European Review for Medical and Pharmacological Sciences 2019; 23: 1742-1750

Effect of estrogen on right ventricular remodeling of monocrotaline-induced pulmonary arterial hypertension in rats and its mechanism

Z. LIU[1], Y.-L. DUAN[2], S.-L. GE[1], C.-X. ZHANG[1], W.-H. GONG[1], J.-J. XU[1]

[1]Department of Cardiac Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, China
[2]Medical Clinical Laboratory, The Second Affiliated Hospital of Anhui Medical University, Hefei, China

Abstract. – OBJECTIVE: To elucidate the effect of estrogen on right ventricular remodeling of pulmonary arterial hypertension (PAH) induced by monocrotaline (MCT) in rats and its underlying mechanism.

MATERIALS AND METHODS: Male Sprague-Dawley (SD) rats were adaptively fed for one week, and then, randomly divided into control group, MCT group, MCT+17-β estradiol (50 mg·kg^{-1}·d^{-1}) group, and MCT+17-β estradiol (100 mg·kg^{-1}·d^{-1}) group, with 8 rats in each group. PAH rat model was constructed by subcutaneous injection of 60 mg·kg^{-1} MCT for consecutive 4 weeks. The right external jugular vein was intubated to monitor rat RVSP (right ventricular systolic pressure) and mPAP (mean pulmonary arterial pressure). After animal procedures, RV (right ventricle) and LV+S (left ventricle+ventricular septum) of rat were harvested and weighed. Rat tibia was collected and recorded for its length, followed by calculation of RV/(LV+S) and RV/length of the tibia. HE staining and Masson staining were conducted to observe the pathological change and collagen deposition in RV, respectively. RV was prepared for homogenate, followed by detection of total antioxidant capacity (T-AOC) and malondialdehyde (MDA) activities. Expression levels of collagen I, collagen III, NOX4, and NF-κB in RV of PAH rats were detected by qRT-PCR and Western blot.

RESULTS: Lower RVSP, mPAP, RV/(LV+S) and RV/length of the tibia were observed in rats of MCT+17-β estradiol (50 mg·kg^{-1}·d^{-1}) group and MCT+17-β estradiol (100 mg·kg^{-1}·d^{-1}) group compared with those of MCT group. Injection of 17-β estradiol (50 mg·kg^{-1}·d^{-1} and 100 mg·kg^{-1}·d^{-1}) in PAH rats remarkably alleviated pathological changes and collagen deposition in RV. T-AOC activity increased, whereas MDA activity decreased in PAH rats injected with 17-β estradiol. Expression levels of collagen I, collagen III, NOX4, and NF-κB in RV of PAH rats were all downregulated by 17-β estradiol treatment.

CONCLUSIONS: 17-β estradiol could remarkably alleviate MCT-induced right ventricular remodeling of PAH rats. It is suggested that 17-β estradiol exerts its protective role in PAH by inhibiting NOX4-mediated oxidative stress and NF-κB-mediated collagen deposition.

Key Words
Estrogen, PAH, Right ventricular remodeling, NOX4, NF-κB.

Introduction

Pulmonary arterial hypertension (PAH) is a cardiovascular disease characterized by progressively increased pulmonary vascular resistance accompanied by irreversible vascular reconstruction. Persistent increase in pulmonary vascular resistance and pulmonary arterial pressure eventually lead to respiratory failure, pulmonary heart disease, heart failure, and even death. Prevention and treatment of PAH and subsequent right ventricular remodeling are of great significance[1,2]. Scholars[3,4] have shown that oxidative stress exerts an important role in PAH-induced right ventricular remodeling. Previous researches have shown the upregulated expression of NOX4 in RV (right ventricle) of monocrotaline (MCT)-induced PAH rats. Also, NF-κB activation is involved in PAH-induced cardiovascular remodeling. NOX4-mediated oxidative stress may enhance the dissociation of IκB, further activating downstream transcription factors of NF-κB[5]. Subsequently, expressions of relative cytokines, growth factors, chemokines, inflammatory mediators, and adhesion molecules are stimulated, thereafter promoting cell interstitial fibrosis[6].

