Original Article
The role of Notch signaling pathway in mechanical stimulation of infrasound altering activities of bone marrow mesenchymal stem cells

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Abstract: Mechanical stimulation played a vital role in changing cell biology activities such as proliferation and apoptosis etc. Previous studies had proved that mechanical stimulation of infrasound could improve proliferation and inhibit apoptosis of bone marrow mesenchymal stem cells (BMSCs). This study aims to explore the role of Notch signaling pathway in mechanical stimulation of infrasound altering BMSCs activities. Primary BMSCs were derived from Sprague Dawley rats. The BMSCs, used at passage three, were divided into experiment groups which received infrasound intervention and γ-secretase inhibitor to inhibit Notch signaling pathway or γ-secretase inhibitor only, and control groups which received infrasound intervention only or no intervention. MMT was used to detect the cell viability. The mRNA and protein of Notch signaling pathway including Jagged1, Notch1 and Hes1 were assayed by qPCR and Western blot respectively. Infrasound could enhance BMSCs viability and significantly promote the expression of mRNA and protein of Jagged1, Notch1 and Hes1. However, mRNA and protein of Jagged1, Notch1 and Hes1 showed no difference regardless of infrasound intervention after inhibition of Notch signaling pathway. Notch signaling pathway seems to involve in mechanical stimulation of infrasound altering BMSCs activities and be activated by infrasound.

Keywords: Infrasound, Notch signaling pathway, BMSCs, Mechanical stimulation

Introduction

Stem cells are defined as cells having self-renewing ability to differentiate into other cell types, which make it possible to treat incurable diseases such as degenerative diseases and immunological diseased. Stem cells can be categorized as embryonic stem cells, tissue specific progenitor stem cells, umbilical cord stem cells and mesenchymal stem cells etc according to regenerative application [1]. Besides multilineage differentiation property of stem cell, Bone marrow mesenchymal stem cells (BMSCS) can be easily isolated from a small aspirate of bone marrow and have few ethical limitations, which make BMSCs to be one of most promising stem cells to be used in regenerative medicine [2]. It is urgent elements in clinical use to promote proliferation and induce lineage-specific differentiation of BMSCs. It has been reported that IGF-1 could promote proliferation and inhibit apoptosis of BMSCs [3] and glutathi-one monoethyl ester could also improve aged rat BMSCs properties and enhance the performance of aged people's BMSCs [4]. BMSCs containing progenitor cells of all tissues make it possible to differentiate into many cell types such as cardiomyocyte, neuronal subtype and osteoblast and so on [5, 6]. So far, promoting proliferation and inducing differentiation of BMSCs is a hot area of research.

Previous studies have demonstrated a better understanding on the control of proliferation and differentiation of BMSCs. But most studies focus on how biochemical composition change the proliferation and differentiation of BMSCs. Present researches show that mechanical intervention plays a vital role in regulating the proliferation and differentiation of BMSCs [7], particularly in osteogenic differentiation. Certain studies have found that mechanical stretch can stimulate the proliferation of human BMSCs [8]. Furthermore, reducing the fluid flow rate

while maintaining a constant peak fluid shear stress is associated with alterations in BMSCs' proliferation [9]. Chemotransport is an important way to improve BMSCs' proliferation treated by oscillatory fluid flow [9]. With regarding to differentiation, studies demonstrate that high-frequency vibration can increase osteogenic differentiation of BMSCs [10]. It is also evidenced that a repeated application of shear stress can stimulate late phenotypic markers of osteoblastic differentiation of BMSCs [11, 12]. Less study has been made to explore the effects of sound mechanical stimulation which is used widely in diagnosis and treatment on BMSCs. So the biologic effects of mechanical stimulation on BMSCs should be explored further.

