Department of Cardiovascular Surgery, The First Affiliated Hospital of Xinxiang Medical University, Weihui, Henan, China

Sildenafil improves the function of endothelial cells in patients suffering from congenital heart disease with pulmonary hypertension

Chaoyuan Zhou, Xiaoliang Guo, Qintao Cui, Xiaochen Liu, Guobao Su, Jie Zhang

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Jie Zhang, Department of Cardiovascular Surgery, The First Affiliated Hospital of Xinxiang Medical University, No.88, Jiankang Road, Weihui 453100, Henan, China.
zhangjie55371@126.com

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Aim of this study was to investigate the potential effects of sildenafil on the function of endothelial cells from patients with congenital heart disease with pulmonary hypertension (CHDPH). Patients who are diagnosed as CHD with PH (n=30) or without PH (n=30), and 30 healthy persons (control) were enrolled in this study. The 30 CHDPH cases were separated into two groups, one was given aspirin while the other received aspirin and sildenafil. An ELISA assay was used to detect the biological indexes for endothelial cells. Furthermore, 24 male New Zealand white rabbits were used to construct the CHDPH model. The signal pathway-related protein expression was analyzed using RT-PCR and western blotting. Compared to that in healthy people, levels for flow-mediated dilatation (FDM), NO, and adiponectin (APN) were significantly decreased while endothelin (ET-1) was significantly increased in CHD patients, while their levels were drastically changed in CHDPH patients ($P<0.01$). Besides, no significant differences for expression levels including FDM, APN, NO, and ET-1 was observed in CHDPH patients receiving aspirin. But the levels for FDM, APN, NO, and ET-1 were significantly changed in CHDPH patients after treatment with sildenafil for 3 months ($P<0.01$). The mRNA and protein levels for JNK1/2, MAPK, and NF-κB were significantly increased in CHDPH rabbits compared to the control ($P<0.01$), but their levels were significantly suppressed by the sildenafil application compared to the CHDPH group ($P<0.01$). Taken together, our study suggested that sildenafil may play a protective role on endothelial function via suppressing the JNK and NF-κB signal pathways in CHDPH patients.

1. Introduction

Pulmonary hypertension (PH) is one of the complications of the left-to-right shunting congenital heart disease (CHD) (Weiford 2015). Serious PH after CHD surgery, is leading to a high morbidity during the perioperative period (Toole et al. 2014). Various factors are involved in the pathogenesis of CHDPH, including the reconstruction of pulmonary vascular structure under the blood mechanical stress and enhanced reaction of pulmonary vessels (Cool et al. 1999; Humbert et al. 2004). Therefore, to deepen the understanding of the pathogenesis of CHDPH will be of great significance for the clinical treatment of CHDPH.

Sildenafil is a phosphodiesterase selective inhibitor, which has been demonstrated to play a role in a variety of diseases, including cardiovascular diseases, femoral head necrosis, and prostate-associated diseases (Das et al. 2005; Guimarães et al. 1999; Kim 2007). Sildenafil also acts as an useful drug against PH in CHD patients, but the mechanism is complicated (Gaetano et al. 2011; Nemoto et al. 2009). For example, Fraisse et al. (2011) proved that intravenous sildenafil reduced pulmonary artery pressure and shortened the time to extubation and intensive care unit stay in children with CHDPH, while Lu et al. (2010) say that oral sildenafil is safe and effective for the CHDPH treatment in adult patients. Thomas et al. (2007) showed that sildenafil dilated blood vessels through increasing the cGMP in pulmonary vessels and by enhancing the endogenous NO. On the other hand, abnormal vascular endothelial cells (VECs) play a pivotal role in the pathogenesis of CHD with PH (Lévy et al. 2007; Yi-Fei and Pang 2008). Even though numerous studies have reported the mechanism for sildenafil in the treatment of CHD with PH, the pathogenesis still remains incompletely understood.

In the current study, we determined the biological indexes of the endothelial cells from CHDPH patients before and after sildenafil. Subsequently, various biological methods were used to investigate the molecular mechanism in a CHDPH model in rabbits and to detect the signal pathway-related protein expression. This study aimed to investigate the effects of sildenafil on endothelial cell function in CHDPH patients and to reveal its mechanism of action.