It is found that the incidence of PAH in females is higher than that of males, especially in young women[7]. Researchers speculated whether estrogen may explain the incidence difference between females and males. However, the effects of estrogen on the intravascular balance of cardiovascu-

Corresponding Author: Shenglin Ge, MM; e-mail: aydgsl@sina.com

lar disease are not fully elucidated. 17β-estradiol (E2) possesses the strongest biological activity among the three natural estrogens in the body and is thought to play a major physiological role[8]. Previous investigations[9-12] have demonstrated that estrogen can improve hemodynamics and gas exchange in PAH by regulating pulmonary vascular endothelial function, promoting vasodilation and inhibiting smooth muscle cell proliferation. Estrogen is also capable of suppressing pulmonary revascularization after PAH. To our best knowledge, the protective role of estrogen in PAH-induced right ventricular remodeling is rarely reported. In the present work, we first constructed a PAH rat model by MCT administration. We aim to explore whether estrogen could protect right ventricular remodeling and its underlying mechanism.

Materials and Methods

Experimental Animals and Sample Collection

Male Sprague-Dawley (SD) rats weighing 180-220 g in SPF (specific pathogen-free) level were provided by JSJ Laboratory Animal, Shanghai, China (license key, SCXK, 2013-0006, Shanghai, China). SD rats were adaptively fed for one week and randomly divided into control group, MCT group, MCT+17-β estradiol (50 $mg \cdot kg^{-1} \cdot d^{-1}$) group, and MCT+17-β estradiol (100 $mg \cdot kg^{-1} \cdot d^{-1}$) group, with 8 rats in each group. PAH rat model was constructed by subcutaneous injection of 60 $mg \cdot kg^{-1}$ MCT. 17-β estradiol was subcutaneously injected for consecutive 4 weeks. All rats were given to free access to food and water. They were housed in an environment with relative humidity of 40-70%, temperature of 20±3°C, and light cycle of 12/24 h. This study was approved by the Animal Ethical Committee of Anhui Medical University.

4 weeks later, rats were anesthetized by intraperitoneal injection of 60 $mg \cdot kg^{-1}$ pentobarbital sodium. A polyethylene catheter with a pressure transducer (0.5 mm in diameter) was inserted into the right ventricle and pulmonary artery through the right external jugular vein of the rat. RVSP and mPAP were recorded using a MedLab polyphysiograph (MedLabTA6008)[11].

Subsequently, blood samples were collected through the carotid artery and rats were sacrificed for isolating the heart. RV (right ventricle) and LV+S (left ventricle+ventricular septum) of rat were harvested and weighed. Rat tibia was collected and recorded for its length, followed by calculation of RV/(LV+S) and RV/length of tibia[3,12]. The right ventricular apex of the rat was harvested and fixed in 4% paraformaldehyde (PFA) overnight. 100 mg RV was weighed, ground in liquid nitrogen and incubated with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA). The remaining RV tissues were lysed on ice for 30 min, followed by centrifugation at 4°C, 12 000 $r \cdot min^{-1}$ for 10 min. The supernatant was collected for protein extraction.

Reagents

17-β estradiol (purity≥98%) were obtained from Pureonebio (Shanghai, China); MCT was provided by Sigma-Aldrich (St. Louis, MO, USA); Masson staining kit was obtained from Keygen (Nanjing, China); Total antioxidant capacity (T-AOC), malondialdehyde (MDA), and bicinchoninic acid (BCA) determination kits were provided by Beyotime (Shanghai, China); TRIzol RNA extraction kit was obtained from Invitrogen (Carlsbad, CA, USA); PrimeScriptTM RT reagent Kit and Power SYBR Green PCR Master Mix were obtained from TaKaRa (Otsu, Shiga, Japan); Primers used in the study were constructed by Sangon (Shanghai, China); Primary antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA); LunimataTM Crescendo was obtained from Merck Millipore (Billerica, MA, USA).

Masson Staining and HE Staining

RV tissues were fixed with 4% polyfluoroalkoxy (PFA) for 48 h and paraffin-embedded. Masson staining and HE staining were performed based on the instructions of relative commercial kits.

Determination of T-AOC and MDA Activities

RV tissues were cut into small pieces for tissue lysis on ice. After centrifugation, RV tissues were prepared for homogenate. T-AOC (total antioxidant capacity) and MDA (malondialdehyde) activities in RV homogenate were determined using relative commercial kits.

Immunohistochemistry Staining

RV tissues were fixed with 10% formaldehyde, paraffin-embedded and sliced into 4 μm sections. After antigen retrieval for 20 min, sections were blocked at room temperature for 1 h. Subsequently, RV sections were incubated with the primary and secondary antibodies. Tissues were then restained with hematoxylin and eosin. The immunohistochemistry staining was finally observed using a microscope.

Table I. Primer sequences used for determination of NOX4, NF-κB, and collagen I and collagen III gene expression.