Infrasound refers to inaudible noise at a frequency <20 Hz and may be considered to consist of mechanical vibrations that are difficult to detect with the human ear [13]. The effects of infrasound are disputed, with some researchers suggesting that infrasound is hazardous to the human body. Infrasound frequencies at 8 Hz, 130 dB, have been demonstrated to impair the rat myocardium and induce elevated levels of intracellular calcium ions in the hippocampal cells of the rat brain [14, 15]. However, a previous study had shown that infrasound with the sound pressure level <90 dB improved the motor function of rats following middle cerebral artery occlusion [16]. With regard to BMSCs, our previous study makes a conclusion that infrasound with the sound pressure level <90 dB can alter the growth of BMSCs [17]. However, the signaling mechanisms involved in the infrasound altering activity of BMSC remain unclear.

The Notch signaling pathway is highly conserved to regulate cell proliferation, apoptosis and differentiation. Notch signaling pathway is initiated when Notch ligands like Jagged1 bind to cell surface Notch receptors like Notch1 on neighboring cells [18]. So it is a common method to regulate cell activities via regulation of Notch signaling pathway. It is reported that Jagged1, Notch1 and Hes1 among Notch signaling pathway was mostly correlated with proliferation of BMSCs [19]. Other study show equilibrium between Notch ligands will determine the angiogenesis [20]. The study on Notch signaling pathway involved in differentiation find that down-regulation of Notch signaling

pathway can induce differentiation of BMSCs into neurons, while activation inducing differentiation of BMSCs into endothelial cell and osteoblast [21-23]. Few studies make an explanation on the effectiveness of mechanical stimulation on Notch signaling pathway of BMSCs. In this study, we will focus on the changes of Notch signaling pathway of BMSCs intervened by the infrasound.

Materials and methods

Infrasound device

The infrasound device (Chi-8™) used in the present study was manufactured by the Chi Institute (San Juan Capistrano, CA, USA). The device consisted of two sections containing the transmitting probe and the mainframe. The infrasound device consists of three options, for which option three was used for this study, producing a frequency of 4-20 Hz and a sound pressure level of 79-86 dB.

Animal experiments and ethical approval

BMSCs were harvested from female Sprague Dawley rats (weight, 100 g; age, 4 weeks) provided by the Animal Center of the Southern Medical University (Guangzhou, China). All experimental procedures on the rats were approved by the Animal Ethics Committee of Nanfang Hospital (Permit number: NFYY20-120128; Guangzhou, China). The rats were anesthetized with 10% chloral hydrate and then sacrificed by cervical dislocation.

BMSC harvest and culture

The rat femora and tibiae were aseptically excised and the epiphyses of the bones were removed. The bone marrow was flushed from the shaft with Dulbecco's modified Eagle's medium (DMEM)/F12 (Hyclone, Waltham, MA, USA) using a 20-gauge needle. The bone marrow suspension was disaggregated by pipetting several times and the cells were collected by centrifugation (2,200*g, 5 min) [17]. The cells were then cultured in DMEM/F12 with 10% fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) containing 100 μl/ml penicillin-streptomycin in 25 cm^2 cell culture flasks (Corning Inc., Acton, MA, USA). The cells were incubated at 37°C in a 5% CO_2 atmosphere. After 48 h, non-adherent cells were removed using phosphate

Table 1. Primers used for qPCR

Gene	Primer sequence	Length (bp)	Accession No
β-actin	Forward: 5'-TTGGTGGCTCTATCCTGGCCTC-3'	131	NM_031144.2
	Reverse: 5'-AAACGCAGCTCAGTAACAGTCCG-3'		
Jagged1	Forward: 5'-GAAGGATGGGATAGGCTTCTTC-3'	137	NM_019147.1
	Reverse: 5'-GCCTCAGTCAGTCCTTAGAATG-3'		
Notch1	Forward: 5'-CTACTACCTCTGCTTATGCCTC-3'	120	NM_001105721.1
	Reverse: 5'-TCGTAGCCATCGATCTTGTC-3'		
Hes1	Forward: 5'-GGAAATGACAGTGAAGCACCTC-3'	118	NM_024360.3
	Reverse: 5'-CTCGTTCATGCACTCGCTG-3'		
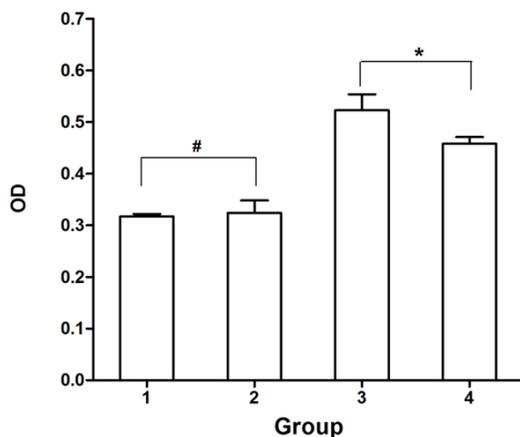
Figure 1. The cell viability. BMSCs of group 1 received γ-secretase inhibitor; group 2 received γ-secretase inhibitor and infrasound; group 3 received infrasound; group 4 received no treatments. MTT was used to evaluate the BMSCs viability. OD value of group 2 was higher than it of group 1, but no significance. OD value of group 3 was significantly higher than it of group 4. Infrasound could significantly improve the BMSCs proliferation. #; $P>0.05$, *; $P<0.05$.

