

# JNK and NF- $\kappa$ B signaling pathways are involved in cytokine changes in patients with congenital heart disease prior to and after transcatheter closure

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**Abstract.** Congenital heart disease (CHD) is a problem in the structure of the heart that is present at birth. Due to advances in interventional cardiology, CHD may currently be without surgery. The present study aimed to explore the molecular mechanism underlying CHD. A total of 200 cases of CHD treated by transcatheter closure as well as 200 controls were retrospectively assessed. Serum cytokines prior to and after treatment were assessed by reverse-transcription quantitative polymerase chain reaction analysis. Furthermore, the levels of proteins associated with c-Jun N-terminal kinase (JNK) and nuclear factor (NF)- $\kappa$ B were assessed by western blot analysis and immunohistochemistry. Furthermore, an animal model of CHD in young pigs was successfully constructed and treated with inhibitors of JNK and/or NF- $\kappa$ B to investigate the roles of these pathways in CHD. The results revealed that tumor necrosis factor- $\alpha$ , interleukin (IL)-6 and IL-8 were significantly elevated in the experimental group following transcatheter closure treatment, compared with those in the healthy control group, and the serum levels of the anti-inflammatory cytokine IL-10 were significantly reduced. Phosphorylated c-Jun and p65 levels in the experimental group were notably higher in the experimental group compared with the control group, but were restored to normal levels following transcatheter closure treatment. Similar results were also obtained in the pig model. The results of the present study suggested that the CHD-associated

changes in cytokines, as well as their recovery following transcatheter closure treatment were associated with the JNK and NF- $\kappa$ B signaling pathways. The present study may provide further understanding of the underlying molecular mechanisms in CHD and propose a potential novel target for the treatment of CHD.

## Introduction

Congenital heart disease (CHD), also known as a congenital heart anomaly, is a structural aberration of the heart that exists at birth (1). Its symptoms depend on the specific type of anomaly and therefore symptoms may vary widely, ranging from none to those of life-threatening severity (2). CHD is also a major cause of infant mortality worldwide (3). Previous studies have demonstrated that 28% of all major congenital anomalies consist of heart defects (4) and the most practical measurement of CHD occurrence is birth prevalence per 1,000 live births (5). The surveillance of birth defects in China revealed that in 1996 CHD was the 5th most common birth defect and it became the leading birth defect in 2009 (6-8).

At present, CHD is thought to be induced by genetic, life-style and environmental factors, including maternal obesity, diabetes, toxicant exposure and alterations in anti-oxidant capacity (9). These well-known risk factors are also associated with immune dysregulation (10). Due to advances in interventional cardiology, certain congenital heart anomalies no longer require surgical treatment and are treated via percutaneous interventions (11,12). There have been numerous studies on CHD, however studies reporting on changes of inflammatory cytokines in patients with CHD following transcatheter closure treatment are lacking. It has been reported that CHD patients with a broad spectrum of pathologies demonstrated certain clinical features and a typical pattern of neurohormonal activation, which is characteristically found in chronic heart failure (CHF) (13). It still remains elusive whether the similarities between adult CHD and CHF extend to the inflammatory cytokine system (14). Certain studies reported that tumor necrosis factor (TNF)- $\alpha$  levels were elevated in a small pediatric cohort with CHD (15). However, only few

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studies have investigated the level of inflammatory cytokine system activity in CHD patients (16-23).

## Materials and methods

**Ethics statement.** The present study was approved by the Ethics Committee of the Third Affiliated Hospital of Zhengzhou University (Zhengzhou, China). Written informed consent was provided by all subjects.

**Subjects.** A total of 200 consecutive patients with CHD [107 males, 93 females; age, 13-41 years ( $29.6 \pm 15.2$ )] admitted to the Third Affiliated Hospital of Zhengzhou University (Zhengzhou, China) between September 2013 and February 2014 were included in this retrospective study. None of the patients had any unassociated abnormalities (not CDH-specific) or pulmonary hypertension. The cardiac diagnoses of all patients were made on the basis of clinical and laboratory examinations, including telecardiography, electrocardiography and echocardiography. The cases treated with transcatheter closure were retrospectively analyzed. Results prior to treatment as well as those at 6 and 12 months were included. Control data were taken from 200 normal individuals [110 males and 90 females; age, 12-39 years ( $25.42 \pm 13.28$ )] who received a physical examination at the Third Affiliated Hospital of Zhengzhou University between September 2013 and February 2014.