2. Investigations and results

2.1. Endothelial cells were damaged in patients with CHDPH

The 60 cases who are enrolled in this study were separated into two groups based on a diagnostic criteria, namely patients with CHD only and patients with CHDPH. Subsequently, indexes for the endothelial cells function were detected using ELISA (Fig. 1). Compared to healthy people, FDM, NO, and APN in CHD patients were significantly decreased ($P<0.01$), whereas the ET-1 level was significantly increased ($P<0.01$), suggesting that endothelial cells were damaged in CHD patients. Furthermore, the levels for FDM, NO, and APN in CHDPH patients were drastically lower than those in CHD patients or in healthy persons ($P<0.01$), which is opposite to the results of ET-1 level determination in CHDPH patients ($P<0.01$), indicating a dysfunction of endothelial cells in CHDPH patients.

2.2. Sildenafil improved the functions of endothelial cells in CHDPH patients

The patients who were diagnosed as CHDPH in our hospital, were randomly separated into two groups, namely the aspirin group (15 cases) and the sildenafil group (given aspirin and sildenafil, 15 cases). The indexes for the endothelial cells function were observed before and after drug was given for 3 months (Fig. 2). The results showed that no significant difference for FDM, NO, APN and ET-1 existed between the values before and the after

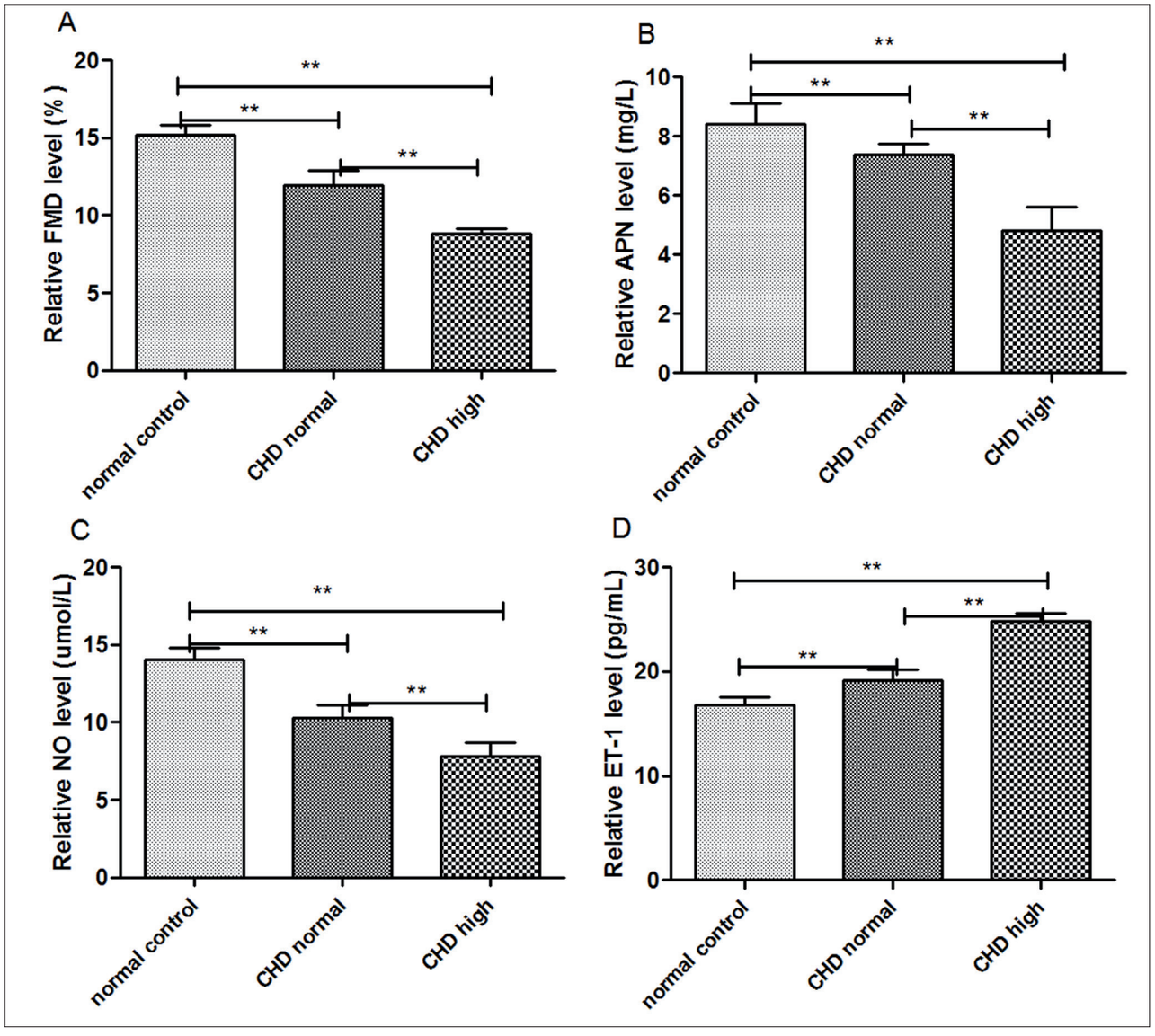

Fig. 1: Indexes of endothelial cells function in patients with congenital heart disease (CHD). A: compared with the healthy persons (n=30), flow-mediated dilatation (FMD) level in endothelial cells from CHD patients (n=30) was significantly decreased, while its level was drastically decreased in endothelial cells from CHDPH patients (n=30); **B**: compared with the healthy persons (n=30), adiponectin (APN) level in endothelial cells from CHD patients (n=30) was significantly decreased, while its level was drastically decreased in endothelial cells from CHDPH patients (n=30); **C**: NO in endothelial cells from CHD patients was lower than that in healthy persons, and its level in CHDPH patients was even less than that in healthy persons; **D**: endothelin (ET-1) level was significantly increased in CHD patients compared to the normal control (healthy person), while its level in CHDPH patients was even more than that in CHD patients. **: P<0.01.