buffer saline (PBS; Boster Biological Tech Ltd, Wuhan, China) to rinse the cells. The culture medium was replaced every second or third day. When the cells reached 85% confluence, the primary culture was subcultured 1:2; third-passage BMSCs were used in the present study.

Groups and treatments

The third passage of BMSCs was randomly divided into four groups with different treatments as follow: group 1 (γ-secretase inhibitor without infrasound), group 2 (γ-secretase inhibitor with infrasound), group 3 (infrasound without γ-secretase inhibitor) and group 4 (without any treatments). BMSCs of group 1 and group 2 were used γ-secretase inhibitor (DAPT, Selleck, China) to inhibit Notch signaling pathway. And

then, four groups were detached with 0.25% trypsin and resuspended in a 5 ml tube with DMEM/F12 in 10% fetal bovine. group 2 and group 3 were exposed to infrasound for 60 min; while group 1 and group 4 exposed to air for 60 min. Following the interventions, the cells were immediately incubated at 37°C in a CO_2 atmosphere for 72 h. BMSCs were seeded in 6-well plates for quantitative polymerase chain reaction (qPCR), 6 cm^2 plates for western blot and 96-well plates for MMT. Same amount of cells were ensured to analyze the results of different groups.

MTT for the cell viability

3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay was used to measure the proliferation of BMSCs. 20 μl MTT (Sigma) solution was added in each well at the concentration of 5 mg/ml (pH 7.4). After 4 h of incubation for MTT formazane formation, supernatant was removed and 150 μl dimethyl sulphoxide (DMSO) was added with fully vibration to dissolve the formazane. Absorbance was measured at a major wavelength of 490 nm to obtain OD value and repeat it three times.

qPCR for mRNA

The BMSCs were harvested and homogenized for RNA extraction with TRIzol™ reagent (Invitrogen Life Technologies). Messenger RNA was reverse-transcribed to cDNA using Primescript RT Reagent kit (Takara Bio, Inc., Shiga, Japan). qPCR was then conducted to measure the mRNA expression level. The expression levels of the β-actin housekeeping gene were measured as a control. The primer sequences are shown in **Table 1**. A volume of 2 μl total cDNA from each sample was amplified in a final volume of 25 μl reaction mixture containing SYBR® Green I (Takara Bio Inc., Shiga, Japan)

