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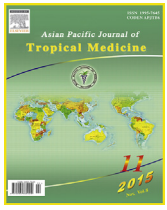

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Expression of Wnt and NCX1 and its correlation with cardiomyocyte apoptosis in mouse with myocardial hypertrophy

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ABSTRACT

Objective: To study the correlation between expression of Wnt and NCX1 and cardiomyocyte apoptosis in mouse with myocardial hypertrophy.
Methods: C57B/16 male mice were given the subcutaneous injection of 1 mg/kg isoprenaline to build the myocardial hypertrophy model. After 14 d of model building, mice were executed by cervical vertebra luxation. The ratio of heart weight/body weight (HW/BW) and heart weight/tibia length (HW/TL) was observed and proved using HE staining that detected the size of cardiomyocytes. 40 male C57B/16 mice were randomly divided into the sham group (normal saline) and model group (isoprenaline), with 20 mice in each group. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling was applied to detect the cardiomyocyte apoptosis; while Western blot and immunohistochemistry were employed to detect the expression of Wnt and NCX1. Meanwhile, the correlation between these two proteins and cardiomyocyte apoptosis was explored.
Results: Compared with the sham group, the ratio of HW/BW and HW/TL was increased in the model group, as well as the bigger and hypertrophied cardiomyocytes, decreased number and increased apoptosis of cardiomyocytes, and increased positive expression of Wnt3a, Wnt5a and NCX1 in the cardiac muscle tissue. Besides, there was positive correlation between the expression of Wnt and NCX1 and the cardiomyocyte apoptosis.
Conclusion: The expression of Wnt3a, Wnt5a and NCX1 in mouse with myocardial hypertrophy is increased and positively correlated with the cardiomyocyte apoptosis.

1. Introduction

The myocardial hypertrophy is the hypertrophied and larger heart that is caused by the increased volume of cardiomyocytes, as the adaptive response of heart to many kinds of pathological stimuli. Besides, the symptoms that are caused by the pathological stimuli, such as the aortic stenosis and hypertension, will be transduced through a series of cell signaling pathways, which will finally reduce the number of cardiomyocytes, result in the fibrosis, cardiac insufficiency and then the heart failure, arrhythmia and sudden death [1]. But the myocardial hypertrophy is also a reversible pathological process. When all kinds of pathological stimuli are removed, the myocardial hypertrophy can be improved. Therefore, the discussion on the expression of signaling proteins in the cardiac muscle tissue after myocardial hypertrophy will contribute to the ventricular remodeling because of myocardial hypertrophy and even the heart failure.

Many signaling pathways played the key role in the myocardial hypertrophy [2]. According to plenty of previous researches, the Wnt signaling pathway after myocardial hypertrophy that was caused by many pathological factors was continuously active, which would accelerate the ventricular remodeling and the generation of arrhythmia and heart failure [3]. After the signal is sent in the Wnt signaling pathway through the upstream signal molecule disheveled (DVL) protein, it can be divided into the Wnt/β-catenin classical pathway and Wnt/β-catenin non-classical pathway. Wnt classical and non-classical signaling pathways play a vital role in the

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heart failure because of myocardial hypertrophy, which can become the new strategy for the treatment of myocardial hypertrophy [4].

Ca^{2+} can regulate the contraction of cardiomyocytes, but the myocardial hypertrophy can lead to the overload of Ca^{2+} in cells that will cause the failure of contraction and finally the death of cardiomyocytes [5]. Na^{+}/Ca^{2+} exchanger (NCX) is some kind of membrane protein that exists in cell membranes of heart, brain, vascular smooth muscle and skeletal muscle, which can regulate the concentration of Ca^{2+} in cells. In normal physiological situation, NCX1 is the main Ca^{2+} pump on the membrane of cardiomyocytes and it can transfer Ca^{2+} outside the cardiomyocytes in the relaxation process. But in case of pressure overload caused by the stimulation of many pathological factors, NCX1 can reversely transfer Ca^{2+} into cells to result in the overload of Ca^{2+} in cells, which will lead to the cardiomyocyte apoptosis and heart failure [6]. According to previous researches, it's reported that the expression of NCX1 was increased in the myocardial hypertrophy [7–9]. It indicates that the Wnt signaling pathway and NCX1 are similar in the biological performance after myocardial hypertrophy, but its specific relationship has been unknown. Therefore, in this study, the subcutaneous injection isoprenaline (ISO) is employed to induce the myocardial hypertrophy model of mice and thus discuss the expression of Wnt and NCX1 after myocardial hypertrophy and the correlation with cardiomyocyte apoptosis.

