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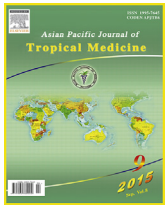

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Effect of CXCR4 pretreated with ultrasound-exposed microbubbles on accelerating homing of bone marrow mesenchymal stem cells to ischemic myocardium in AMI rats

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ABSTRACT

Objective: To investigate the role and potential mechanism of CXCR4 in promoting targeted homing of bone marrow mesenchymal stem cells (BMSCs) with ultrasound-exposed microbubbles (UM) pretreatment.

Methods: Third generation BMSCs were divided into four groups control group, ultrasound (US) group, UM group and ultrasound-exposed microbubbles plus catalase group. RT-PCR and western blot were performed to determine the levels of CXCR4 mRNA transcription and protein expression, respectively. Third generation BMSCs were labeled with Fluo-α/AM and divided into three groups: control group, US group and UM group, and fluorescence intensities in the cells were observed immediately, 5 min and 15 min after intervention under fluorescence microscope. The calcium iron levels in the cells were analyzed. BMSCs were divided into five group: group A without calcium in the medium, group B, group C, group D and group E containing calcium chloride with concentration of 1 mol, 2 mol, 4 mol, anti-calcium-sensing receptor antibody, respectively. RT-PCR and western blot were performed to determine the levels of CXCR4 mRNA transcription and proteins expression of the third generation BMSCs of each group, respectively.

Results: The levels of CXCR4 mRNA transcription and protein expression between US group and control group had no statistically significant difference ($P > 0.05$) shown by RT-PCR and western blot; the transcription level in the UM group was significantly higher than that in US group and control group ($P < 0.05$); and in the ultrasound-exposed microbubbles plus catalase group, the transcription level was much lower than that in UM group. Fluorescence intensify in the cells of US group had no significant difference compared with that in the cells of the control group ($P > 0.05$), which in the cells of UM group was significantly higher than that in the cells of both US group and control group ($P < 0.05$). Compared to group A, expressions of CXCR4 of group B to D were significantly increased in concentration-dependent manner showed by RT-PCR and western blot ($P < 0.05$). Compared to group C, expressions of CXCR4 of group E were significantly decreased ($P < 0.05$).

Conclusions: UM can promote the influx of calcium in BMSCs and increase mRNA transcription and protein expression of CXCR4. The latter may partly be caused by influx of calcium.

1. Introduction

Acute myocardial infarction (AMI) can lead to a mass of loss of cardiomyocytes and the endogenous regenerative capacity of myocardium is limited; thus, cardiac failure significantly increases after AMI and has a great impact on the life quality as well as anticipated life span of patients after AMI [1,2]. Stem cell transplantation is one of the most potential therapy for AMI [3]. Many researches show that stem cell transplantation can improve the cardiac function after AMI, despite that the improvement is not that satisfactory due to the low homing efficiency of stem cells, *etc.*; and how to accelerate and improve the homing and the therapeutic effect of stem cells remains the one of the current hot issues in research [4].

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Research finds that the injection of bone marrow mesenchymal stem cells (BMSCs) pretreated with ultrasound-exposed microbubbles (UM) via coronary artery plays a significant role in accelerating the homing of BMSCs to damaged myocardium, increasing the neovascularization in the cardiac ischemia area and meanwhile improving the cardiac function compared with that in group without treatment of UM [5]. There into, stromal derived factor 1 (SDF-l)/CXCR4 axis plays an important role in the homing of BMSCs [6,7]. The increasing expression of CXCR4 in BMSCs can accelerate the targeted homing of BMSCs to ischemic myocardium, the angiogenesis as well as myocardium generation so that the cardiac function is significantly improved [8,9]. In addition, the concentration of calcium ion in cells is closely related to the expression of CXR4 in cells, namely, the increase in concentration of calcium ion in cells within certain limits can increase the expression of CXCR4 [10]. However, there is no report about the relevant mechanism and effect of UM on expression of CXCR4 in stem cells for now. Therefore, the research plans to observe the effect of BMSCs pretreated with UM on the transcription and expression of CXCR4 in BMSCs as well as the calcium ion in BMSCs in order to explore the possible mechanism of UM pretreatment accelerating the homing of BMSCs.