A **Notch1 mRNA**

B **Jagged1 mRNA**

C **Hes1 mRNA**
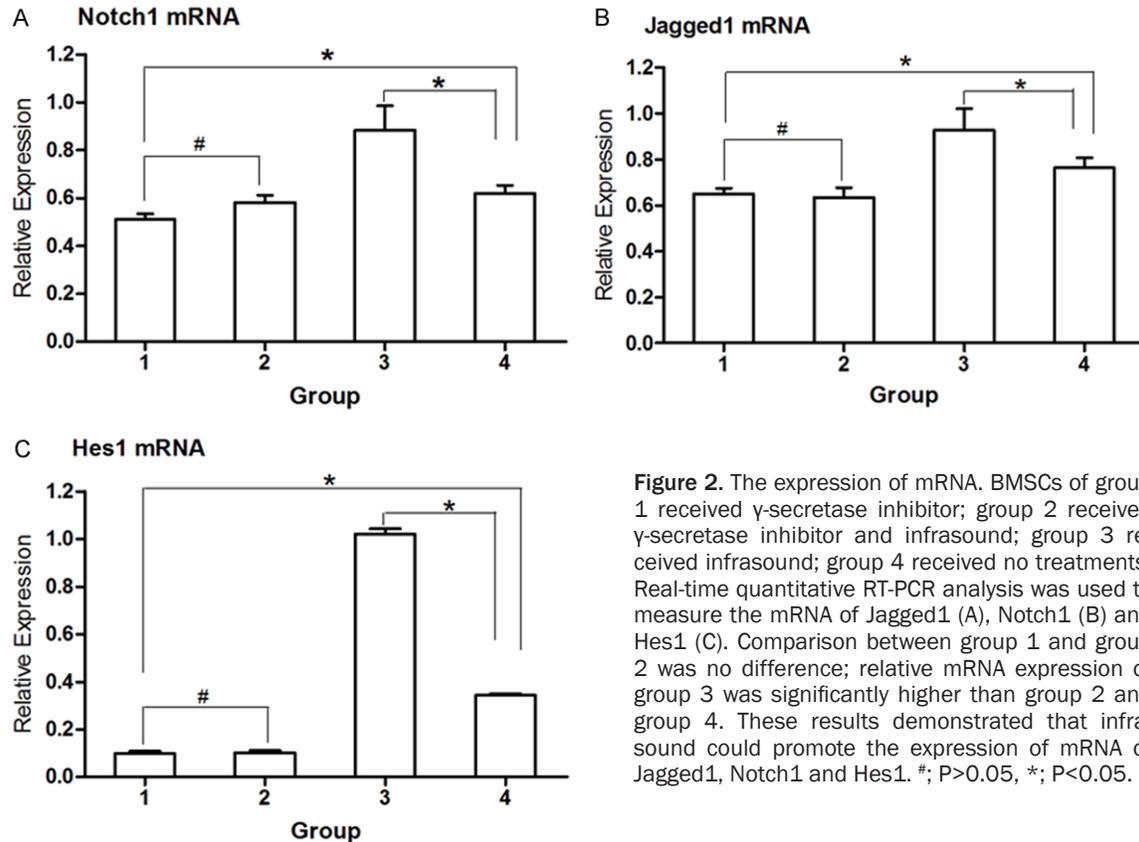
Figure 2. The expression of mRNA. BMSCs of group 1 received γ-secretase inhibitor; group 2 received γ-secretase inhibitor and infrasound; group 3 received infrasound; group 4 received no treatments. Real-time quantitative RT-PCR analysis was used to measure the mRNA of Jagged1 (A), Notch1 (B) and Hes1 (C). Comparison between group 1 and group 2 was no difference; relative mRNA expression of group 3 was significantly higher than group 2 and group 4. These results demonstrated that infrasound could promote the expression of mRNA of Jagged1, Notch1 and Hes1. #; P>0.05, *; P<0.05.

using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Inc., Foster City, CA, USA). The cycling conditions were as follows: 95°C for 30 sec, 40 cycles at 95°C for 5 sec and 34 sec at 60°C.

Western blot for protein

BMSCs were lysed with 200 µl lysis buffer on ice for 20 min. Equal amounts of protein extracts were fractionated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). These nitrocelluloses were incubated with the following primary antibodies: Jagged1 (Abcam, UK) Notch1 (Abcam, UK) and Hes1 (Abcam, UK). The proteins were visualized using enhanced chemiluminescence reagent (Pierce, Rockford, IL) and photographed by Quantity One software (Kodak, 4000R PRO). β-actin (Beyotime, China) was used to be as loading control to normalize the results.

Statistical analysis

All the results are presented as the means ± standard deviation for each group. The data were analyzed using SPSS software (SPSS, Inc., Chicago, IL, USA). The results of MTT, qPCR and western bolt were analyzed with one-way analysis of variance and LSD method was used to analyze the difference between groups. A difference of *P*<0.05 was considered to indicate a statistically significant difference.