Gene	Oligonucleotide primer sequences (5'-3')
NOX4	Forward: 5'-CCAGAATGAGGATCCCAGAA-3' Reverse: 5'-AGCAGCAGCAGCATGTAGAA-3'
NF-κB	Forward: 5'-CCAGGAGAAAGTCAGCCTCCT-3' Reverse: 5'-TCATACCAGGGCTTGAGCTCA-3'
Collagen I	Forward: 5'-CCAACTGAACGTGACCAAAAACCA-3' Reverse: 5'-GAAGGTGCTGGGTAGGGAAGTAGGC-3'
Collagen III	Forward: 5'-ATTCTGCCACCCTGAACTCAAGAGC-3' Reverse: 5'-TCCATGTAGGCAATGCTGTTTTTGC-3'
β-Actin	Forward: 5'-TGTCACCAACTGGGACGATA-3' Reverse: 5'-ACCCTCATAGATGGGCACAG-3'

RT-PCR (Real Time-Polymerase Chain Reaction)

We used TRIzol to extract total RNA for reverse transcription according to the instructions of PrimeScript RT reagent Kit. The specific parameters in PCR were previously described[13]. The expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method and analyzed using 7300 System SDS Study Software. Primers used in RT-PCR were listed in Table I.

Western Blot

RV tissues were added with lysis buffer and shaken on ice for 30 min. The total protein was separated after the centrifugation at 14,000 g/min for 15 min at 4°C. Protein concentration was calculated by the BCA protein assay kit. The extracted proteins were separated on a 12% SDS-PAGE gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. Western blot analysis was performed according to standard procedures.

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA). The quantitative data were represented as mean ± standard deviation ($\bar{x}\pm s$). One-way ANOVA was performed for analyzing differences among groups, followed by Least Significant Difference (LSD) analysis. $p<0.05$ was considered statistically significant.

Results

Effect of Estrogen on Hemodynamic Parameters in PAH Rats

Compared with rats in control group, RVSP and mPAP remarkably elevated in rats of MCT group ($p<0.01$). After injection of 50 $mg\cdot kg^{-1}\cdot d^{-1}$ and 100 $mg\cdot kg^{-1}\cdot d^{-1}$ 17-β estradiol for 4 weeks, RVSP and mPAP remarkably decreased in PAH rats, indicating that estrogen could improve hemodynamic parameters in PAH rats (Table II).

Effect of Estrogen on RV/(LV+S) and RV/Length of Tibia in PAH Rats

The RV/LV+S and RV/length of the tibia are not affected by body weight and left ventricular mass, which are considered as major predictors of right ventricular hypertrophy[3,12]. Compared with the control group, RV/LV+S and RV/length of the tibia in MCT group significantly increased ($p<0.01$), indicating that the right ventricle was hypertrophied after subcutaneous injection of MCT in rats. After 4 weeks injection of different doses of estrogen, the above data were significantly reduced, suggesting that estrogen inhibits right ventricular remodeling in PAH rats (Table III).

Table II. Effect of 17-β estradiol on RVSP and mPAP in monocrotalineinduced pulmonary hypertension of rats. x±s.

Group	n	RVSP/mmHg	mPAP/mmHg
Control	8	34.46 ± 2.38	36.60 ± 2.59
Monocrotaline	7	67.10 ± 5.05**	50.95 ± 1.27**
+ 17-β estradiol (50 $ug\cdot kg^{-1}$)	8	47.91 ±3.60$^{\#}$	46.29 ± 1.61$^{\#}$
+ 17-β estradiol (100 $ug\cdot kg^{-1}$)	8	44.50 ± 3.10$^{\#\#}$	43.79 ± 2.17$^{\#\#}$

**$p<0.01$ *vs.* control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* monocrotaline group. RVSP: Right ventricle systolic pressure; mPAP: Mean pulmonary arterial pressure.

Table III. Effect of 17-β estradiol on RV/LV+S and RV/tibial length in monocrotaline-induced pulmonary hypertension of rats. x±s.

Group	n	RV/LV+S (mg/mg)	RV/tibial length (mg/cm)
Control	8	0.22 ± 0.03	56.11 ± 5.27
Monocrotaline	7	0.43 ± 0.06**	66.65 ± 5.25**
+ 17-β estradiol (50 ug·kg^{-1})	8	0.34 ± 0.05$^{\#}$	74.73 ± 5.62$^{\#}$
+ 17-β estradiol (100 ug·kg^{-1})	8	0.30 ± 0.04$^{\#\#}$	68.57 ± 8.34$^{\#\#}$

**p<0.01 *vs.* control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs.* monocrotaline group. RV: Right ventricle; LV+S: Left ventricle + septum.