2. Materials and methods

2.1. Reagents and equipments

Specific pathogen free male rats were purchased from Shanghai Lab Animal Center. Rats used for BMSCs extraction weighed 100–150 g while the ones for AMI model weighed 200–250 g. L-dulbecco's modified eagle medium (L-DMEM) solution and fetal bovine serum were purchased from Hyclone Company. Trypsin of 0.25% was purchased from Gibco Company. Taq DNA Polyrnerase and 1st-strand cDNA synthesis kit were purchased from Fermantas Company. Catalase was purchased from Sigma Company. Bradford protein assay kit was purchased from Bi Yun Tian Company. CXCR4 antibody and goat anti-rabbit IgG-HRP were purchased from sANTA CRUZ Company. Fluo-4/AM was purchased from Invitrogen Company. Western electrophoresis apparatus were purchased from Bio-Rad Company. DYY-6B nucleic acid electrophoresis apparatus was purchased from Beijing Liuyi Instrument Factory. MultiGene Gradient PCR cycle system was purchased from Labnet Company. Uv-2450 ultraviolet photometer was purchased from SHIMADZU Company. Transwell chamber was purchased from Millipore Company.

2.2. Experimental methods

2.2.1. In vitro extraction and culture of BMSCs

Male SD rats weighed 100–150 g were executed by cervical dislocation and the whole body was immersed for disinfection in 75% of volume fraction of ethyl alcohol for 10 min. Thighbone and tibia were taken on the super clean bench and rinsed with phosphate buffered saline (PBS) three times. The metaphysic of thighbone and tibia was cut off and the marrow cavity was revealed. The marrow was washed out by PBS. According to the proportion of 1:1, the marrow suspension was added along the wall with pipet into the upper layer of centrifuge tube loaded with isopyknic density of 1.073 g/mL Ficoll-Paque separating medium, and centrifuged at 2000 r/min for 20 min. The cells of interface layer (white film layer) was lightly sucked with pipet, put into the centrifuge tube, rinsed with PBS, and centrifuged twice. After that, the cells were resuspended with L-DMEM containing 10% of FBS, inoculated into 25 mL of cell culture bottle and cultured in incubator with 5% CO_2 at 37 °C. About 48 h later, the medium was changed for the first time and the cells not sticking to the wall were abandoned. Then, the medium was changed each 3 d and the growth of cells was observed. When the cell fusion reached 80%–90%, the passage was proceeded after 0.25% trypsinization according to the proportion of 1:2, and *in vitro* expansion was proceeded.

2.2.2. Pretreatment of BMSCs with UM

The third generation of BMSCs after culture were divided into 4 groups, control group, ultrasound (US) group, UM group and ultrasound-exposed microbubbles plus catalase (UMC) group. No intervention was conducted for control group. For US group, the irradiation of ultrasonic of 1 MHz and 1 W/cm^2 was performed for 30 s. For UM group, microbubbles were first added to make the final concentration of 10^6/mL and then the ultrasonic irradiation with the same condition was conducted. For UMC group, catalase (final concentration of 1250 U/mL) was first added to the culture bottle of BMSCs and then microbubbles was added to make the final concentration of 10^6/mL, and ultrasonic irradiation with the same condition was conducted.