Fasting blood samples were obtained and collected from the antecubital vein using a lightly fitting tourniquet. Blood (3 ml) was drawn from each subject after fasting for 12 h. Samples were collected in a sterile tube and sera were obtained immediately after clotting of samples, filled in sterile tubes and stored at  $-80^{\circ}\text{C}$  until use.

**Serum biochemical analyses.** TNF- $\alpha$  (cat. no. DTA00C), interleukin (IL)-6 (cat. no. D6050), IL-8 (cat. no. DY208) and IL-10 (cat. no. D1000B) levels were measured using commercially available ELISA assay kits (R&D Systems, Minneapolis, MN, USA). The results are presented in pg/ml or U/l.

**Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA was extracted from the serum of subjects from the experimental group and the control group using an RNA extraction kit (cat. no. DP412; Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. Detection of the copy number of the mRNA of cytokines was performed as previously described (24).

**Immunohistochemical (IHC) staining.** Randomly selected samples of peripheral blood mononuclear cells were subjected to IHC staining and detection. IHC was performed as previously described (25).

**Western blot analysis.** Total protein was extracted and quantitated using a BCA Protein Assay reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Western blot analysis was performed as previously described (25). The membranes were incubated at  $4^{\circ}\text{C}$  overnight with the following primary antibodies: Anti-c-Jun antibody (1:1,000; ab32137), anti-p-c-Jun antibody (1:1,000; ab32385), anti-IK $\beta$

Table I. Characteristics of patients in the experimental group and control group at baseline.

Characteristic	Experimental group	Control group
Sex		
Male	107	110
Female	93	90
Age (years)	$29.6 \pm 15.2$	$25.42 \pm 13.28$
Height (cm)	$172 \pm 10.13$	$175 \pm 9.26$
Body weight (kg)	$65.2 \pm 10.15$	$68.6 \pm 14.5$
Values are expressed as n or as the mean $\pm$ standard deviation.		

antibody (1:1,000; ab32518), anti-p-IK $\beta$  antibody (1:10,000; ab133462), anti-P65 antibody ( $2.5 \mu\text{g/ml}$ ; ab19870), anti-p-P65 antibody (1:10,000; ab76302) (all from Abcam, Cambridge, MA, USA). This was followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies: anti-rabbit (ab6721) or anti-mouse (ab6785) immunoglobulin G antibodies (both 1:5,000; Beyotime Institute of Biotechnology, Haimen, China) at  $37^{\circ}\text{C}$  for 45 min. The target bands were visualized by using an enhanced chemiluminescence kit (Qihai Biotech, Shanghai, China) and the associated band intensities were analyzed using Gel-Pro-Analyzer software version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).  $\beta$ -actin was used as the internal control.

**Animal model of CHD.** A hybrid method was employed to generate the animal model of CHD in young pigs, as previously described (26). A total of 50 animals (4-8 weeks old; 10-12 kg) were provided by the Third Affiliated Hospital of Zhengzhou University (Zhengzhou, China), CHD was successfully induced in 43 animals and 40 animals were divided into four groups ( $n=10/\text{group}$ ) with 3 animals in reserve. All animals were housed in isolated cages under a 12 h light/dark cycle at  $23-25^{\circ}\text{C}$ , humidity 50-70% with free access to food and water according to the Guide for the Care and Use of Laboratory Animals (27). A group of 10 normal young pigs was used for determination of serum cytokine levels as a blank control group. Over 3 months following surgery, a group of CHD model animals with catheter closure treatment received continuous injection of normal saline as a control group. A second group of the CHD models received continuous injection of pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), a nuclear factor (NF)- $\kappa$ B inhibitor, over 3 months following catheter closure treatment. Another group of CHD models received continuous injection of SP600125 (Sigma-Aldrich; Merck KGaA), a c-Jun N-terminal kinase (JNK) inhibitor, and a further group of CHD models received continuous combined injection of PDTC and SP600125 over 3 months following catheter closure treatment. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Third Affiliated Hospital of Zhengzhou University (Zhengzhou, China).

**Statistical analysis.** Quantitative data are expressed as the mean  $\pm$  standard deviation. SPSS 18.0 (SPSS, Inc., Chicago,

Table II. Serum cytokine levels in patients with congenital heart disease prior to and after transcatheter closure treatment as well as in the control group.