2. Materials and methods

2.1. Animals

A total of 40 C57B/16 male mice with the weight of (20 ± 2) g were purchased from Shanghai SLAC laboratory Animal Co., Ltd., with the qualified certificate of SCXK (Hu)2012-0002. The room temperature was controlled at (23 ± 2) °C and mice were given the diet and water freely.

2.2. Reagents and instruments

The rabbit-anti Wnt3a, Wnt5a and NCX1 antibodies were purchased from Cell Signaling Technology; BCA kit and TUNEL kit from Beyotime Biotechnology; mini vertical electrophoresis tanks, mini Trans-blot electrophoresis tank and ChemiDoc™ XRS gel imaging system from Bio-Rad.

2.3. Modeling and grouping

A total of 40 male C57B/16 mice were randomly divided into the sham group (normal saline) and model group (ISO), with 20 mice in each group. C57B/16 male mice were given the subcutaneous injection of 1 mg/kg ISO to build the myocardial hypertrophy model that lasted for 14 d.

2.4. Ratio of heart weight/body weight (HW/BW) and ratio of heart/tibia length (HW/TL)

After 14 d, mice were weighted and executed by cervical vertebra luxation. The heart was taken by cutting the breast and then it was washed with normal saline to remove the excess connective tissues and vessels. The bibulous paper was used to absorb the water on samples. Then the heart was weighted and put it in the refrigerator at −70 °C for the measurement. Afterwards, the vernier caliper was employed to measure the left tibia length (TL) of mice.

2.5. HE staining

After being immersed in 10% neutral formalin, samples were embedded with paraffin and then cut into slices and dried. They were deparaffinaged using dimethylbenzene and dehydrated with water-free 95% and 80% ethanol. They were washed with running water and immersed in the hematoxylin and hydrochloride alcohol. When being turned to be antiblue, they were stained with eosin. Afterwards, they were washed with running water, dehydrated with ethanol at gradient degree. They were transparented with dimethylbenzene. After being dried, they were mounted with the neutral resin and then observed under the microscope.

2.6. Cardiomyocyte apoptosis detected by TUNEL

Paraffin sections were taken out and dehydrated. 100 μL 20 μL/mL protease K was added for the digestion. They were incubated at the room temperature for 10 min and then washed with PBS twice, lasting for 3 min each time. Afterwards, 50 μL 3% H_2O_2, was added and placed at the room temperature for 20 min. They were washed with PBS twice. TUNEL reaction fluid was added and it was reacted in the incubator in a dark place and at 37 °C for 1 h. After the reaction, 25 μL horseradish peroxidase antibody was added. They were incubated at the room temperature for 30 min and then washed with PBS twice. Afterwards, they were colored using DAB, washed with running water, re-stained with hematoxylin, anti-blued with ammonia, dehydrated with ethanol, transparented with dimethylbenzene and mounted with the neutral resin.

2.7. Immunohistochemistry

Paraffin sections were taken out and deparaffinaged using dimethylbenzene. They were dehydrated with ethanol at gradient degree and washed with running water. After being repaired with the antigen, they were cooled at the room temperature and washed with PBS. The primary antibodies were incubated at the room temperature and washed with PBS; while secondary antibodies were incubated at 37 °C and washed with PBS. Afterwards, they were colored using DAB, re-stained, dehydrated, transparented and mounted with the resin. Primary antibodies were replaced by PBS as the negative control.