2.2.3. Determination of mRNA of CXCR4 in BMSCs by RT-PCR

The transcriptional level of *CXCR4* mRNA in BMSCs was determined by RT-PCR. The primer sequence (353 bp) of actin was: upstream: 5′-GCTCGTCGTCGACAACGGCTC-3′; down stream: 5′-CAAACATGATCTGGGTCATCTTTTC-3′. The prime sequence (257 bp) of CXCR4 was: upstream: 5′-GGGCA ATGGGTTGGTAATC-3′; downstream: 5′-GGACAATGGCA AGGTAGCG3′. After the extraction of total RNA, arbitrary primers were used to synthesize cDNA in reverse. cDNA was used as template for PCR amplification. Primers were designed with Primer 5.0 and Oligo 6.0 software and 6 specific PCR primers were defined after secondary screening. The reaction conditions for PCR primers and internal reference reduced glyceraldehyde-phosphate dehydrogenase were: after 2-min initial denaturation at 94 °C, the following circulation began with 1-min denaturation at 94 °C, 1-min annealing at 62 °C, 1-min extension at 72 °C for 30 cycles. Then gel immunoelectrophoresis was performed after which gray analysis software BandScan 4.3 was used for result analysis.

2.2.4. Determination of expression level of CXCR4 in BMSCs by western blot

About 5×10^6 gastric carcinoma cells at logarithmic phase or post transfection were collected, rinsed with cold PBS twice into which 100 μL of cell lysis buffer was added. After that, the cells were put into ice-bath for 30 min and centrifuged at 12000 *g*/min at 4 °C for 10 min. Total protein was extracted by BCA Protein Assay kit and went through electrophoresis with sodium dodecyl sulfate polyacrylamide gel electrophoresis. Afterwards,

the gel was put under the constant voltage of 80 V and transferred onto nitrocellulose membrane. The membrane was sealed with 10 mL 10% defatted milk and corresponding primary antibodies CXCR4 (1:300) was added. After the overnight incubation at 4 °C, tris buffered saline with tween was used to rinse the membrane. The corresponding second antibody labeled by horse radish peroxidase (1:5000) was diluted with 2% defatted milk powder and incubated at room temperature for 1–2 h. After the membrane rinse with tris buffered saline with tween, coloration was fulfilled with enhanced chemiluminescence. The grey level on protein band was determined by Bandscan 5.0 software and the ratio of grey level on target band and the according value on β-actin was served as the indicator for the comparison of the expression strength of target protein in sample.

2.2.5. Determination of calcium ion in BMSCs

According to the instruction of Fluo-4/AM provided by Invitrogen Company, the concentration of Fluo-4/AM was made into 1 mol/L with dimethylsulfoxide, packed at 5–10 μL and preserved at −20 °C in away from light. The well-grown BMSCs of third generation were went through trypsinization and rinsed with PBS twice. Then, cell suspension was achieved with PBS and adjusted the concentration to 1×10^6/mL. About 2 μL mother solution was added into the 2 mL cell suspension, blown to be well-distributed, incubated at 37 °C for 10 min, centrifuged (1500 r/min, 5 min) and the supernatant was abandoned. The cells were rinsed with PBS twice to make sure that the fluorescence probe which did not enter into cells was completely rinsed out. PBS was again used for resuspension, and 24-well plate was moved in; and 3 groups came into being with control group, US group and UM group. For US group, irradiation of ultrasonic of 1 MHz and 1 W/cm^2 was performed for 30 s. For UM group, microbubbles were added to make the final concentration of 10^8/mL and then the ultrasonic irradiation with the same condition was performed. Immediately, 5 min, and 15 min after the intervention, the fluorescence intensity in cells was determined by fluorescence microscope. The excitation wavelength was 494 nm while the emission wavelength was 516 nm. A random 6 cells in a well was observed and photographed for the fluorescence intensity. The photographs were analyzed for the fluorescence intensity of each cell by Image-Pro Plus 6.0 analysis software. The concentration of calcium ion in BMSCs was expressed by fluorescence intensity and as mean ± standard deviation.

2.2.6. Effect of calcium ion on expression of CXCR4 in BMSCs

The original generation of BMSCs was cultured with calcium ion culture solution with different concentrations in 5 groups. A group: DMEM without calcium ion; B group: DMEM with 1 mL calcium chloride. C group: DMEM with 2 mL calcium chloride; D group: DMEM with 4 mL calcium chloride; E group: culture solution of C group added with CaSR specific antibody (final concentration of 10 pg/mL). After cultured to the third generation, the cells in all groups were determined by RT-PCR and western blot respectively for the transcription level of mRNA in CXCR4 and expression level of protein.