Cytokine	Control	Experimental group		
		Baseline	6 months	12 months
TNF- $\alpha$	877 $\pm$ 129	1,936 $\pm$ 214 <sup>a</sup>	1,311 $\pm$ 211 <sup>a</sup>	866 $\pm$ 242
IL-6	599 $\pm$ 289	1,699 $\pm$ 299 <sup>a</sup>	1,196 $\pm$ 313 <sup>a</sup>	561 $\pm$ 189
IL-8	623 $\pm$ 92	1,268 $\pm$ 104 <sup>a</sup>	972 $\pm$ 272 <sup>a</sup>	413 $\pm$ 113 <sup>a</sup>
IL-10	1,191 $\pm$ 296	499 $\pm$ 211 <sup>a</sup>	791 $\pm$ 363 <sup>a</sup>	1,278 $\pm$ 202

<sup>a</sup>P<0.05 vs. Control. TNF, tumor necrosis factor; IL, interleukin.

IL, USA) was used for all statistical analyses. Comparison between two groups was performed using Student's t-test. Comparisons among three or more groups were performed using one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Clinical characteristics of patients.** All 200 cases of CHD were subjected to transcatheter closure treatment at the Third Affiliated Hospital of Zhengzhou University (Zhengzhou, China) between September 2013 and February 2014. The baseline patient characteristics are presented in Table I. The mean patient age in the experimental group was 29.6 $\pm$ 15.2 years and that in the control group was 65.42 $\pm$ 3.28 years.

**Transcatheter closure treatment restores cytokine levels in CHD patients.** Peripheral blood mononuclear cell samples and serum were obtained from patients in the experimental group and the control group. ELISA demonstrated that serum inflammatory cytokine levels of TNF- $\alpha$ , IL-6 and IL-8 in the experimental group after transcatheter closure treatment were significantly elevated compared with those in the healthy control group without treatment. Conversely, the serum levels of the anti-inflammatory cytokine IL-10 were significantly reduced. Of note, at 6 months and 12 months after transcatheter closure treatment, TNF- $\alpha$ , IL-6 and IL-8 levels in patients' serum were significantly reduced, and IL-10 rose compared with the control. The levels of all of the cytokines were nearly restored (Table II).

To further confirm the changes of cytokines in the patients, RT-qPCR was performed and the results demonstrated that the mRNA levels of TNF- $\alpha$ , IL-6 and IL-8 in the experimental group were significantly upregulated, while those of IL-10 were significantly reduced compared with those in the control group. At 6 and 12 months after transcatheter closure treatment, the mRNA levels of TNF- $\alpha$ , IL-6, IL-8 and IL-10 returned to normal levels (Fig. 1).

To explore the molecular mechanisms associated with the changes in cytokine levels of CHD patients after transcatheter closure treatment, peripheral blood mononuclear cells were subjected to western blot analysis and IHC staining. The western blot results demonstrated that phosphorylated

(p)-c-Jun, p65 and p-I $\kappa$ Ba levels in the experimental group were higher than in the control group, and were restored to normal levels after transcatheter closure treatment (Fig. 2). IHC analysis was conducted 6 months following treatment and the results indicated that the p-c-Jun- and p-65-positive cell rate in peripheral blood mononuclear cells was higher prior to treatment compared with that in the control group and decreased following treatment (Fig. 3). These results indicate that the JNK and NF- $\kappa$ B signaling pathways were activated in patients with CHD, and that transcatheter closure treatment may notably decrease the activation of these pathways.

**Animal model of CHD.** Next, an animal model of CHD was successfully constructed in 40 young pigs via a hybrid method according to standard protocols and divided into four groups. The serum cytokine levels of TNF- $\alpha$ , IL-6 and IL-8 were elevated following transcatheter closure treatment, while the levels of the anti-inflammatory cytokine IL-10 dropped. Injection of PDTC (NF- $\kappa$ B inhibitor) or SP600125 (JNK inhibitor) alone only partly restored TNF- $\alpha$ , IL-6, IL-8 or IL-10 compared with normal levels. Of note, combination treatment with PDTC and SP600125 restored TNF- $\alpha$ , IL-6, IL-8 and IL-10 to normal levels (Table III). These results suggested that the CHD-associated changes in cytokines as well as their recovery after transcatheter closure treatment are associated with the JNK and NF- $\kappa$ B signaling pathways.