aspirin treatment (P<0.01; Fig. 2A, 2B and 2C). However, FDM, NO, and APN levels were significantly increased while ET-1 was drastically decreased when patients had been treated with sildenafil for 3 months compared to initial values (P<0.01; Fig. 2D).

2.3. Effects of sildenafil on JNK and NK-κB pathway-related protein expression

The expression of JNK, MAPK, and NK-κB in endothelial cells from CHDPH rabbits was measured to investigate the potential mechanism for the influence of sildenafil on endothelial cell function (Fig. 3). The results showed that mRNA and protein levels for JNK, MAPK, and NK-κB in endothelial cells from CHDPH rabbits were significantly increased compared to the control (healthy persons) (P<0.01). However, mRNA and protein levels of the three kinds of proteins were significantly decreased in endothelial cells after sildenafil treatment (P<0.01; Fig. 3A, 3B, 3C, and 3D), suggesting a correlation between the protective role of sildenafil on endothelial cells and the JNK and NK-κB signal pathway.

3. Discussion

CHD with PH remains a pivotal reason for the poor prognosis for CHD (Bishop and Oldershaw 1996; Granton and Marlene 2002). The pathogenesis of CHD with PH is complicated, including a damage of endothelial cells resulting in platelet aggregation caused by the direct contact between the circulating in blood and the subendothelial matrix, finally leading to the PH (Cohen et al. 1999; Stolle and Beck 1990). In this study, we investigated the effects of sildenafil on the function of endothelial cells from patients with CHDPH and we further analyzed the possible molecular mechanism in a rabbit CHDPH model. In agreement with previous data (Ciftel et al. 2012; Wei et al. 2011), our results showed that levels for FDM, NO, and APN were drastically decreased while ET-1 was significantly increased in CHDPH patients compared to healthy persons (Fig. 1), suggesting a dysfunction of endothelial cells in CHDPH patients.