2.8. Western blot

Myocardial samples were collected from each group and treated with the homogenizer. Then the appropriate RIPA lysis buffer was added and they were shaken in the vortex every 10 min for 30 s. After 40 min, they were centrifuged at 4 °C and 10000 rpm for 10 min. The supernatant was taken carefully to obtain the total protein. BCA kit was employed to detect the protein concentration. SDS gel electrophoresis was performed on protein samples and then it was transferred with the wet method. Then the film was immersed into the primary antibody solution for the incubation at 4 °C over night. After being washed, it was immersed into the secondary antibody solution

(1:100) for the incubation at the room temperature for 1–2 h. Afterwards, the film was taken out and washed, while ECL reagent was added on the film for the exposure in the gel imaging system. Statistics was performed on the gray value of each antibody band using 'Quantity one' software.

2.9. Data analysis

The results were expressed as mean ± SD. The *t* test was performed for the significant difference and Pearson correlation analysis for the correlation between two factors. $P < 0.05$ was regarded to be significant difference. All data were treated using SPSS 17.0.

3. Results

3.1. Ratio of HW/BW and ratio of HW/TL in sham group and model group

As shown in Table 1, compared with the sham group, the ratios of HW/BW and HW/TL were all increased in the model group, namely increased by 24.33% and 23.14%, with the statistical difference ($P < 0.05$), which indicated the successful building of myocardial hypertrophy model of mice.

Table 1
HW/BW and HW/TL in sham group and model group (mean ± SD).

Group	Amount	HW/BW	HW/TL
Sham group	20	4.89 ± 0.69	75.42 ± 8.21
Model group	20	6.08 ± 0.78*	92.87 ± 7.65*

Note: compared with the sham group, *$P < 0.05$.

3.2. Size of cardiomyocytes in sham group and model group

As shown in Figure 1, compared with the sham group, cardiomyocytes became bigger and hypertrophied in the model group, with the statistical difference ($P < 0.05$), which further indicated the successful building of myocardial hypertrophy model of mice.

3.3. Cardiomyocyte apoptosis in sham group and model group

As shown in Figure 2, the brown-staining of cells indicated the apoptosis. Compared with the sham group, the number of cardiomyocyte apoptosis was increased in the model group, with the statistical difference ($P < 0.05$).

3.4. Expression of Wnt and NCX1 in cardiomyocytes in sham group and model group

As shown in Figure 3, Wnt3a, Wnt5a and NCX1 positive cells were stained to be brown, which indicated the increased expression of Wnt3a, Wnt5a and NCX1. Compared with the sham group, the positive expression of Wnt3a, Wnt5a and NCX1 was all significantly increased.

