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Research Article

Inhibition of Endoplasmic Reticulum Stress by BMSC and Klotho Genes on Cardiac Remodeling in Chronic Hypoxic Rats

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Abstract

Objective: To observe the effects of Bone Marrow Mesenchymal Stem Cells (BMSC) and Klotho gene on cardiac remodeling Endoplasmic Reticulum Stress (ERS) in chronic hypoxic rats.

Methods: The model of cardiac remodeling in rats with chronic hypoxia for 28 days was established. The rats were divided into normal group, model group, BMSC group and Klotho group. Rat BMSC was prepared and identified. The recombinant lentivirus mediated Klotho gene. After the model was established, BMSC and Klotho genes were injected into the tail vein respectively. After 4 weeks, the protein expression of HSP47, CHOP and AKT in rat myocardium was detected by Western Blot. The myocardial collagen content was detected by Masson staining and the apoptosis rate of cardiomyocytes was detected by Tunel.

Results: After 4 weeks of BMSC and Klotho gene treatment, the myocardial collagen volume fraction (CVF%), myocardial apoptosis rate, HSP47 and CHOP protein expression were significantly decreased ($P<0.01$), and AKT protein expression was significantly increased ($P<0.01$). The apoptotic rate of cardiomyocytes in BMSC group was lower than that in Klotho group ($P<0.01$). The expression of HSP47 and CHOP protein in Klotho group was lower than that in BMSC group ($P<0.05$), while the expression of AKT protein was higher ($P<0.05$).

Conclusion: Both BMSC and Klotho gene transplantation can reverse cardiac remodeling by inhibiting ERS.

Keywords: Myocardial fibrosis; Cardiomyocyte apoptosis; Bone marrow mesenchymal stem cells; Klotho gene; Endoplasmic reticulum stress

Introduction

Cardiac Remodeling (CR) is a common pathological mechanism of heart failure and atrial fibrillation caused by various pathological factors. Cardiac fibrosis and cardiomyocyte apoptosis play an important role in the pathological changes of structural remodeling [1]. Endoplasmic Reticulum Stress (ERS) can be activated by continuous ischemia, hypoxia, infection, etc., and various signal pathways are involved in tissue fibrosis and apoptosis [2]. The study found that Bone-marrow Mesenchymal Stem Cells (BMSC) can differentiate into cardiomyocytes in a specific environment, and can secrete a variety of cytokines involved in the regulation of signaling pathways, inhibit cardiomyocyte apoptosis, and reduce Matrix collagen deposition [3,4]. The Klotho gene is a newly discovered anti-aging gene with anti-oxidative stress, inhibition of cellular inflammation, anti-apoptosis and inhibition of myocardial fibrosis [5,6]. In this study, the 28-day cardiac remodeling model of chronic hypoxia in SD rats was used to study the effects of BMSC and Klotho genes on ERS, and to explore the mechanism of their protective effects on the heart.

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Materials and Methods

Grouping and Modeling of Experimental Animals 30 male SPF male Sprague-Dawley rats (production license number: SCXK (Yunnan) 2011-0004) were established, and they were randomly divided into model group, BMSC group and Klotho group. There were 8 in each group and the other 6 were normal groups. The normal group was not given anoxic treatment, and the other groups were placed in an anoxic tank. The hypoxia tank was modified from the experiment method of Wang J et al. [5] and the oxygen concentration in the anoxic chamber was set to 10%, which was continuously lacking in the box. At 28 d of oxygen, 7 rats died during the modeling period, 5 in the remaining model group, 6 in the BMSC group, and 6 in the Klotho group. The experimental procedures of all SD rats follow the National Laboratory Animal Management Regulations and the Regulations for the Administration of Laboratory Animals.

Preparation and labeling of BMSC *in vitro* the cultured BMSC was isolated and cultured at 37°C, saturated humidity and 5% CO_2. The third generation BMSC was used for the experiment. Adipogenic induction and osteogenic induction were performed, and staining with oil red O and alizarin red working solution. Identification of lipid droplet formation and calcium nodules. The surface markers CD34, CD44 and CD90 of BMSC were identified by flow cytometry. Lentivirus-mediated Green Fluorescent Protein (GFP) was transfected into BMSC for fluorescent labeling.

After BMSC and Klotho gene transplantation, BMSC was transplanted into the BMSC group of chronic hypoxia model rats by tail vein injection at 5×10^6/ml, and the Klotho gene was injected into

the model rat according to 1 × 10^9 TU/ml. In the Klotho group, the animals were anesthetized 4 weeks later.