2.3. Statistical analysis

SPSS 19.0 statistical software was used for data analysis. Measurement data were expressed by mean ± standard deviation. t test was used for comparison between groups while ANOVA for among groups. $P < 0.05$ was considered as statistically significant difference.

3. Results

3.1. Effect of UM on mRNA transcription of CXCR4 in BMSCs

RT-PCR results showed that there was no significant difference in mRNA transcription level of *CXCR4* between US group and control group ($P > 0.05$) while the mRNA transcription level in UM group was significantly higher than that in US group and control group ($P < 0.05$). However, the mRNA transcription level of *CXCR4* in UMC group was significantly lower than that in UM group ($P < 0.05$) (Figure 1).

3.2. Effect of UM on protein expression of CXCR4 in BMSCs

Western blot results showed that the protein expression of CXCR4 in US group showed no significant difference with control group ($P > 0.05$) while the expression level in UM group was significantly higher than that in US group and control group ($P < 0.05$). However, the protein expression level of CXCR4 in UMC group was significantly lower than that in UM group ($P < 0.05$) (Figure 2).

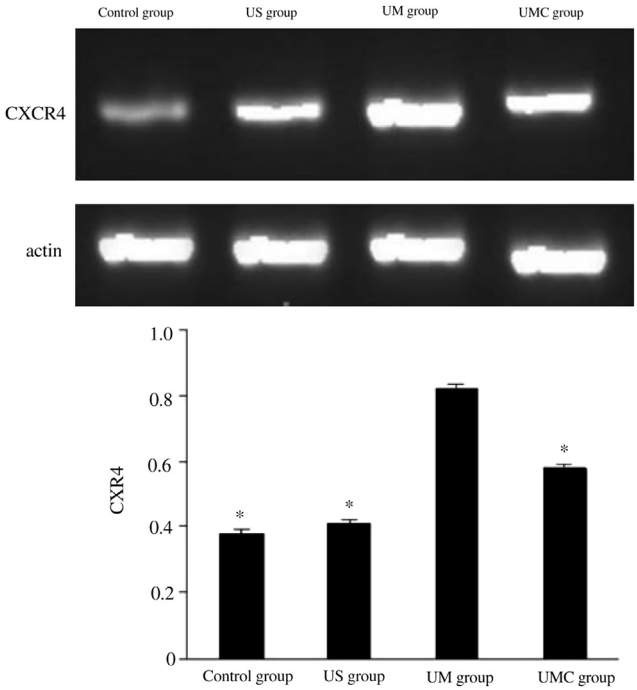

Figure 1. Effect of UM on mRNA transcription of *CXCR4* in BMSCs. *$P < 0.05$ compared with UM group.

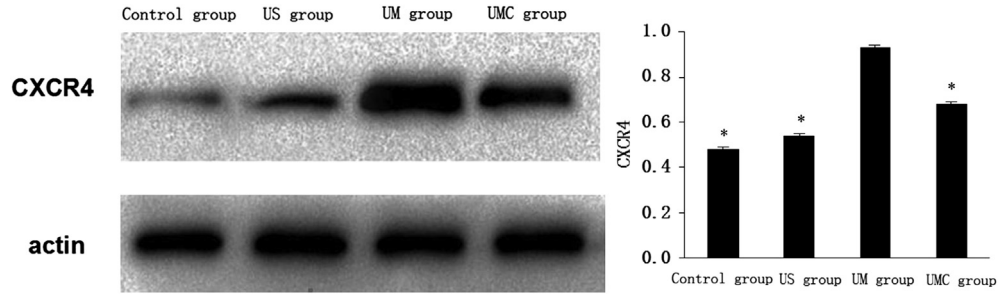

Figure 2. Effect of UM on protein expression of CXCR4 in BMSCs.
*$P < 0.05$ compared with UM group.