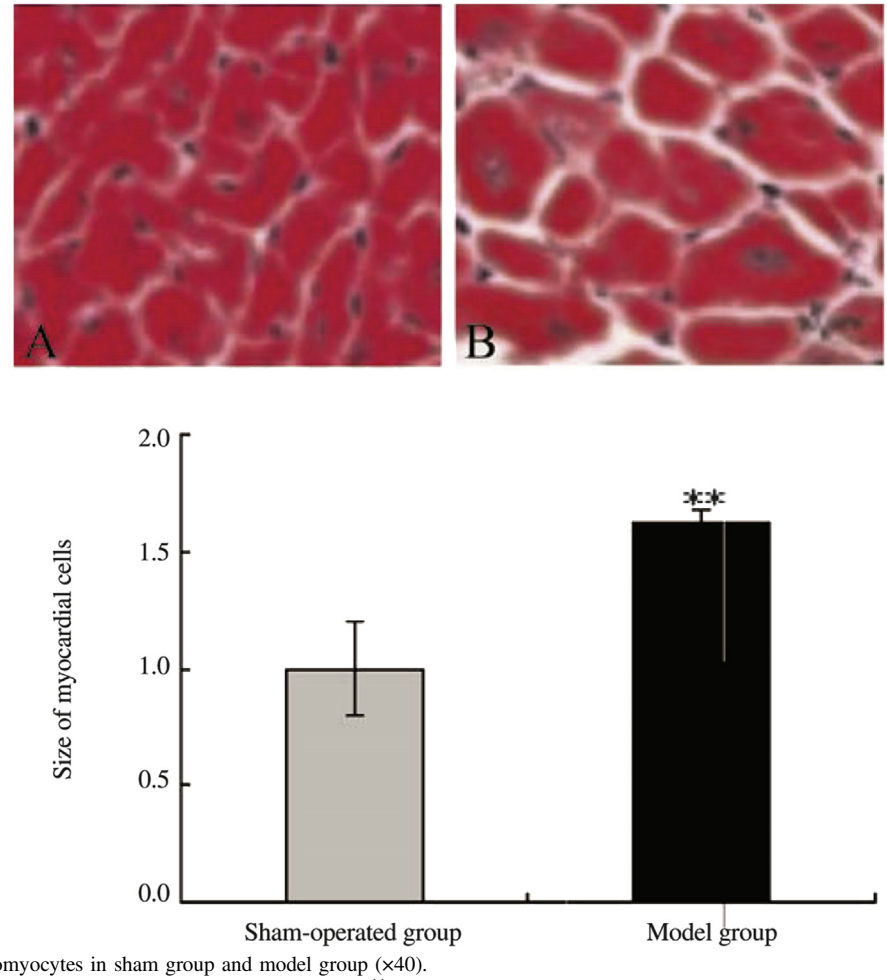

Figure 1. Size of cardiomyocytes in sham group and model group (×40).
A: Sham group; B: Model group. Compared with the sham group, **$P < 0.01$.

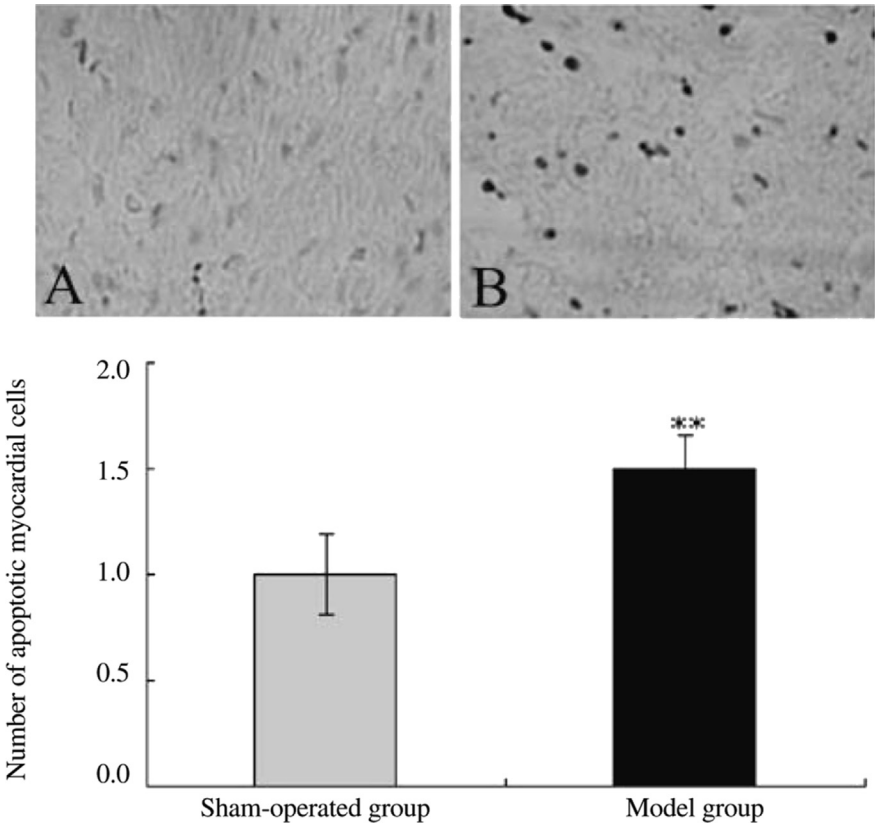

Figure 2. Cardiomyocyte apoptosis in sham group and model group (×40).
A: sham group; B: model group. Compared with the sham group, $^{**}P < 0.01$.