Klotho mRNA expression in myocardium the total RNA of BMSCs was extracted by Trizol method. The cDNA was obtained by reverse transcription of 1ug RNA according to the instructions of the reverse transcription kit, and the target fragment was amplified by using cDNA as a template. The PCR primers were synthesized by Shanghai Shenggong Biological Co., Ltd. After the end of the PCR, the Ct value was obtained, and 2-ΔΔCt was calculated, and the expression difference was analyzed (Table 1).

Analysis of CHOP, HSP47 and AKT expression in myocardium the rat myocardium was ground with liquid nitrogen, and the supernatant was collected by centrifugation with RIPA lysate. After protein quantification and denaturation, SDS-PAGE electrophoresis, transfer to PVDF membrane, block at room temperature, add primary antibody at 4°C overnight, incubate in the dark at room temperature, and analyze each image with Image-pro plus 6.0 image analysis software. The value of the gray value of the specific gray scale strip, combined with the calibration error of the gray value of the internal reference GAPDH, the result is the relative expression of the target protein.

Myocardial histological observation Changes were observed under the microscope using Masson staining and Tunel. Image-pro plus 6.0 software was used to calculate the ratio of Masson-stained blue collagen fibers to the total tissue area, i.e., myocardial collagen volume fraction (CVF%). Calculate the percentage of apoptotic cells and the total number of cells in the Tunel assay, which is the rate of cardiomyocyte apoptosis.

Statistical analysis Data analysis was performed using SPSS 17.0 statistical software. Measurement data were expressed as mean ± standard deviation, and comparison between groups was performed by one-way analysis of variance or Tamhane analysis of variance.

Results

Preparation of BMSC: BMSC morphology Primary cells are more than 90% confluent in about 10 days, which is a long spindle-shaped cell group with uniform distribution of swirls. After passage, cell proliferation time was shortened, and an average of more than 85% of fusion was achieved in 5 days (Figure 1).

BMSC adipogenic and osteogenic differentiation BMSCs were induced by lipid formation and stained with oil red O in orange-red. After osteogenic induction, staining with alizarin red was dark red, indicating that BMSC has multi-directional differentiation (Figure 2).

Immunophenotype of BMSC Flow cytometry analysis, BMSC expressed CD44, CD90, etc.; CD34 was negative, indicating BMSC (Figure 3).

BMSC fluorescence transfection and localization BMSCs carrying GFP were observed under fluorescent microscope after 48 hours of lentiviral-mediated Green Fluorescent Protein (GFP) transfection. The tail vein was transplanted into the model rats, and the fluorescence microscope was used to track the distribution of BMSC in the myocardium (Figure 4).

Klotho mRNA: Expression in the myocardium of each group was 1.80±0.5 in the normal group, 0.78±0.14 in the model group and 4.86±0.61 in the Klotho group. The relative expression of Klotho mRNA in the Klotho group was significantly increased (P<0.05).

The protein expressions of CHOP, HSP47 and AKT in the myocardium of each group were significantly higher than those in the normal group. The expression of CHOP and HSP47 protein in the model group was significantly increased (P<0.01), and the expression of AKT protein was significantly decreased (P<0.01). Compared with the model group, the expressions of CHOP and HSP47 in the BMSC group and Klotho group were significantly decreased (P<0.01), and the expression of AKT protein was significantly increased (P<0.01). The Klotho group was more in the BMSC group than in the BMSC group. The expression of CHOP and HSP47 protein was significantly decreased (P<0.05), and the expression of AKT protein was significantly increased (P<0.05) (Figure 5) (Table 2).

Myocardial tissue Masson staining and CVF% staining: The collagen fibers are blue, and the muscle and cellulose are red (Figure 6).

The CVF% results showed that compared with the normal group, the CVF% of the model group, Klotho group and BMSC group were significantly increased (P<0.01); Compared with the model group, the Klotho group and the BMSC group were significantly lower (P<0.01); the BMSC group was lower than the Klotho group, but there was no significant difference (P>0.05) (Table 3).

Myelin tissue detection and cardiomyocyte apoptosis ratE: DAPI stained all nuclei and appeared blue under UV excitation. The positive apoptotic nuclei were labeled with Tunel fluorescein and green under green light excitation (Figure 7).

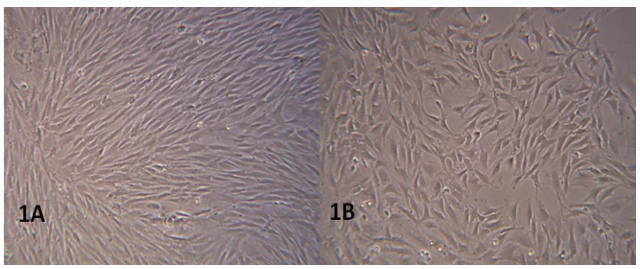

Figure 1: Bone marrow mesenchymal stem cell morphology (× 100). 1A is a primary BMSC cultured for 10 days, and 1B is a 3rd generation BMSC.