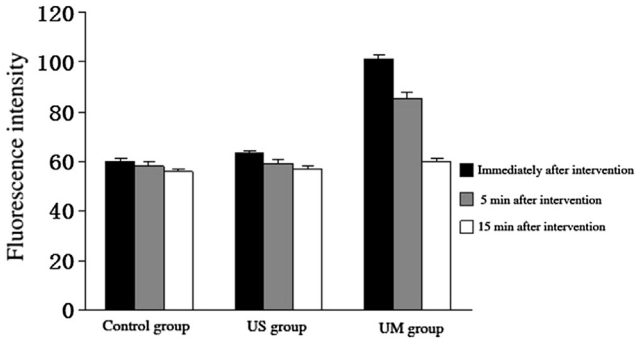

Figure 3. Effect of UM on calcium ion in BMSCs.

3.3. Effect of UM on calcium ion in BMSCs

Immediately, 5 min and 15 min after the US intervention, the fluorescence intensity in US group and control group showed no statistically significant difference ($P > 0.05$), suggesting that US intervention could not significantly increase the concentration of calcium ion in BMSCs. Immediately and 5 min after the UM intervention, the fluorescence intensity in UM group was significantly higher than that in US group and control group ($P < 0.05$), suggesting that UM could make significant increase in concentration of calcium ion in BMSCs. However, as the time went, the fluorescence intensity decreased; and in UM group, the fluorescence intensity 15 min after UM intervention was significantly lower than that immediately after intervention ($P < 0.05$), and no statistically significant difference was observed in comparison between UM group and US group, control group ($P > 0.05$) (Figure 3).

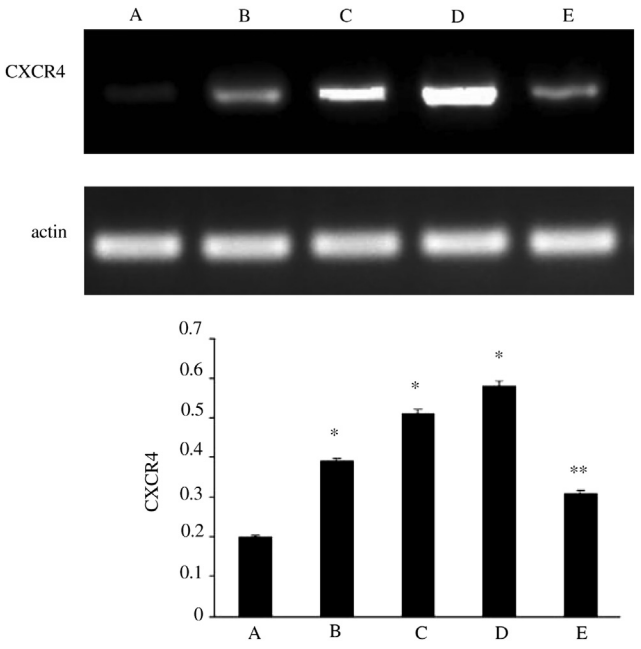

Figure 4. Effect of culture solution of calcium ion at various concentrations on mRNA transcription of *CXCR4* in BMSCs.
The concentrations of calcium ion in groups A, B, C and D were 0, 1, 2, 4 mol respectively; E group: culture solution of C group with calcium-sensing receptor antibody; *$P < 0.05$ in comparison with A group, with statistically significant difference; **$P < 0.05$ in comparison with C group, with statistically significant difference.

3.4. Effect of calcium ion on mRNA transcription of CXCR4 in BMSCs

The determination results of *CXCR4* transcription in BMSCs cultured with culture solution of calcium ion at different concentrations by RT-PCR showed that mRNA level in *CXCR4* was the lowest in culture solution without calcium and addition of 1 mol calcium chloride significantly increased the *CXCR4* transcription with concentration-dependent increase. The *CXCR4* expression reached higher after addition of 2 mol calcium chloride, and the increasing extent of *CXCR4* expression became small when 4 mol calcium chloride was added, with continuous rise ($P < 0.05$). The effect could be significantly inhibited by CaSR specific antibody ($P < 0.05$) (Figure 4).