Accordingly, we analyzed the effects of sildenafil on the expression levels of FDM, NO, APN, and ET-1. The results showed that when CHDPH patients were given sildenafil for 3 months, FDM, NO, and APN were significantly increased while ET-1 was signifi-

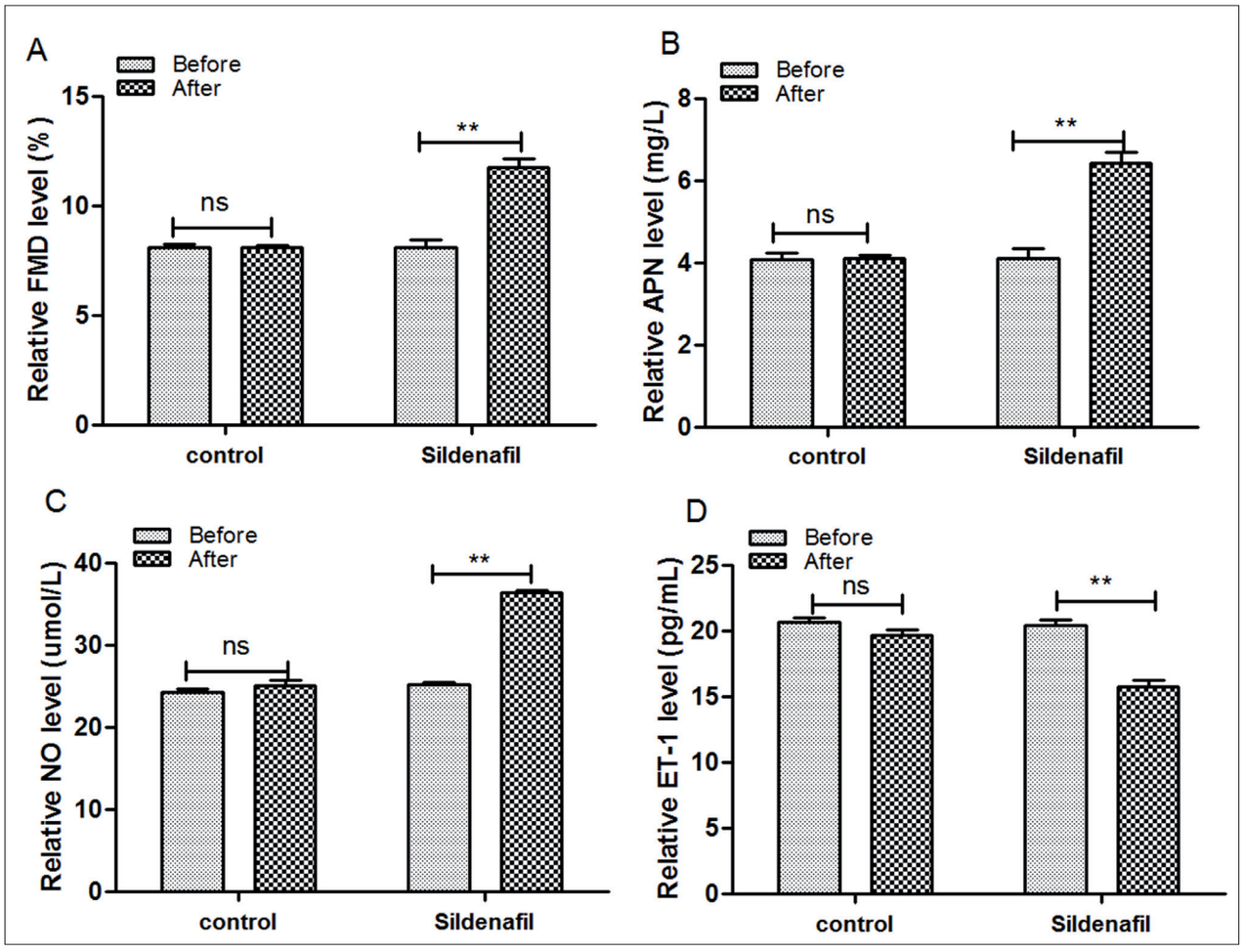

Fig. 2: Influence of sildenafil on endothelial cell function in congenital heart disease with pulmonary hypertension (CHDPH) patients. **A-C**: after being given sildenafil for 3 months, the FMD, APN, or NO level was significantly increased compared with that in patients (n=15) without giving sildenafil; **D**: ET-1 level in CHDPH patients who are given sildenafil for 3 months was significantly decreased compared to the before given sildenafil. In control group, no significant difference was found between the CHDPH patients before given aspirin and the patients after given aspirin (n=15). NS: no significant difference among two groups, **: P<0.01.