## Discussion

TNF- $\alpha$ , IL-6, IL-8 and IL-10 are important inflammatory or anti-inflammatory cytokines, which contribute to numerous signaling pathways (JNK, NF- $\kappa$ B) to regulate certain biological processes (28-33). The present study provided a better understanding of the roles of cytokines in patients with CHD; the results indicated the presence of a certain degree of immune activation, causing an increase in the levels of inflammatory cytokines and a decrease in anti-inflammatory cytokine levels to result in inflammation. Following catheter closure treatment, the patients' immune and inflammatory status was gradually restored to normal levels.

An increasing number of studies have demonstrated that the occurrence of inflammation is closely associated with the expression of genes linked to inflammation (34,35). The

Table III. Levels of cytokines in the model of congenital heart disease in young pigs.

			Experimental group		
Cytokine	Control	Vehicle treated	PDTC	SP600125	PDTC + SP600125
TNF- $\alpha$					
Baseline	689 $\pm$ 172	1,312 $\pm$ 191 <sup>a</sup>	1,311 $\pm$ 211 <sup>a</sup>	1,196 $\pm$ 313 <sup>a</sup>	1,301 $\pm$ 328 <sup>a</sup>
6 months	691 $\pm$ 296	472 $\pm$ 133	984 $\pm$ 191 <sup>a</sup>	967 $\pm$ 211 <sup>a</sup>	456 $\pm$ 98
IL-6					
Baseline	531 $\pm$ 298	1,699 $\pm$ 299 <sup>a</sup>	1,531 $\pm$ 298 <sup>a</sup>	1,588 $\pm$ 139 <sup>a</sup>	1,761 $\pm$ 189 <sup>a</sup>
6 months	581 $\pm$ 112	482 $\pm$ 133	884 $\pm$ 11 <sup>a</sup>	867 $\pm$ 111 <sup>a</sup>	456 $\pm$ 98
IL-8					
Baseline	431 $\pm$ 298	1,788 $\pm$ 299 <sup>a</sup>	1,453 $\pm$ 298 <sup>a</sup>	1,688 $\pm$ 139 <sup>a</sup>	1,861 $\pm$ 189 <sup>a</sup>
6 months	481 $\pm$ 112	482 $\pm$ 133	974 $\pm$ 11 <sup>a</sup>	967 $\pm$ 111 <sup>a</sup>	356 $\pm$ 98
IL-10					
Baseline	1,431 $\pm$ 298	288 $\pm$ 29 <sup>a</sup>	453 $\pm$ 298 <sup>a</sup>	488 $\pm$ 139 <sup>a</sup>	461 $\pm$ 189 <sup>a</sup>
6 months	1,481 $\pm$ 112	1,482 $\pm$ 133	974 $\pm$ 113 <sup>a</sup>	967 $\pm$ 111 <sup>a</sup>	1,356 $\pm$ 98

<sup>a</sup>P<0.05 vs. Control. TNF, tumor necrosis factor; IL, interleukin; PDTC, pyrrolidine dithiocarbamate.

