group(Fig.2:A1-D1). The COL II green staining fluorescent and the DAPI fluorescent staining of the blue stained fluorescent particles in LIPUS group ($P=0.0054<0.01$), LIPUS+Noggin group($P=0.0078<0.01$) increased significantly compared with control group(Fig.2:A2-D2). But the expression of COL II in LIPUS + Noggin group ($P= 0.0419<0.01$) was decreased than that of LIPUS group(Fig.2:C1,D1).

Fig.2 Immunofluorescence staining for COL II, DAPI and Alcian blue staining results are shown after 3 weeks of chondrogenic induction: Control group (A); LIPUS group (B); Noggin group (C); Noggin+LIPUS group (D).

3.3.2 Quantitative RT-PCR detected the mRNA expression of COL II, GAG and Sox9

After continuous in vitro chondrogenesis induction culture and LIPUS loading with hUMSCs for 3 w, The mRNA expression of COL II, GAG and Sox9 were detected by RT-PCR. Compared with the control group, the expression of COL II ($P=0.0187<0.05$), GAG($P=0.0093<0.01$) and Sox9($P=0.0042<0.01$) were significantly increased in the LIPUS group. When hUCMSCs were stimulated with LIPUS 50mW/cm² intensity and BMP2 specific blocker Noggin, the expression of COL II($P=0.0212<0.05$), GAG($P=0.019<0.05$) and Sox-9($P=0.0308<0.001$) in LIPUS + Noggin group was significantly reduced than those of LIPUS group. However, after the addition of Noggin blocker, the expression of COL II($P=0.053>0.05$), GAG($P=0.012<0.05$) and Sox-9($P=0.033<0.05$) in Noggin group was significantly decreased than control

group.

3.3 Quantitative RT-PCR detected the mRNA expression levels of BMP2 signaling pathway related genes

Quantitative RT-PCR test results showed that the mRNA expression of BMP2($P=0.0172<0.05$), smad1($P=0.0061<0.01$), smad5($P=0.0166<0.05$), and smad9($P=0.0317<0.05$) in the LIPUS group was significantly higher than those in control group, And the expression levels of smad1($P=0.005<0.01$), smad5($P=0.018<0.05$) and smad9($P=0.034<0.05$) in the LIPUS +Noggin group was significantly less than those of LIPUS group, those in the Noggin group were the lowest among the experience group. But compared with the control group, Smad1($P=0.013<0.05$) and smad9($P=0.009<0.05$) in Noggin group are statistically significant increased, while there was no significant difference in smad5($P=0.067>0.05$) expression.

4. DISCUSSION AND CONCLUSIONS

At present, there is controversy about LIPUS irradiation can promote cell proliferation. Yoon JH et al has demonstrated that LIPUS can promote hUCMSCs proliferation, but no specific experimental screening of the radiation intensity parameters that promote cell proliferation was carried out [21]. On the contrary, Tien YC et al found that LIPUS stimulation did not promote the proliferation of

chondrocytes [22]. Aliabouzar M et al study has shown that the optimum frequency of sound wave, pulse repetition and duty cycle are 1.5 Mhz, 1 Khz and 20% respectively[23]. Our experimental studies found that optical irradiation with (50w/cm², 5min/d) intensity can significant promote the proliferation of hUCMSCs cells, but with the increase of irradiation intensity and the extension of irradiation time, the cell proliferation ability decreases instead, cell proliferation is obviously lowest with (80w/cm², 20min/d) intensity.

These results suggest that LIPUS irradiation has a "double-edged sword" effect to promote and inhibit cell proliferation. What are the reasons for the disparity in results between studies? We consider whether this is related to different pulse equipment parameters, pulse intensity, pulse action time, cell type, cell culture method, and number of cells seeded in different studies. So, further in vitro and in vivo experimental studies are needed for verification.

The effects of mechanical stimulation of LIPUS in cells are complex and interrelated. Numerous studies have demonstrated that BMP2, an important subtype of bone morphogenetic proteins (BMPs) of TGF β superfamily, plays an important role in inducing MSCs to differentiate into cartilage. BMP2 mainly transmits the signal from the cell membrane to the nucleus by activating the Smad/1/5/9 proteins, thereby promoting the differentiation of cartilage and maintaining the phenotype of

chondrocytes, chondrocytes lack of Smad1, Smad5 can cause severe dysplasia of cartilage. Noggin is a specific extracellular antagonist of BMP2 signaling, which can specifically bind BMP2 to prevent BMP2 from binding to its cell surface receptors, thereby inhibiting the effects of BMP2 biological function .

Our quantitative RT-PCR detection results showed that the expression of BMP2 and its key downstream signaling molecules Smad1, Smad5 and Smad9 in the LIPUS group were significantly up-regulated, and the expression levels of extracellular cartilage matrix GAG, COL II, and Sox9 were highest compared to the other groups after chondrogenic induction for 3W. When the exogenous BMP2 antagonist Noggin is added, the expression levels of COL II, GAG, and Sox-9 in the LIPUS+Noggin group is significantly reduced compared with the LIPUS group, and BMP2 and Smad1, Smad5, Smad9 were significantly down-regulated, indicating that Noggin can inhibit the chondrogenic differentiation of hUMSCs under the effect of LIPUS to a certain extent. These results suggested that the BMP2 /smads signaling pathway has an important positive regulatory role in the process of hUMSCS chondrogenic differentiation under LIPUS irradiation.

We observed that the expression of COL II, GAG and Sox9, and the expression of BMP2, Smad1, Smad5 and Smad9 in the Noggin group were significantly lower than that of the LIPUS group, LIPUS+Noggin group. However, compared with the control

group, the expression of smad1 and smad9 increased rather than decreased, while the expression of smad5 was no significant difference. Is it possible that the addition of the Noggin, an exogenous BMP2 inhibitor, may not completely inhibit the activation of BMP2? More work needs to be done to further explored. In addition, we found that the expression of Sox-9, COL II, and GAG in LIPUS+Noggin group was higher than the control group. Whether this may be related to the activation of other signaling pathways in the process of LIPUS promoting cartilage differentiation. More studies have verified that the interaction between multiple signaling pathways in the process of chondrogenic differentiation [24, 25].

In conclusion, LIPUS irradiation with suitable parameters can promotes the proliferation and chondrogenesis of hUMSCs, and the BMP2 /smads signaling pathway plays an important positive regulatory role in the process. Our findings uncover the mechanism of the effects of LIPUS on hUMSCs and provide some promising treatment for articular cartilage defects in the future.

Declarations:**Ethics approval and consent to participate:**

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of

Affiliated Haikou Hospital of Xianya Medical College. Written informed consent was obtained from individual or guardian participants.

Consent for publication:

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HJW and XHJ participated in study design conducted all experiments and sample analysis. XFC and JJC conceived this study and participated in the design of the experiments, provided assistance for data analysis and statistical analysis and reviewed the manuscript. ZMZ and SX participated in the development of the study design and data analysis. DGH contributed to the technical work of the model used in the study. All authors read and approved the final manuscript.

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Figures

Figure 1

After culture for 24, 48, 72, 96, and 120 hours, the effect of LIPUS on hUCMSCs proliferation capacity was tested by CCK8 assay at 5min/d, (B) 10min/d, (C) 20min/d. Note: *P < 0.05.

Figure 2

Immunofluorescence staining for COL II, DAPI and Alcian blue staining results are shown after 3 weeks of chondrogenic induction: Control group (A); LIPUS group (B); Noggin group (C); Noggin+LIPUS group (D).