cantly decreased compared to the control group (Fig. 2). ET-1 is a kind of shrink blood vessels peptide and promotes vascular smooth muscle cell division secreted by endothelial cells, and is involved in various kinds of cardiovascular diseases (Lutz et al. 1999), whereas NO is a strong pulmonary vasodilator, which can relax the pulmonary vascular smooth by activating cAMP (Barnett and Machado 2006). It is widely known that ET-1 levels are high in CHD patients with PH either in children or in adults (Ding et al. 2003), but FDM, NO, and APN are lower in CHD patients with PH (Wei et al. 2011). Based on our results, we hypothesize that sildenafil may play certain protective role on the function of endothelial cells in patients with CHDPH.

Meanwhile, our results presented that the JNK1/2, MAPK, and NF-κB levels were high in endothelial cells from CHDPH patients, but they were significantly decreased by the sildenafil treatment (Fig. 3). NF-κB is a nucleoprotein factor isolated from the B lymphocyte nucleus extract, which can play important roles in various kinds of cells including vessel endothelial cells, smooth muscle cells and myocardial cells (Jules et al. 2010). The JNK signal pathway belongs to the MAPK family signal pathway involved in regulating cell biology including apoptosis and proliferation (Yang et al. 2014). The JNK signal pathway is activated in lung injury induced by high oxygen (Li et al. 2007). Similar results were also observed by Rahman et al. (2004), where activated NF-κB resulted in the damage of endothelial cells in pulmonary arterial vessels. In addition, the protective role of sildenafil on endothelial cell function and proliferation has been reported by Whitehouse et al. (2002). Based on our results, we hypothesize that sildenafil may protect the endothelial cells from being damaged by suppressing the JNK and NF-κB signal pathways.

Our study may provide a theoretical basis for the application of sildenafil in the clinical treatment of CHDPH.

4. Experimental

4.1. Patients and samples

A total of 60 specimens who were diagnosed as left-to-right shunting in adult CHD at the First Affiliated Hospital of Xinxiang Medical University from July 2013 to July 2015 were enrolled in this study. Thirty cases were diagnosed as CHD with pulmonary hypertension (CHDPH), and 30 cases had CHD without pulmonary hypertension. Additionally, 30 healthy persons who had a physical examination in our hospital were chosen as the normal control (NC). All the patients were informed and consented. In the procedures in this study were approved by the hospital's protection of human ethics committee, the First Affiliated Hospital of Xinxiang Medical University.

4.2. Enzyme-linked immunosorbent assay (ELISA)

The flow-mediated dilatation (FDM), NO, endothelin (ET-1), and adiponectin (APN) levels in serum were measured using the ELISA according to the manufacture's protocol (Al-Qahtani et al. 2005). Briefly, pre-treated microtitre plates were coated with 50 μg/mL calf thymus FDM, NO, ET-1, and APN (Sigma, USA) for 2 h at 37 °C, and then placed overnight at 4 °C. After that, plates were washed with PBS buffer containing 0.05% Tween-20 (PBST) for three times, followed by blocked with 5% goat serum in PBST for 1 h. Then serum samples (diluted at 1:100 in PBST containing 10% calf serum and 5% goat serum) were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, (Sigma) for 2 h at 37 °C. Then the reaction was blocked with 2 N H_2SO_4 and the absorbance was measured using a microplate reader (Bio-Rad) at 492 nm.