Results

Results of cell viability

OD value of group 2 (0.324±0.024) was higher than it of group 1 (0.317±0.005), but no significance. OD value of group 3 (0.523±0.031) was significantly higher than it of group 4 (0.458±0.013) (P<0.05) (Supplementary Data). These results showed that infrasound could enhance BMSCs viability effectively, but the effect could be impaired by inhibiting the Notch signaling pathway (**Figure 1**).

The results of qPCR

Notch1 mRAN of group 1, group 2, group 3 and group 4 were 0.510±0.023, 0.581±0.030, 0.882±0.103 and 0.619±0.034 respectively.
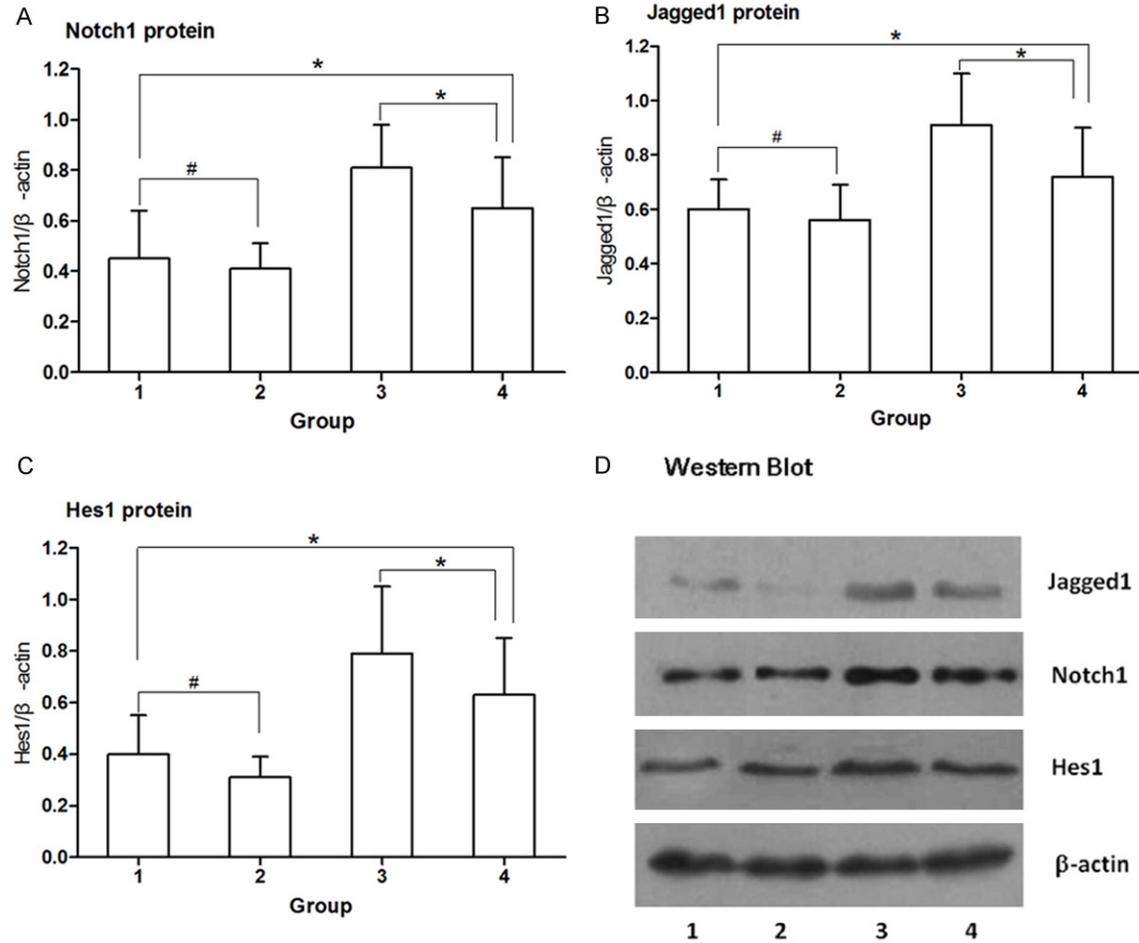
Figure 3. The expression of protein. BMSCs of group 1 received γ-secretase inhibitor; group 2 received γ-secretase inhibitor and infrasound; group 3 received infrasound; group 4 received no treatments. Western Blot was used to measure protein expression of Jagged1 (A), Notch1 (B) and Hes1 (C). (D) was the protein sample measured by WB. Protein expression of group 1 was no significant difference compared with group 2; relative expression of group 3 was significantly higher than group 2 and group 4. #; P>0.05, *; P<0.05.