Effect of Estrogen on RV Structure Change in PAH Rats

HE staining was conducted to observe the pathological change in RV of PAH rats. Right ventricular cardiomyocytes were hypertrophied and arranged disorderly in rats of MCT group. Besides, swelling of cytoplasm and widening of intercellular space of myocardium were also observed (Figure 1). By comparison, pathological lesions in RV were remarkably alleviated in MCT+17-β estradiol (50 mg·kg^{-1}·d^{-1}) group and MCT+17-β estradiol (100 mg·kg^{-1}·d^{-1}) group.

Effect of Estrogen on Collagen Deposition in RV of PAH Rats

Masson staining showed that the collagen fibers were stained blue. In control group, the myocardial cell gap in RV was clearly visible, and the paravascular collagen distribution was sparse with light color staining. Compared with rats in MCT group, MCT+17-β estradiol (50 mg·kg^{-1}·d^{-1}) group and MCT+17-β estradiol (100 mg·kg^{-1}·d^{-1}) group showed less blue staining in RV, indicating that estrogen has some improvement on myocardial collagen deposition in the RV of PAH rats

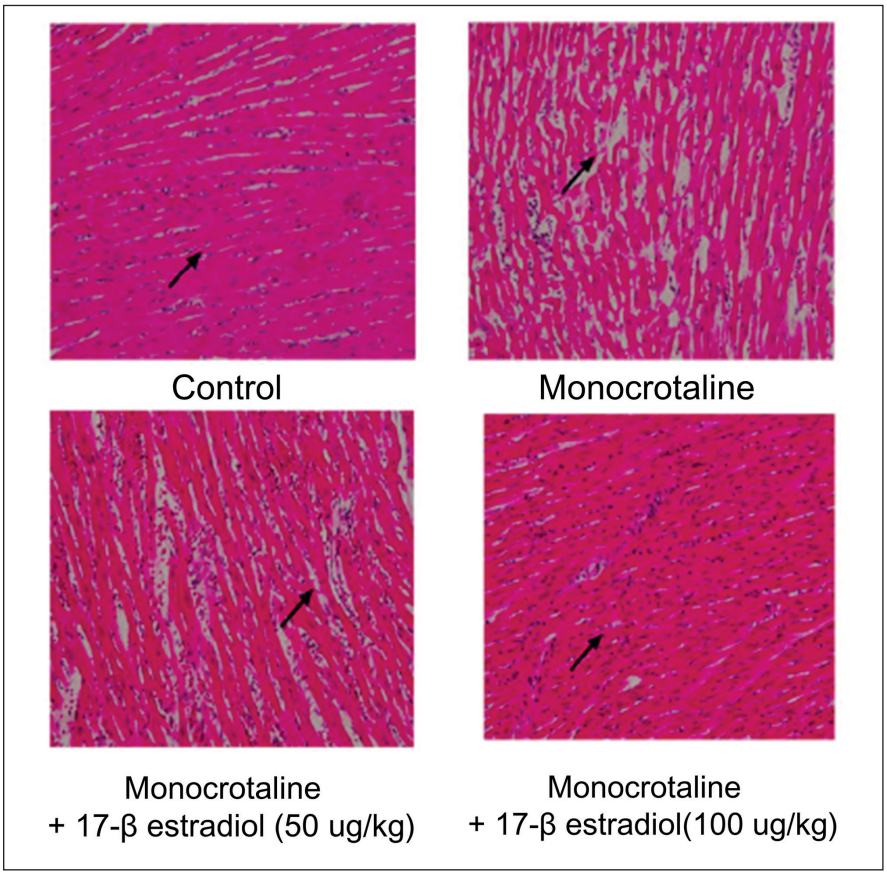

Figure 1. Hematoxylin-eosin staining of the right ventricle in MCT-induced PAH rats (magnification ×400).