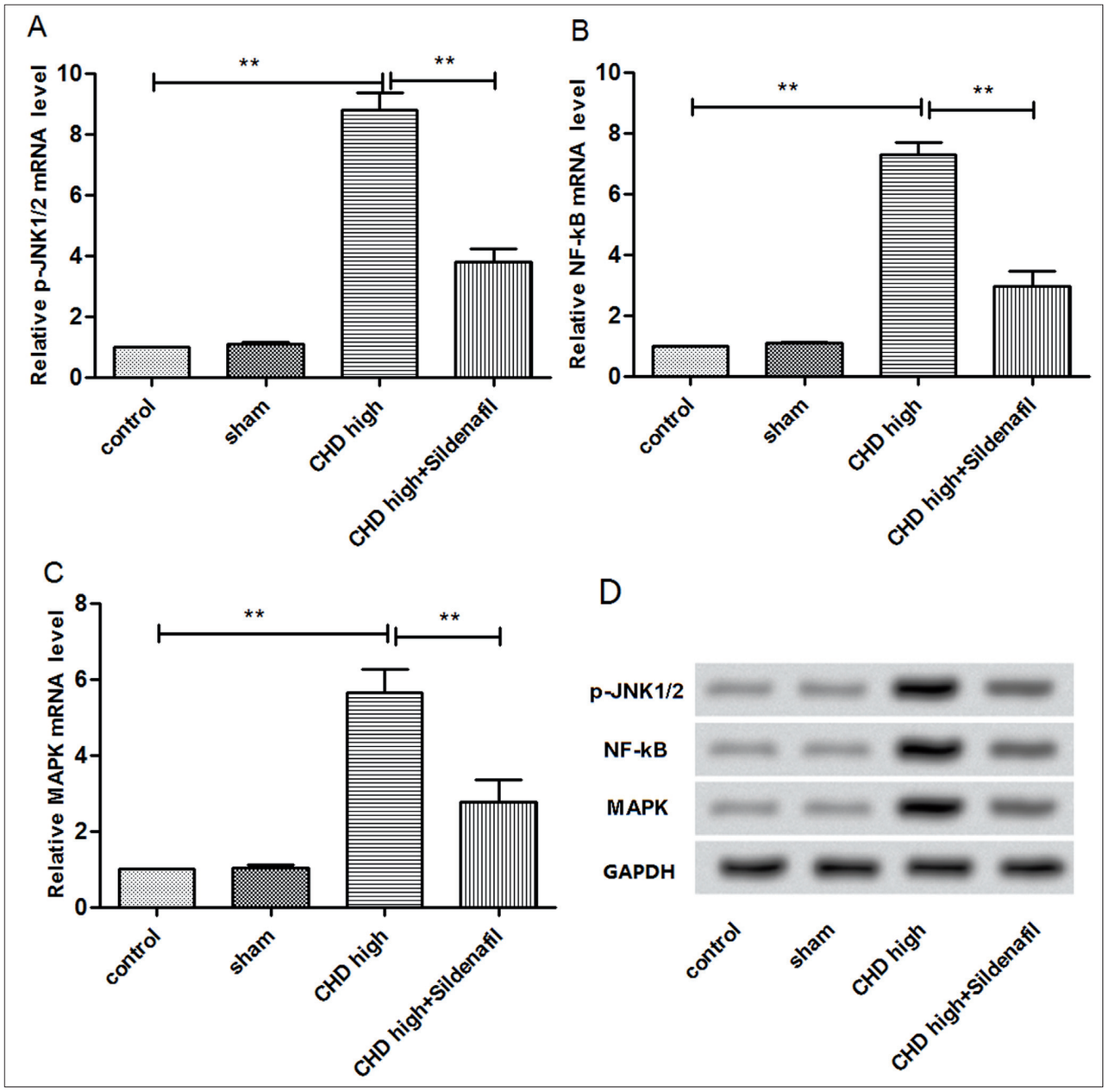

Fig. 3: Effects of sildenafil on the signal pathway-related protein expression in endothelial cells from congenital heart disease with pulmonary hypertension (CHDPH) rabbits. **A-C**: the relative mRNA level for protein including p-JNK1/2, NF-κB, or MAPK in CHDPH rabbits was significantly increased compared to that in control group (healthy rabbits, n=12), however, their levels in cells treated with sildenafil were drastically decreased compared with that in CHDPH rabbits (without sildenafil, n=12); **D**: the protein level for p-JNK1/2, NF-κB, or MAPK in cells from different group was similar to that with their relative mRNA level. **: P<0.01.