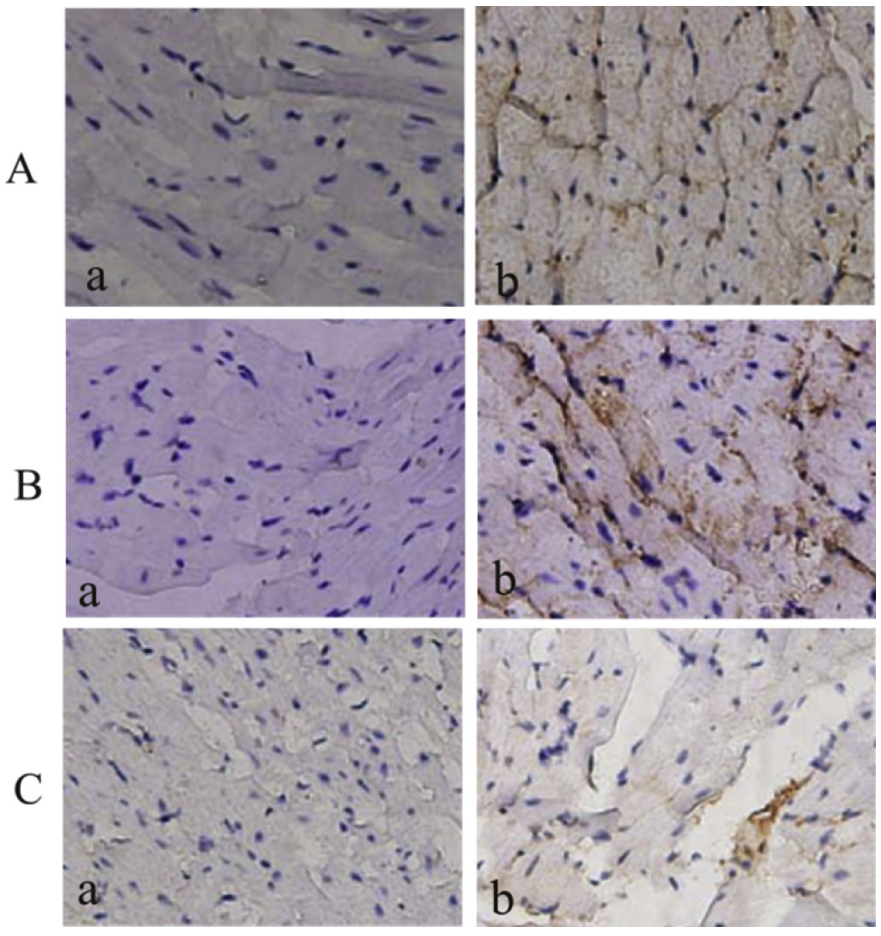

Figure 3. Immunohistochemistry to detect the expression of Wnt and NCX1 in cardiomyocytes in sham group and model group.
A: Wnt3a, B: Wnt5a, C: NCX1; a: sham group, b: model group.