3.5. Effect of calcium ion on protein expression of CXCR4 in BMSCs

The determination results of protein expression of CXCR4 in BMSCs cultured with culture solution of calcium ion at different concentrations by western blot showed that the protein expression level of CXCR4 was the lowest in culture solution without calcium and addition of 1 mol calcium chloride significantly increased the protein expression, with concentration-dependent increase. The CXCR4 expression reached higher after addition of 2 mol calcium chloride, and the increasing extent of CXCR4 expression became small when 4 mol calcium chloride was added, with continuous rise ($P < 0.05$). The effect can be significantly inhibited by CaSR specific antibody ($P < 0.05$) (Figure 5).

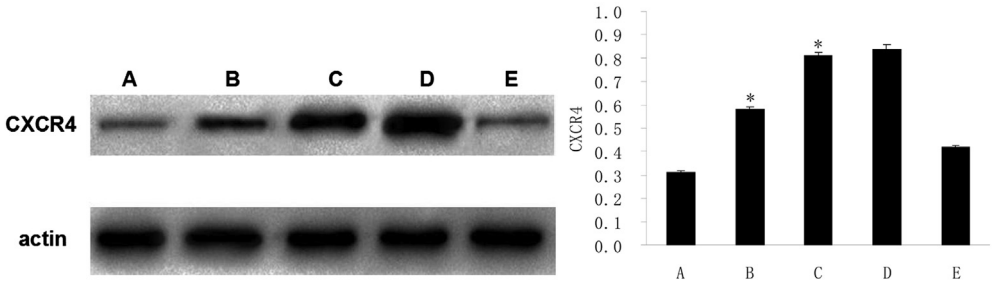

Figure 5. Effect of culture solution of calcium ion at various concentrations on protein expression of CXCR4 in BMSCs.
The concentrations of calcium ion in groups A, B, C and D were 0, 1, 2, 4 mol respectively; E group: culture solution of C group with calcium-sensing receptor antibody; $^{*}P < 0.05$ in comparison with A group, with statistically significant difference; $^{**}P < 0.05$ in comparison with C group, with statistically significant difference.

4. Discussion

4.1. Pretreatment with UM accelerating the homing of BMSCs by increasing CXCR4 expression

As the extensive and intensive researches on UM are started, UM have reached the many aspects of diagnosis and treatment of heart disease. The application in diagnosis aspect includes the evaluation in left ventricular function, heart infusion, myocardial infarction area and of whether ischemic myocardium is live, *etc.*, while in treatment aspect includes thrombolytic activity, targeting drug & gene delivery, *etc*. The present research explores the effect of pretreatment with UM on CXCR4 expression in BMSCs and it is found that after BMSCs are pretreated with UM, the mRNA transcription and protein expression in CXCR4 significantly increase, suggesting that at least part of the increasing CXCR4 expression mediates the rising curative effect of BMSCs pretreated with UM on AMI.

CXCR4 is the specific receptor of SDF-1. Many recent researches show that SDF-1/CXCR4 axis plays a key role in mediating the homing of stem cells to infarcted myocardium. The injection of BMSCs and microbubbles into coronary artery of pigs died of infarction with US irradiation on chest can accelerate the targeted homing of BMSCs [11,12]. Wang *et al.* found in their experiment that stem cells *in vitro* incubated with CXCR4 antagonist could significantly inhibit the homing of stem cells to ischemic tissues [10]. Bhakta *et al.* found in the *in vitro* experiment that the chemotaxis of BMSCs with overexpressed *CXCR4* after gene transfection to SDF-1 significantly increased [13]. Cheng *et al.* injected the stem cells of overexpressed *CXCR4* after gene transfection into AMI rats and found that compared with untransfected *CXCR4* group, the number of stem cells homing to partly damaged myocardium significantly increased in experimental group, and the cardiac function and ventricular remodeling after infarction were significantly improved [14]. Kyriakou *et al.* also attained the similar results [15]. The aforementioned experiments confirm from all aspects that the increasing expression of CXCR4 can accelerate the homing of BMSCs, which verifies our assumption.