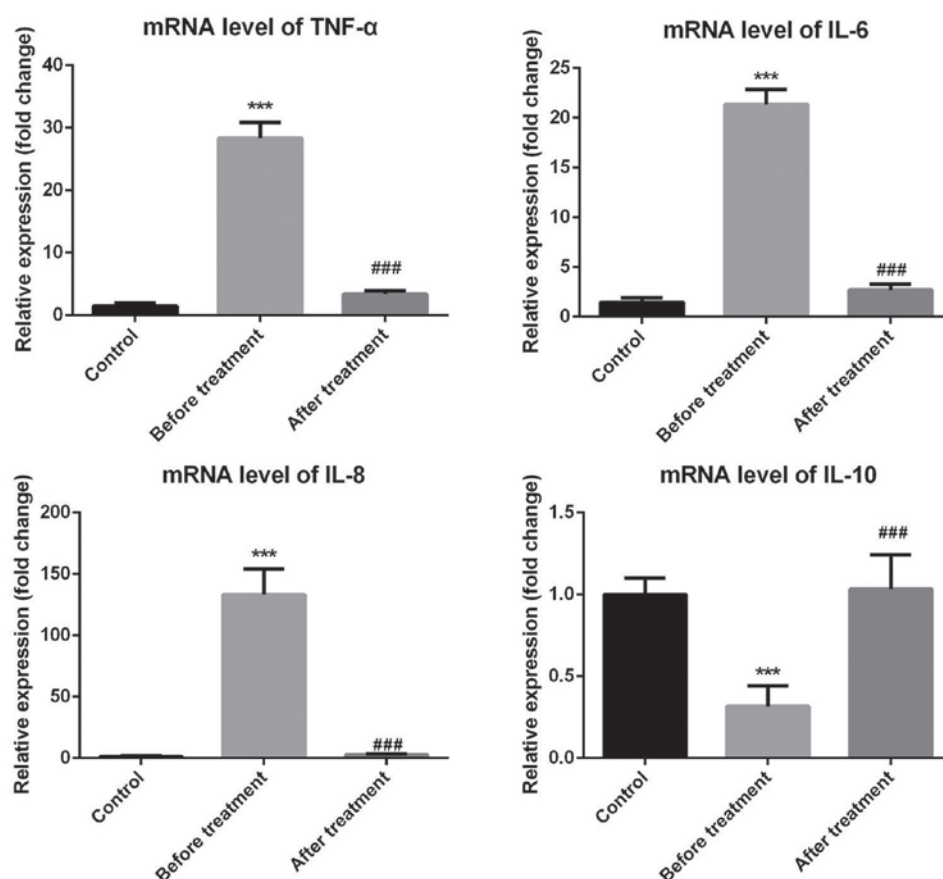


Figure 1. Cytokine mRNA levels prior to and following transcatheter closure treatment as well as in the control group. TNF, tumor necrosis factor; IL, interleukin. \*\*\*P<0.001 vs. the control group; ###P<0.001 vs. the before treatment group.

JNK (36-41) and NF- $\kappa$ B (42-47) signaling pathways are two important pathways that have roles in the occurrence and development of inflammation and are associated with heart diseases such as heart failure (48). Cytokine changes

caused by CHD may synergistically occur via these two pathways (48,49). Changes of cytokines in patients with CHD may be due to the coordination of members of the NF- $\kappa$ B and the JNK signaling pathway. Wang *et al* (50) demonstrated