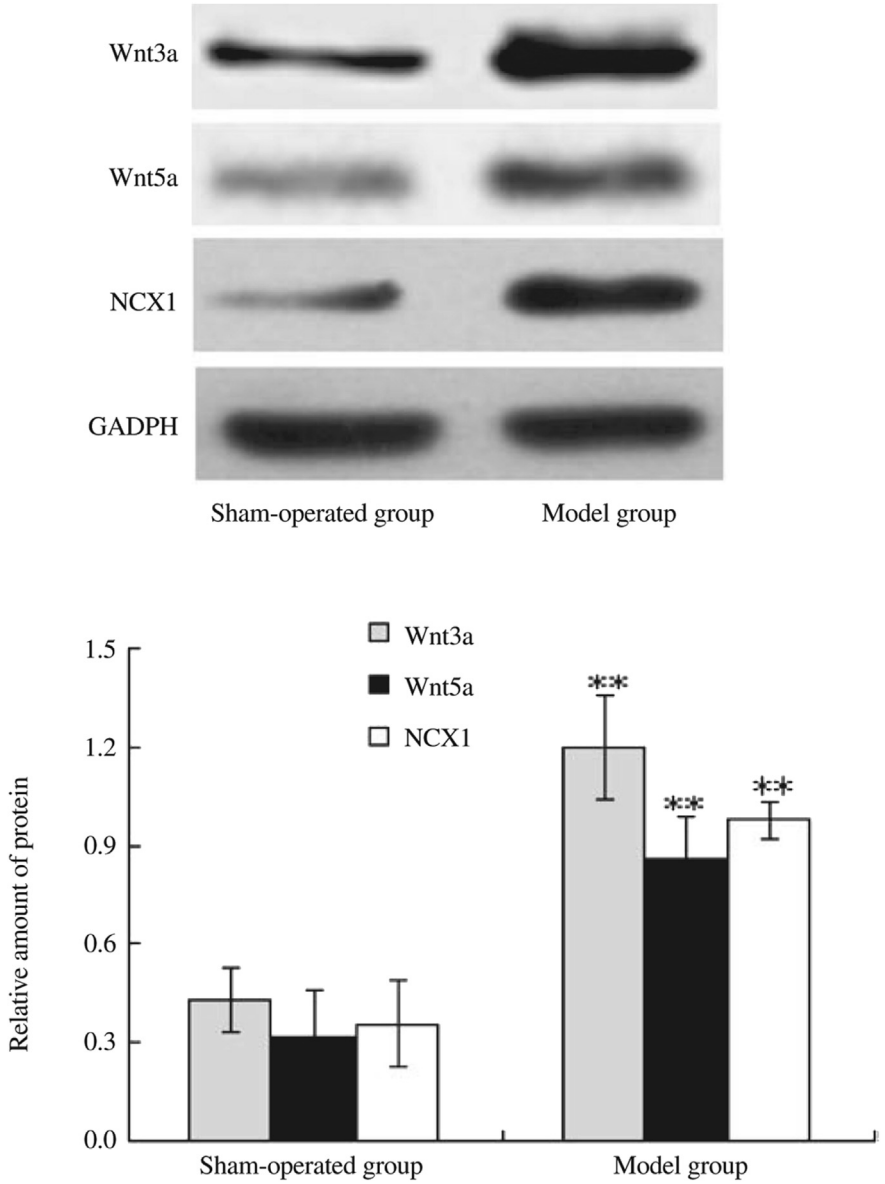

Figure 4. Expression of Wnt and NCX1 in cardiomyocytes in sham group and model group. Compared with the sham group, $^{**}P < 0.01$.

3.5. Expression of Wnt and NCX1 in cardiomyocytes in sham group and model group

As shown in Figure 4, similar with the results of immunohistochemistry, results of Western blot showed that, compared with the sham group, the positive expression of Wnt3a, Wnt5a and NCX1 was all significantly increased in the model group, with the statistical difference ($P < 0.05$).

3.6. Correlation between expression of Wnt and NCX1 and cardiomyocyte apoptosis after myocardial hypertrophy of mice

As shown in Table 2, according to Pearson correlation analysis, after the myocardial hypertrophy, the expression of Wnt was positively correlated with the cardiomyocyte apoptosis, as well as the one between the expression of NCX1 and cardiomyocyte apoptosis and the expression of Wnt and one of NCX1, with the statistical difference ($P < 0.05$).

Table 2
Correlation between expression of Wnt and NCX1 and cardiomyocyte apoptosis after myocardial hypertrophy of mice (mean ± SD).