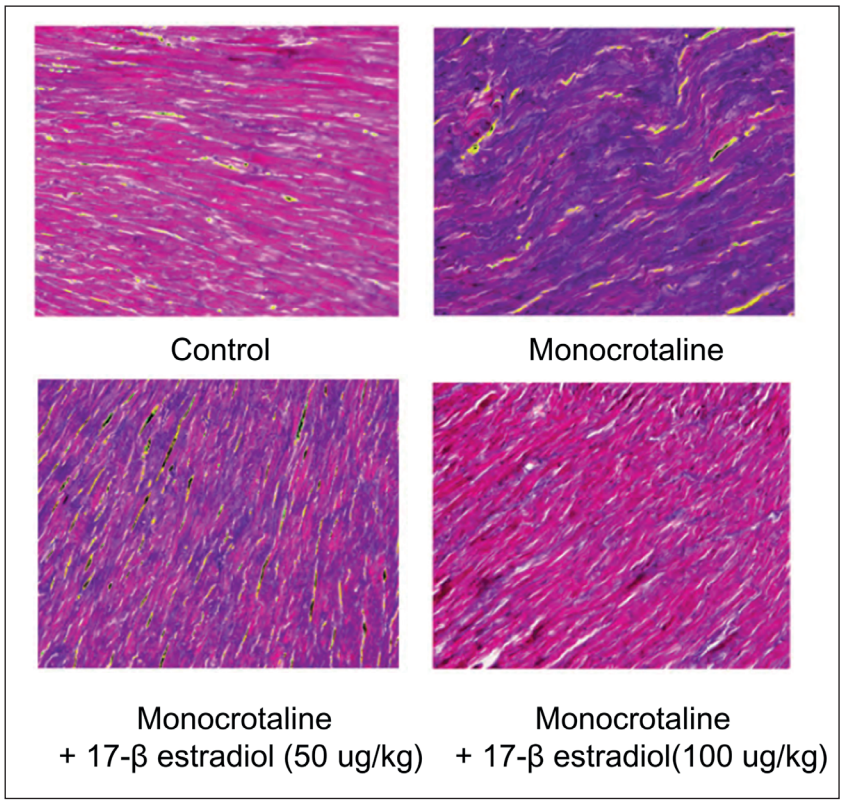

Figure 2. Effect of 17-β estradiol on collagen deposition of the right ventricle in MCT-induced PAH rats (magnification ×400).

(Figure 2). Subsequently, we detected mRNA and protein levels of collagen I and collagen III in rat RV. MCT group showed higher expression levels of collagen I and collagen III compared with those of control group. However, 17-β estradiol injection markedly decreased their expressions (Figure 3).

Effect of Estrogen on NOX4 Expression in RV of PAH Rats

NOX4 expression in RV was determined by qRT-PCR, Western blot, and immunohistochemistry staining, respectively. We found higher mRNA and protein levels of NOX4 in MCT group than those of control group, which were remarkably downregulated after 17-β estradiol treatment (Figure 4A and 4B). Similarly, immunohistochemistry staining indicated fewer NOX4-positive cells in MCT+17-β estradiol (50 $mg \cdot kg^{-1} \cdot d^{-1}$) group and MCT+17-β estradiol (100 $mg \cdot kg^{-1} \cdot d^{-1}$) group compared with that of MCT group (Figure 4C).

Effect of Estrogen on Activities of MDA and T-AOC in PAH Rats

MDA activity increased, whereas T-AOC activity decreased in MCT group than control group ($p<0.01$), which were remarkably reversed by 17-β estradiol administration. It is indicated that estrogen protects oxidative stress in PAH rats (Table IV).

Furthermore, we determined NF-κB expression in RV of PAH rats. The data showed that both mRNA and protein levels of NF-κB are upregulated after MCT induction ($p<0.01$). However, rats in MCT+17-β estradiol (50 $mg \cdot kg^{-1} \cdot d^{-1}$) group and MCT+17-β estradiol (100 $mg \cdot kg^{-1} \cdot d^{-1}$) group showed downregulated NF-κB expression in RV (Figure 5).

Discussion

Ventetuolo et al[14] constructed PAH model in rats using estrogen receptor antagonist and estrogen metabolic converting enzyme inhibitor (COMT). The results showed that the expression of estrogen receptor (ER) β is up-regulated in pulmonary vessels of the hypoxic group, while ERα expression does not alter. In the non-selective ER inhibitor and selective ERα inhibitor intervention group, the beneficial effects of E2 on the cardiopulmonary hemodynamics were attenuated. Although COMT interfered with E2 metabolism, the protective role of E2 on PAH still existed.