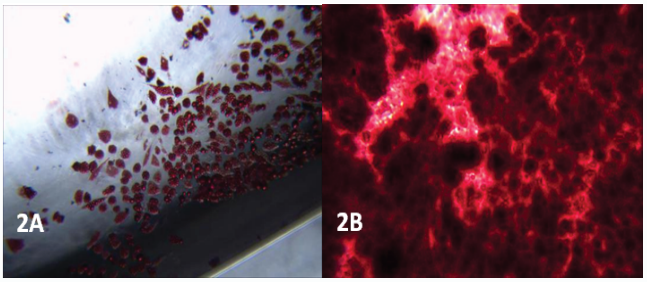

Figure 2: Adipogenic and osteogenic differentiation of bone marrow mesenchymal stem cells (× 100) 2A is adipogenic and 2B is osteogenesis.

Table 1: After the end of the PCR, the Ct value was obtained, and 2-ΔΔCt was calculated, and the expression difference was analyzed.

Primer	Upstream	Downstream
Klotho	5'-CAATGGCTTCCCTCCTTTACCT-3'	5'-TTCTCTTCTTGGCTACAACCCC-3'
GAPDH	5'-TTCCTACCCCCAATGTATCCG-3'	5'-CATGAGGTCCACCACCCTGTT-3'

Table 2: CHOP, HSP47, AKT protein expression in each group of myocardium (`x ± s, n=5).

Group	CHOP	HSP47	AKT
Normal group	25.25 ± 3.50	35.32 ± 10.01	546.65 ± 32.30
Model Group	652.91 ± 72.59**	735.21 ± 153.42**	46.53 ± 4.19**
Klotho group	78.43 ± 9.35**##Δ	147.51 ± 35.84**##Δ	424.94 ± 25.58**##Δ
BMSC Group	186.01 ± 64.77**##	236.75 ± 17.53**##	274.82 ± 11.57**##

Note: Compared with the normal group, **P<0.01; compared with the model group, ##P<0.01; compared with the BMSCs group, ΔP<0.05

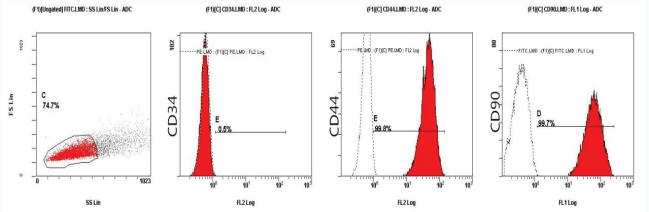

Figure 3: Phenotypic analysis of bone marrow mesenchymal stem cells (CD34 0.5%; CD44 99.8%, CD90 99.7%).

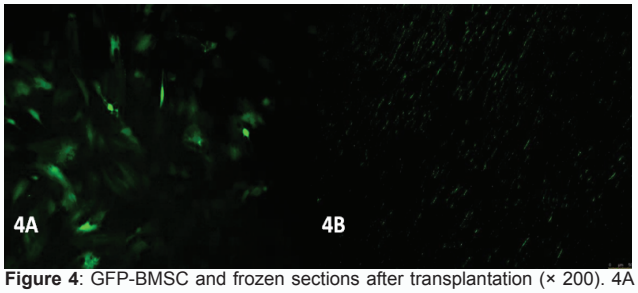

Figure 4: GFP-BMSC and frozen sections after transplantation (× 200). 4A was transfected for 48h; 4B was after GFP-BMSC transplantation.

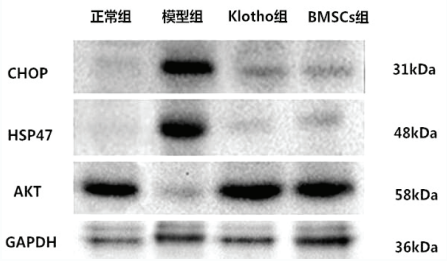

Figure 5: CHOP, HSP47, AKT protein band imaging in each group of myocardium.

The percentage of cardiomyocyte apoptosis rate showed that the apoptosis rate of cardiomyocytes in model group, BMSCs group and Klotho group was significantly higher than that in normal group (P<0.01). Compared with model group, BMSCs group and Klotho group were significantly lower (P<0.01); compared with the Klotho group, the BMSCs group was significantly lower (P<0.01) (Table 4).