Jagged1 mRAN of group 1, group 2, group 3 and group 4 were 0.649±0.025, 0.633±0.043, 0.926±0.094 and 0.763±0.044 respectively. Hes1 mRAN of group 1, group 2, group 3 and group 4 were 0.099±0.009, 0.101±0.008, 1.021±0.022 and 0.342±0.009 respectively (Supplementary Data). Comparison of Jagged1, Notch1 and Hes1 mRNA between group 1 and group 4 were made to observe the effects of γ-secretase inhibitor on Notch signaling pathway. Those of group 1 were significantly less than those of group 4, especially Hes1, which indicated that γ-secretase inhibitor inhibited the Notch signaling pathway successfully. In order to know the effects of infrasound on BMSCs, the difference between group 3 and group 4 was analyzed. The Jagged1, Notch1 and Hes1 mRNA of group 3 were increased sig-nificantly compared with those of group 4. It demonstrated that infrasound seemed to up-regulate the Notch signaling pathway. After inhibition of Notch signaling pathway, Jagged1, Notch1 and Hes1 mRNA between group 1 and group 2 showed no significant difference (**Figure 2**).

The results of western blot

Notch1 protein of group 1, group 2, group 3 and group 4 were 0.45±0.19, 0.4±0.10, 0.81±0.17 and 0.65±0.20 respectively. Jagged1 protein of group 1, group 2, group 3 and group 4 were 0.60±0.11, 0.56±0.13, 0.91±0.19 and 0.72± 0.18 respectively (Supplementary Data). Hes1 mRAN of group 1, group 2, group 3 and group 4 were 0.40±0.15, 0.31±0.08, 0.79±0.26 and

0.63±0.22 respectively. After inhibition of Notch signaling pathway, Jagged1, Notch1 and Hes1 protein of group 1 were significantly decreased compared with those of group 4. We found that BMSCs of group 3 expressed Jagged1, Notch1 and Hes1 protein more than those of group 4 after infrasound treatment. When analyzing the difference between group 1 and group 2, we also found no significant difference between two groups. These results also demonstrated that infrasound seemed to activate Notch signaling pathway (**Figure 3**).

Discussion

Mechanical stimulation plays a vital role in cell biology including proliferation, differentiation and gene and protein expression via transforming mechanical stimuli into biochemical signal [24]. It is a critical factor to form micromechanical environment to influence cell biology [25]. Micromechanical environment can transform mechanical signal into biochemical signal and then result in subsequent genetic or protein changes which is the key to lead to biological activities changes [26]. Some studies show that cyclic compression can improve the proliferation of BMSCs [27]; hydraulic pressure can induce BMSCs into chondrogenic differentiation [28]; equiaxial cyclic stimulation on BMSCs can promote GATA4 expression which can help to form functional cells in cardiovascular engineering [29]. So when we explore the influence factors of cell biology, micromechanical environment shouldn't be ignored. Mechanical stimulation of infrasound is a controversial therapeutic method. Organisms may be considered as mechanical vibration systems and certain parts of the body exhibit natural frequencies within the range of infrasound frequency; thus infrasound may produce resonance reactions in the body, resulting in physical changes and chemical reactions [30]. Infrasound can also affect cells through forming mechanical vibration environment. This study demonstrated that infrasound could promote the proliferation of BMSCs, but the effect could be impaired by inhibiting the Notch signaling pathway. So we should move forward to concrete effects of infrasound on signaling pathway.