4.3. Rabbit model

All the procedures conducted in this study were in accordance with guidelines of the local animal care and protocols were approved by the local experimental ethics committee. Twenty four male New Zealand white rabbits (animal center of Xinxiang Medical University) weighing 2.4-2.8 kg were used for the CHD model construction. In brief, 2.5% sodium pentobarbital was intravenously injected into the margin of the rabbit ear. After anesthesia, rabbits were fixed onto the operating table. Hair on the neck was removed and then disinfected with 75% alcohol, then the remote site under the first vein outside the neck was ligated. The blood close to the heart was blocked using a small vascular clamp around the right common arotid artery, then the common carotid artery was ligated about 0.5 cm below the bifurcation between the internal and the external arteries and then was cut in diagonal line. A small vascular clamp was used to block the blood at the vein close to heart of the external carotid, followed by the medial incision on the external carotid vein. Then 7.0 Prolene was used to suture the carotid artery and the jugular vein under a microscope. The volatility in the bright red blood close to the heart of the external carotid stands for the anastomotic was unobstructed. The two blood vessels were separated instead of ligation and anastomotic in rabbits from the sham group. Penicillin was put at the anastomotic site to prevent bacterial infection. Rabbits characterized with > 30 mmHg pulmonary artery systolic pressure and > 20 mmHg mean pulmonary artery pressure indicate that the model was successfully constructed.

4.4. Real-time PCR

Total mRNA was isolated from the blood samples as previously described (Mackay 2004). Complementary DNA (cDNA) was produced using reverse transcriptase (iScript™ cDNA Synthesis Kit; Bio-Rad Laboratories). The expression levels of mRNAs were measured by SYBR green-based quantitative RT-PCR (SYBR Green Master mix; Thermo Scientific, Waltham, MA, USA). Primers used for the target gene amplification are shown in the Table.

4.5. Western blotting

Cells were lapped with RIPA assay (radioimmunoprecipitation, Sangon Biotech, China) lysate containing PMSF (phenylmethanesufonyl fluoride, Sigma, USA), and then were centrifuged at 12,000 rpm for 10 min at 4 °C. Supertanant was collected for the measurement of protein concentrations using BCA protein assay kit (Pierce, Rochford, IL). For Western blotting (Mabuchi et al. 2007), 50 μg protein per cell lysate was subjected onto a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferred onto a polyvinylidencefluoride (PVDF) membrane (Mippore). Then the PVDF membranes were blocked in tris-buffered saline Tween (TBST) containing 5% non-fat milk for 1 h at room temperature. Then the membranes were incubated with rabbit anti-human antibodies (MAPK, JNK1/2, and NF-κB, 1:100 dilution, Invitrogen, USA)

Table 1: Primers used for target amplification in this study

Target	primer	Sequence (5'-3')
GAPDH	Sense	GGGTGGAGCCAAACGGGTC
	Antisense	GGAGTTGCTGTTGAAGTCGCA
MAPK	Sense	GCCATGGAGTCAATTAGCTG
	Antisense	TGCCCAAGAAGAAGCCGACC
JNK	Sense	GTG CAG CACCCG CGG CTG CA
	Antisense	TGCAACTGCTGCGTTAGCATG
NF-κB	Sense	ACGATCTGTTTCCCCTCATCT
	Antisense	TGCTTCTCTCCCCAGGAATA

overnight at 4 °C. Then membrane was incubated with horseradish-peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the PVDF membranes were washed 3 times with 1× TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control.

4.6. Statistical analysis

All experiments were repeated three times independently. The data were expressed as the mean±SD in this study. The significance for data was calculated using the SPSS 16.0. Independent sample t-test was used for the paired data significance calculation. Post-hoc Turkey-test was used to calculate the difference among groups. $P<0.05$ was considered statistically significant in comparison.

Conflict of interest: None declared.

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