Discussion

The results of this study showed that the expression of HSP47 and CHOP protein was lower and the expression of AKT protein was higher in the normal group. The expression of HSP47 and CHOP protein in the model group was significantly increased, the expression of AKT protein was significantly decreased, and the apoptosis rate of CVF% and cardiomyocytes were significantly higher. Elevation, indicating that hypoxia can activate ERS production, induces myocardial fibrosis and cardiomyocyte apoptosis rate by mediating protein expression of HSP47, CHOP and AKT. After BMSC and

Table 3: Collagen Volume Fraction (CVF%) in each group of myocardium (`x ± s, n=5).

Group	CVF%
Normal group	0.06 ± 0.03
Model group	33.51±10.66**
Klotho group	8.13 ± 4.94**##
BMSC group	7.55 ± 2.18**##

Note: Compared with the normal group, **P<0.01; compared with the model group, ##P<0.01

Table 4: % apoptotic rate of cardiomyocytes in each group (`x ± s, n=5).

Group	Cardiomyocyte apoptosis rate (%)
Normal group	0.12 ± 0.05
Model group	67.72 ± 10.73**
BMSC group	10.19 ± 2.03**##
Klotho group	32.43 ± 2.74**##ΔΔ

Note: Compared with the normal group, **P<0.01; compared with the model group, ##P<0.01; compared with the BMSCs group, ΔΔP<0.01

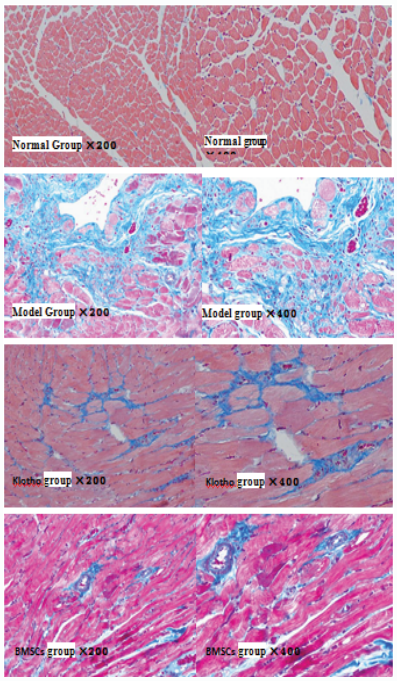

Figure 6: Masson staining of each group of myocardial tissue (× 200, × 400).

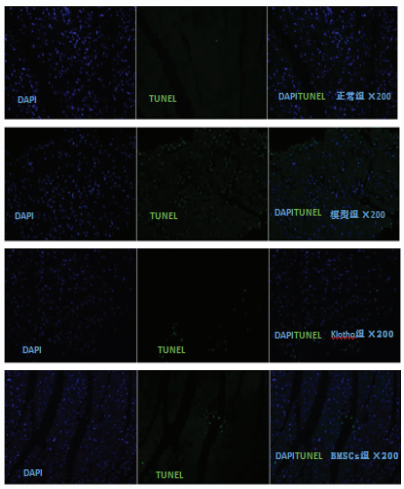

Figure 7: Tunel detection of myocardial tissue in each group × 200.

Klotho gene therapy, the CVF% was significantly decreased, and the expression of HSP47 protein was significantly decreased. Therefore, both BMSC and Klotho genes can alleviate myocardial fibrosis by inhibiting ERS-mediated HSP47 protein expression. On the other hand, the model group ERS promoted the expression of CHOP protein and decreased the expression of AKT protein. The apoptosis rate of cardiomyocytes decreased significantly after BMSC and Klotho gene transplantation, and the expression of CHOP protein decreased significantly and the expression of AKT protein increased significantly. The results indicated that both BMSC and Klotho genes can attenuate myocardial fibrosis and reduce cardiomyocyte apoptosis by inhibiting the expression of ERS-mediated collagen chaperones and related proapoptotic signaling pathways, and play a role in reversing cardiac remodeling [4,5].

By comparing the BMSC group with the Klotho group, the Klotho group was more effective than the BMSCs group in inhibiting HSP47, CHOP protein expression and increasing AKT protein activity, indicating that the Klotho gene inhibited ERS significantly more than BMSC. In myocardial collagen deposition, the BMSC group was slightly lower than the Klotho group, but there was no significant difference. However, in the apoptotic rate of cardiomyocytes, the BMSC group was significantly lower than the Klotho group. We speculated that BMSC could homing to the myocardium through vein transplantation and had the effect of differentiating into cardiomyocytes to replace apoptotic cells. Klotho gene can inhibit cardiomyocyte apoptosis only by inhibiting ERS regulatory signaling pathway, and the effect of inhibiting ERS is more significant than that of BMSC, suggesting that combined BMSC and Klotho genes have better effects on reversing cardiac remodeling.

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