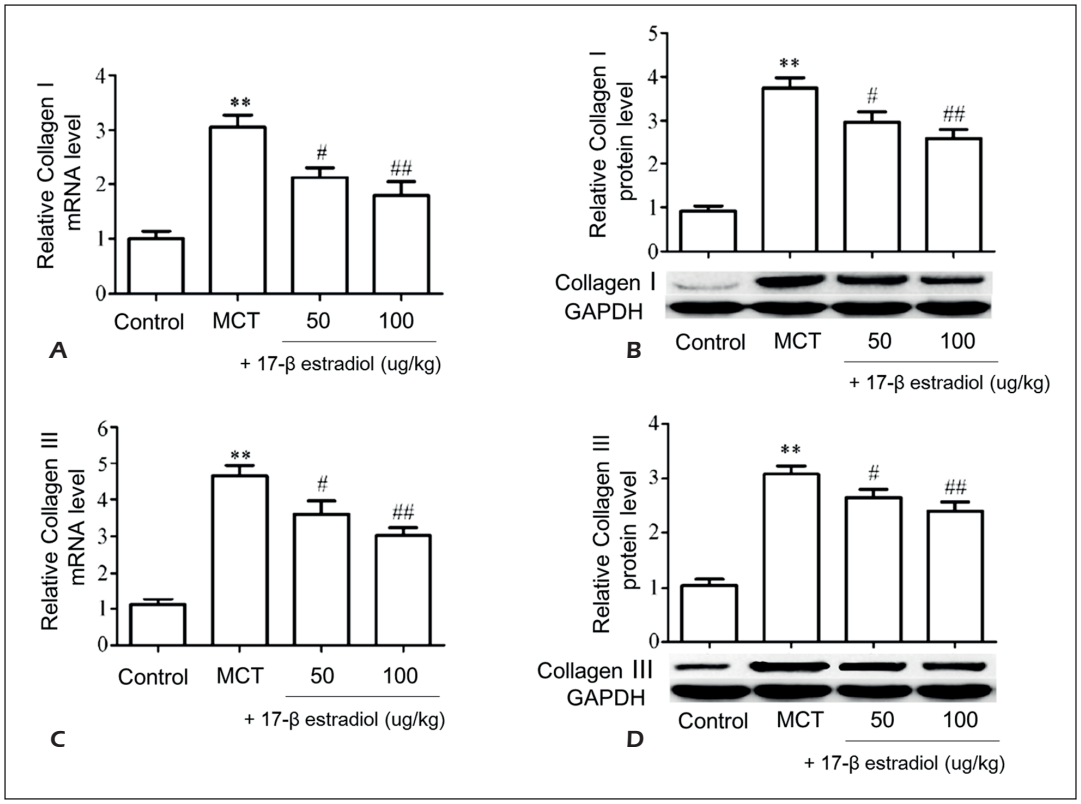

Figure 3. Effect of 17-β estradiol on the collagen I and collagen III mRNA and proteins expression of the right ventricle in MCT-induced PAH rats. **A** and **C**, The mRNA expressions of collagen I and collagen III. **B** and **D**, The protein expressions of collagen I and collagen III. n=7-8. $^{**}p<0.01$ vs. control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. MCT group. MCT: Monocrotaline.

It is suggested that E2-mediated anti-proliferation directly protects PAH development. In the present study, the injection of 17-β estradiol in PAH rats remarkably alleviated pathological changes and collagen deposition in RV. We concluded that estrogen is capable of reducing pulmonary artery pressure, right ventricular remodeling, and fibrosis in PAH rats.

The pathogenic mechanism of PAH-induced right ventricular remodeling has not been fully elucidated. A large number of studies have shown that NOX-induced oxidative stress markedly impairs pulmonary vascular remodeling and right ventricular remodeling[15]. Liu et al[3] have found that resveratrol and its derivatives inhibit hypoxia-induced pulmonary ventricular remodeling by inhibiting the expressions of NOX2 and NOX4. Li et al[4] have found that the expression of NOX4 in the right ventricle of MCT-induced PAH rats is significantly upregulated. We suggested that NOX4-mediated oxidative stress may influence right ventricular remodeling after PAH. After 4-week estrogen administration, the expression of NOX4 in the right ventricle of PAH rats significantly decreased. Besides, MDA activity significantly decreased, whereas T-AOC activity increased. These results further indicated that estrogen has an antioxidant effect, showing a protective effect on right ventricular remodeling in PAH rats through downregulating NOX4 expression.

NF-κB is a nuclear transcription factor that is ubiquitous in eukaryotes and participates in many important physiological processes in the body. Oxidative stress activates NF-κB, thus participating in the occurrence of PAH[16,17]. Under normal conditions, NF-κB binds to its inhibitor IκB and is present in the cytoplasm in an inactive state. Oxidative stress could activate intracellular IκB kinase, which can phosphorylate and dissociate IκB. NF-κB immediately translocates into the cell nucleus, further initiating interstitial fibrosis and participating in the cardiovascular remodeling of PAH[5,6]. It is reported[18,19] that the protective effects of estrogen on inhibiting inflammation, oxidative stress, cell apoptosis, and proliferation are mainly achieved by inhibiting NF-κB activation. We found that the expression of NF-κB in the right