Group	*r*
Expression of Wnt3a and NCX1	0.752
Expression of Wnt5a and NCX1	0.728
Expression of Wnt and cardiomyocyte apoptosis	0.804
Expression of NCX1 and cardiomyocyte apoptosis	0.821

4. Discussion

The myocardial hypertrophy is some kind of adaptive reaction, showing the larger and heavier cardiomyocytes. The decreased in the number of cardiomyocytes, namely the increase in the number of cardiomyocyte apoptosis, is of compensatory significance in the early stage. However, the long-term myocardial hypertrophy will lead to the arrhythmia, sudden death and heart failure. In this study, the subcutaneous injection

of ISO was employed to build the myocardial hypertrophy model of mice. By measuring HW/BW, HW/TL and size of cardiomyocytes, it's found that in mice with myocardial hypertrophy, the ratios of HW/BW and HW/TL were all increased and cardiomyocytes become larger and hypertrophied, which indicated the successful building of model and were consistent with findings of Mu [10] and Peng *et al*. [11].

Wnt was found by Nusse *et al*. in 1982 when inducing the mouse mammary carcinoma using the papillomavirus. Wnt signaling pathway is a highly conserved pathway in multicellular eukaryotes, which regulates the process of many life activities. If being exposed to the external stimulus, Wnt signaling pathway that activates DVL protein and relies on the involvement of axin protein will inhibit the phosphorylation activity of downstream glycogen synthase kinase-3β (GSK-3β) and activate the β-catenin-induced classical signaling pathway. In this way, β-catenin will be accumulated in the cytoplasm and transferred to the nucleus to regulate the expression of downstream genes such as the cell cycle related protein (*CyclinD1*) and *C-myc*. Malekar *et al*. [3] found that in the transgenic model of rats with the overexpression of DVL-1, the expression of CyclinD1 and C-myc was up-regulated, which resulted in the myocardial hypertrophy. In the transgenic model of mice with the overexpression of DVL-1 in Wnt signaling pathway, the ratio of HW/BW was increased, being accompanied by the larger cardiomyocytes, increased expression of atrial natriuretic peptide, enlarged left ventricle, reduced ejection fraction, death before the age of 6 months and disappeared effect of β-epinephrine induced myocardial hypertrophy after the knockdown of *DVL* gene [3]. According to Tateishi *et al*. [12], in the myocardial hypertrophy model of mice induced by aorta banding, LiCl could inhibit the expression of GSK-3β and thus up-regulate the expression of β-catenin and aggravate the myocardial hypertrophy; while in transgenic rats with the overexpression of GSK-3β, the incidence of myocardial hypertrophy was inhibited. In the rat model with the knockdown of *β-catenin* gene and horizontal aorta banding-induced over pressure load, the ratio of HW/BW was decreased [13]. The transgenic mice with the overexpression of Dapper-1 might have the myocardial hypertrophy, increased ratio of HW/TL, cross-sectional area of muscle cells and hypertrophied cardiomyocytes, which was related to the up-regulated expression of Wnt3a. Besides, after the knockdown of Dapper-1, the expression of Wnt3a and phenylephrine-induced myocardial hypertrophy were all decreased [14], which indicated that Wnt classical pathway played a key role in the myocardial hypertrophy and the expression of major proteins was increased. According to results of this study, it also found the increased expression of Wnt3a in the ISO-induced myocardial hypertrophy model of mice.

Wnt signaling pathway can activate the DVL protein and also the non-classical pathway, such as Wnt/Ca^{2+} signaling pathway. In this way, it can lead to the accumulation of Ca^{2+} in cells and then stimulate Ca^{2+} related enzymes such as CaMKII and CaN. The activated CaN can promote the introduction of downstream cell nuclear factors into the nucleus to regulate the expression of downstream target genes and thus be involved in the process of development and differentiation of heart. The inhibition of CaMKII and CaN and their downstream transcription factors can inhibit the myocardial hypertrophy and weaken the left ventricular remodeling [15]. The knockdown of CaMKIIδγ in the myocardial model of transgenic mice with the overexpression of DVL-1 can show the normal myocardial form, without the myocardial hypertrophy, fibrosis or cardiomyocyte apoptosis. Besides, CaMKIIδγ can be integrated with histone deacetylase 4 and myocyte enhancer factor-2 of Wnt non-classical pathway [16], which indicated that Wnt non-classical pathway Wnt/Ca^{2+} also plays an important role in myocardial hypertrophy and the expression of key proteins was increased. In this study, it also found the increased expression of Wnt5a in ISO-induced myocardial hypertrophy model of mice.