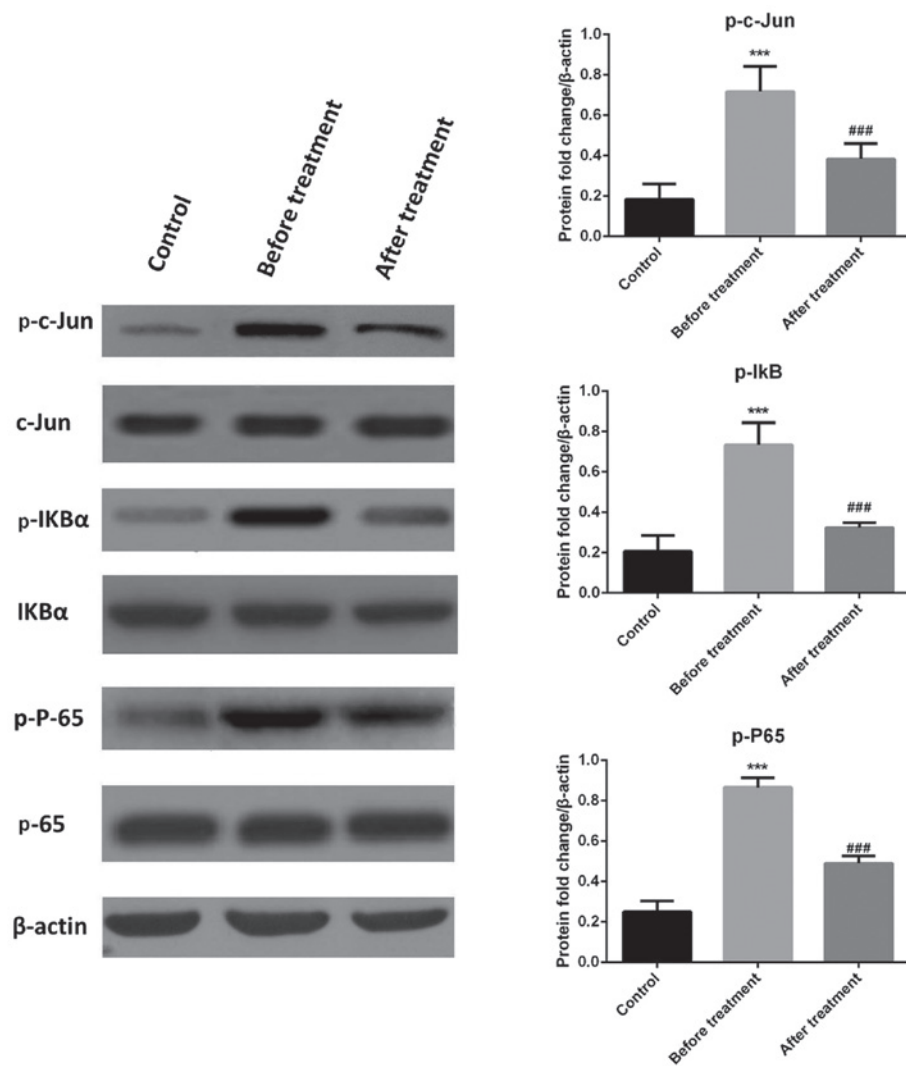


Figure 2. Levels of c-Jun N-terminal kinase and nuclear factor-κB pathway proteins prior to and after transcatheter closure treatment as well as in the control group. p-IkB, phosphorylated inhibitor of nuclear factor κB. \*\*\*P<0.001 vs. the control group; ###P<0.001 vs. the before treatment group.

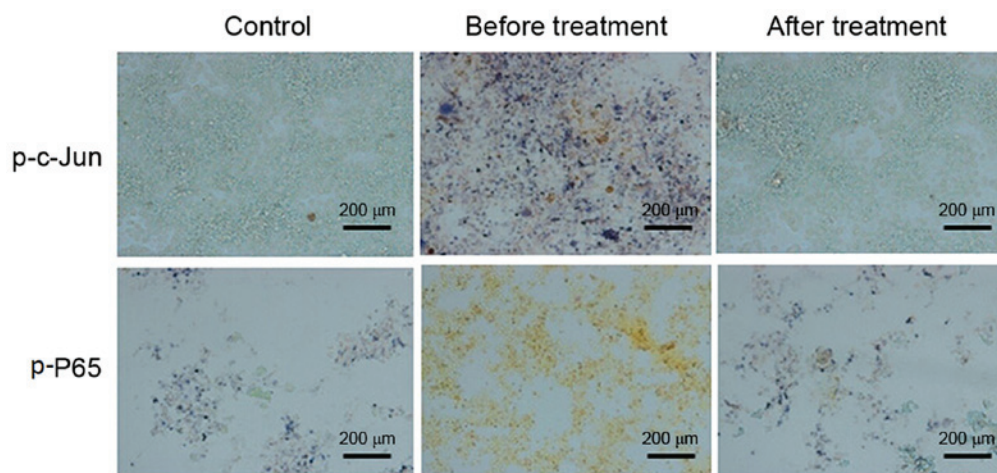


Figure 3. Immunostaining for p-P65 and p-c-Jun in the experimental and control groups 6 months following transcatheter closure treatment (scale bar, 200 μm). p, phosphorylated.

that fasudil hydrochloride hydrate, a rho-kinase inhibitor, suppressed isoproterenol-induced heart failure in rats via the JNK and Extracellular signal-regulated kinase 1/2 signaling

pathways. Gu *et al* (51) demonstrated that hypoxia-induced SOCS3 inhibited NF-κB activation in congenital heart disease.

Of note, the NF- $\kappa$ B and JNK signaling pathways were not activated in patients with CHD following transcatheter closure treatment. To a certain degree, the abnormal changes of cytokines in patients with CHD and their recovery following transcatheter closure treatment are dependent on the coordination of JNK and NF- $\kappa$ B signaling pathways.

The present study had certain limitations. Sampling of a larger amount of serum from the same patient with CHD would have been more beneficial to assess more cytokines to better elucidate the potential underlying mechanism.

In conclusion, the results of the present study suggested a certain degree of immune activation in patients with CHD, resulting in an increase in inflammatory cytokine levels and a decrease in anti-inflammatory cytokine levels. Transcatheter closure treatment gradually restored the patients' inflammatory status. Changes in the levels of cytokines in patients with CHD may be regulated by the NF- $\kappa$ B and JNK signaling pathways. Taken together, the results of the present study indicated that CHD is associated with an upregulated inflammatory response, providing a theoretical basis for further research.

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