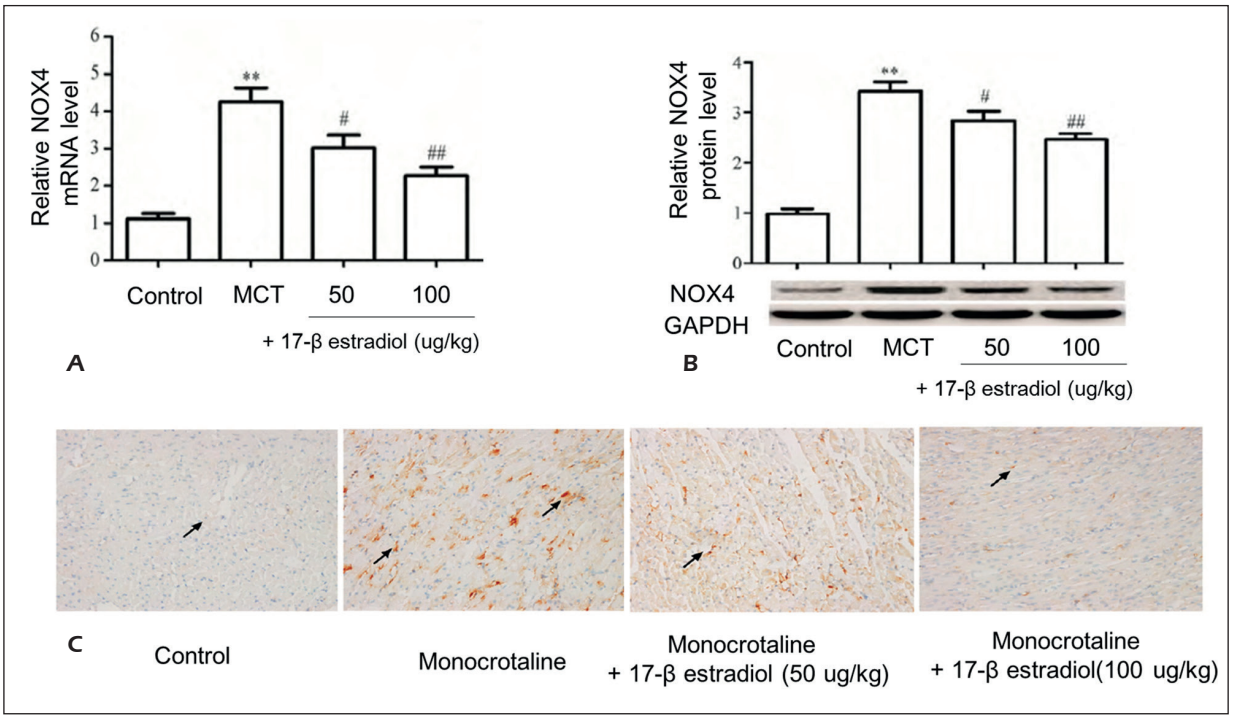

Figure 4. Effect of 17-β estradiol on the NOX4 expression of the right ventricle in MCT-induced PAH rats. **A**, The expression of NOX4 in right ventricle was determined by qPCR. **B**, The expression of NOX4 in right ventricle was determined by Western blot. **C**, The expression of NOX4 in right ventricle was determined with immunohistochemistry staining (arrows indicated positive staining of NOX4). n = 7-8. **p<0.01 vs. control group; $^{\#}p$< 0.05, $^{\#\#}p$<0.01 vs. MCT group. MCT: Monocrotaline; NOX4: NADPH oxidase 4.

ventricle of PAH rats significantly increased. After 4 weeks of estrogen administration, the mRNA and protein levels of NF-κB in the right ventricle of PAH rats were downregulated. We confirmed that estrogen inhibits right ventricular remodeling after PAH by inhibiting NF-κB activation.

In summary, estrogen can alleviate the right ventricular remodeling in PAH rats. The mechanism may be related to the inhibition of NOX4 expression and NF-κB activation. Through literature review[3], NOX2 upregulation in the myocardium may also participate in the occurrence and progression of right ventricular remodeling after PAH. Whether estrogen could inhibit NOX2-induced oxidative stress in PAH requires further investigation.

Conclusions

We found that the 17-β estradiol could remarkably alleviate MCT-induced right ventricular remodeling of PAH rats. It is suggested that 17-β estradiol exerts its protective role in PAH by inhibiting NOX4-mediated oxidative stress and NF-κB-mediated collagen deposition.

Table IV. Effect of chrysin on MDA and T-AOC level in right ventricle of monocrotaline-induced pulmonary hypertension of rats. x ± s.