Different cell activities depend on different mechanisms which signaling pathway involve in. Li et al study [31] indicates that p38MAPK

signaling is important in transduction of mechanical signals during chondrogenesis and BMP signaling play a role in process of dynamic compressive stress increasing cell viability [32]. Wu and Chen's [33] study shows that mechanical signals stimulate the differentiation extent by increasing the peak of marker synthesis instead of altering the speed of differentiation. Other study demonstrate that FAK-ERK1/2 signaling pathway involve in promoting BMSCs migration [34]. Thus when we explore the mechanical stimulation on cell activities, analysis of signaling pathway should be also included. Notch signaling pathway is well known to control cell fate through cells interaction. Notch signaling pathway is required for neuron and glia formation to involve in neurogenesis, especially the expression of Notch1 [35]. When Notch signaling pathway is activated, neighboring stem cells will switch from neurogenesis to gliogenesis [36]. Up-regulation of Notch activity resulted from neuronal contacts lead to restriction of neuronal growth in mature cerebral cortex. Activation of Notch signaling pathway involve in Simvastatin enhancing BMSCs differentiation into endothelial cells [22]. Mechanical force can act on Notch signaling pathway via transendocytosis of the Notch extracellular domain into the neighboring ligand-bearing cell and cause conformational changes of ligand interaction site [37]. It is reported that mechanical pulling force generated by endocytosis of Notch-bound ligand can change the Notch receptor and facilitates its sequential proteolytic cleavage [37].

This study showed that Notch signaling pathway involved in the change of BMSCs activities treated by infrasound. In this study, we found that the expression of Jagged1, Notch1 and Hes1 mRNA and Protein of BMSCs were significantly increasing under only intervention of infrasound. The result indicated that infrasound could alter the Notch signaling pathway of BMSCs and induce its up-regulation. γ-secretase inhibitor plays an important role in inhibiting Notch signaling pathway and other processes that are involved in cells fate decisions [38]. So our study chose γ-secretase inhibitor to inhibit Notch signaling pathway to observe the effect of infrasound on it. Since γ-secretase inhibitor blocks the release of NICD, the signal couldn't transmit to cell nucleus to activate target gene like Hes1 [39]. After using γ-secretase

inhibitor, we observed that the Jagged1, Notch1 and Hes1 mRNA and Protein were all decreased obviously, particularly the remarkable reduction of Jagged1. The results demonstrated that γ-secretase inhibitor reached to the purpose that Notch signaling was inhibited in our study, which many studies had also proved [40]. When Notch signaling was inhibited, infrasound couldn't decrease or increase Jagged1, Notch1 and Hes1 protein and mRNA of BMSCs significantly. So we made a conclusion that Notch signaling pathway seemed to involve in mechanical stimulation of infrasound altering activity of BMSCs and infrasound could induce the up-regulation of Notch signaling of BMSCs. Conformational changes caused by mechanical stimulation are responsible for cells signaling change. We consider that infrasound can also give rise to conformational changes to lead to up-regulation of Notch signaling pathway. When further analysis were made on the difference of increasing level among Jagged1, Notch1 and Hes1 mRNA and protein of BMSCs treated by infrasound, we found that infrasound induced the most up-expression of Hes1 mRNA and protein. So we infer that infrasound may act on the signaling pathway which transmits signaling from cell membrane to cell nucleus. However, this study didn't make a continuous exploration on specific acting points of infrasound in signaling pathway, which should be elucidated in next step.

In conclusion, this study demonstrates that infrasound can promote the BMSCs proliferation, which we also prove previously. After inhibition of Notch signaling pathway, the increasing viability of BMSCs induced by infrasound can be impaired, which indicate that Notch signaling pathway involve in the BMSCs proliferation. Through analyzing the expression of mRNA and protein of Jagged1, Notch1 and Hes1, We find that infrasound can activate the Notch signaling pathway, which should be one of mechanisms to increase the viability of BMSCs induced by infrasound.

Acknowledgements

Disclosure of conflict of interest

None.

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