4.2. Possible mechanism of pretreatment with UM accelerating BMSCs migration

After cells undergo the ultrasonic irradiation and suspension, the permeability of cytomembrane can be increased and the effect significantly increase when there are microbubbles, and meanwhile the material exchange between inside and outside cells can be caused, including the flow of ions, among which calcium ion is the one that has been studied the most [16,17]. In the present research, there was no statistically significant difference observed in fluorescence intensity of BMSCs immediately, 5 min and 15 min after US irradiation, compared with control group ($P > 0.05$). However, the fluorescence intensity after US intervention in UM group at the corresponding time was significantly higher than that in both US group and control group ($P < 0.05$), suggesting that US intervention can not significantly increase the concentration of calcium ion in BMSCs while the UM can. With time going by, the fluorescence intensity decreased, and in UM group, the fluorescence intensity 15 min after intervention was significantly lower than that immediately after intervention.

Park *et al.* found that under the effect of microbubbles irradiated by US, the permeability of cytomembrane of microvascular endothelial cells in rat brain and human umbilical vein endothelial cells for calcium ion increased and the calcium influx also increased [18]. On the other hand, Tsukarnoto *et al.* found that UM can increase the concentration of intracytoplasmic calcium ion in fibroblast [19]. In addition, under the function of UM, the calcium ion in myocardial cells of rats increased and without the intervention of microbubbles or US, this effect disappeared [20]. These researches all suggest that UM can accelerate the opening of calcium channel of cell membrane and make the concentration of calcium ion in cells increase. In the present research, under the intervention of UM, the calcium ion in BMSCs increased transiently with time going by. The permeability of cell membrane increased under the function of UM and can return to the state before intervention within 30 s after US intervention. During the time, the calcium ion in cells increased continuously and later the calcium homeostasis was established through overflow as well as the intake of intracellular calcium pool, and so forth. The whole process was completed within about 3 min.

Moreover, when BMSCs was cultured *in vitro*, the increasing concentration of calcium ion in culture solution can increase the expression of CXCR4. The effect can be inhibited by CaSR specific antibody, suggesting that the increase in concentration of calcium ion in BMSCs can increase the expression of CXCR4, and accelerate the migration of SDF-1 so that the targeted homing of BMSCs to ischemic myocardium *in vivo* may be accelerated. Cencioni *et al.* pretreated BMSCs with acidification, as a result, the expression of CXCR4 increased with calcium ion-dependent increase [14]. Wu *et al.* pretreated BMSCs with calcium chloride

and found the calcium influx increased and meanwhile the expression of CXCR4 increased, and migration to SDF-1 increased; the effect can be inhibited with CaSR antibody [21]. At the same time, after bone marrow derived stem cells were pretreated with calcium, the homing to ischemic tissues increased and the revascularization of ischemic limbs in rats was strengthen. The results of all the above experiments are in accord with the ones in the present research.

Therefore, under the function of UM, the permeability of cytomembrane in BMSCs increase, the influx of calcium ion is accelerated and the calcium ion in cells increase. The increase in concentration of calcium ion in cells can both increase the expression of CXCR4 in BMSCs and accelerate the migration of BMSCs to SDF-1, suggesting that after BMSCs is pretreated with UM, the migration of BMSCs to SDF-1 can be accelerated *in vitro* and the targeted homing of BMSCs to ischemic myocardium can be accelerated *in vivo*, through the possible mechanism of UM-calcium ion-CXCR4 axis. For sure, UM can cause transmembrane flow of various objects in BMSCs and the intracellular change in concentration of calcium ion can also lead to kinds of biological effects, like apoptosis, *etc*. Thus, the specific mechanism and signaling pathway involved require further research.

Conflict of interest statement

We declare that we have no conflict of interest.

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