The excess load of heart caused by any pathological stimuli can lead to the myocyte hypertrophy and abnormal expression of NCX1, which will show the reverse transfer of NCX1, transfer Na^{+} outside the cells and pump Ca^{2+} insides. In this way, it will cause the overload of Ca^{2+} in cells and thus promote the incidence of apoptosis [6]. Studer *et al*. [17] found that, compared with the control group, levels of NCX and mRNA in patients with end-stage dilated cardiomyopathy and ones with coronary heart disease accompanied by heart failure were increased by 55% and 41% respectively. Meanwhile, the expression of NCX1 was increased in LPS-induced myocardial hypertrophy [6], as well as the expression of NCX1 in myocardial hypertrophy and heart failure [7–9] and the expression of *NCX1* mRNA in myocardial hypertrophy caused by 50% coarctation of the abdominal aorta [18]. In cardiac myoblasts, the inhibition of NCX1 on the cell membrane can inhibit the endoplasmic reticulum stress and cardiomyocyte apoptosis that are caused by the anoxia and reoxygenation injury [19]. In the situation of intermittent hypoxia, compared with wild mice, the mice with cardiac-specific NCX1 knockdown had the relieved left ventricular dysfunction and reduced cardiomyocyte apoptosis [20]. Results showed the increased expression of NCX1 after myocardial hypertrophy. In this study, the expression of NCX1 was also increased in ISO-induced myocardial hypertrophy model of mice.

Furthermore, this study also found the positive correlation between Wnt3a, Wnt5a, NCX1 and cardiomyocyte apoptosis in case of hypertrophied cardiomyocytes of mice. The hypertrophied cardiomyocytes can activate Wnt classical and non-classical pathways, especially the activation of Wnt/Ca^{2+} signaling pathway, which can promote the accumulation of Ca^{2+} in cells, stimulate the activity of Ca^{2+} related enzymes. The activated CaN can promote the introduction of downstream cell nuclear factors into the nucleus to regulate the expression of related proteins and induce cardiomyocyte apoptosis. In the rat cardiomyocytes H9c2 with anoxia and reoxygenation induced overload of Ca^{2+}, there was the significant increase in the expression of Wnt5a, as well as cardiomyocyte apoptosis [21]. NCX1 is the regulator of Ca^{2+} concentration. In case of myocardial hypertrophy, it can reversely introduce Ca^{2+} into cells to cause the overload of Ca^{2+} in cells and thus result in the cardiomyocyte apoptosis. In mice during ischemia reperfusion period, the increased expression of NCX1 can reversely transfer Ca^{2+} to cause the overload of Ca^{2+} in cells and thus result in the cardiomyocyte apoptosis [22,23]. It indicates that Wnt is connected with NCX1 by affecting Ca^{2+} concentration and also play a key role in myocardial hypertrophy, with the positive correlation between them.

In conclusion, ISO is used to build the myocardial hypertrophy model of mice. According to results of immunohistochemistry and Western blot, compared with the sham group, the positive expression of Wnt3a, Wnt5a and NCX1 was increased in the cardiac muscle tissue after myocardial hypertrophy of

mice. Besides, after myocardial hypertrophy, the cardiomyocyte apoptosis also increased, showing the positive correlation among three of them, which lay a theoretical foundation for the clinical treatment of myocardial hypertrophy.

Conflict of interest statement

We declare that we have no conflict of interest.

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