Group	n	T-AOC/μmol·mg-1 (protein)	T-AOC/μmol·mg-1 (protein)/cm)
Control	8	69.35 ± 3.73	10.76 ± 3.65
Monocrotaline	7	35.83 ± 2.74**	46.63 ± 3.78**
+ 17-β estradiol (50 ug·kg^{-1})	8	45.90 ± 4.88$^{\#}$	24.26 ± 4.95$^{\#}$
+ 17-β estradiol (100 ug·kg^{-1})	8	51.36 ± 4.25$^{\#\#}$	25.86 ± 5.44$^{\#\#}$

**p<0.01 *vs.* control group; $^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs.* monocrotaline group. RV: Right ventricle; LV+S: Left ventricle + septum.

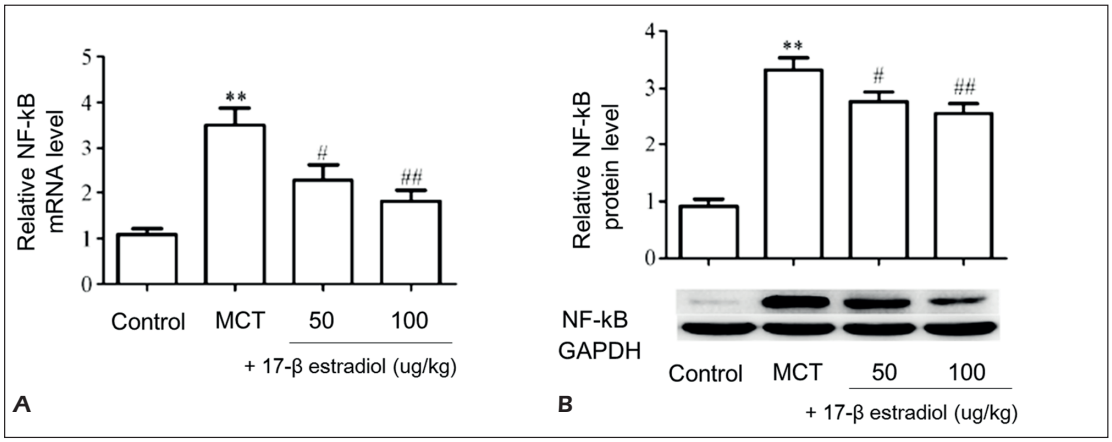

Figure 5. Effect of 17-β estradiol on the NF-κB expression of right ventricle in MCT-induced PAH rats. **A**, The expression of NF-κB in right ventricle was determined by qPCR. **B**, The expression of NF-κB in right ventricle was deter mined by Western blot.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Chin KM, Rubin LJ. Pulmonary arterial hypertension. J Am Coll Cardiol 2008; 51: 1527-1538.
2) Li WJ, Hu K, Yang JP, Xu XY, Li N, Wen ZP, Wang H. HMGB1 affects the development of pulmonary arterial hypertension via RAGE. Eur Rev Med Pharmacol Sci 2017; 21: 3950-3958.
3) Liu B, Luo XJ, Yang ZB, Zhang JJ, Li TB, Zhang XJ, Ma QL, Zhang GG, Hu CP, Peng J. Inhibition of NOX/VPO1 pathway and inflammatory reaction by trimethoxystilbene in prevention of cardiovascular remodeling in hypoxia-induced pulmonary hypertensive rats. J Cardiovasc Pharmacol 2014; 63: 567-576.
4) Li XW, Wang XM, Li S, Yang JR. [Effects of rutaecarpine on right ventricular remodeling in rats with monocrotaline-induced pulmonary hypertension]. Zhongguo Ying Yong Sheng Li Xue Za Zhi 2014; 30: 405-410.
5) Sawada H, Mitani Y, Maruyama J, Jiang BH, Ikeyama Y, Dida FA, Yamamoto H, Imanaka-Yoshida K, Shimpo H, Mizoguchi A, Maruyama K, Komada Y. A nuclear factor-kappaB inhibitor pyrrolidine dithiocarbamate ameliorates pulmonary hypertension in rats. Chest 2007; 132: 1265-1274.
6) Huang J, Kaminski PM, Edwards JG, Yeh A, Wolin MS, Frishman WH, Gewitz MH, Mathew R. Pyrrolidine dithiocarbamate restores endothelial cell membrane integrity and attenuates monocrotaline-induced pulmonary artery hypertension. Am J Physiol Lung Cell Mol Physiol 2008; 294: L1250-L1259.
7) Thenappan T, Shah SJ, Rich S, Gomberg-Maitland M. A USA-based registry for pulmonary arterial hypertension: 1982-2006. Eur Respir J 2007; 30: 1103-1110.
8) Lakhani NJ, Sarkar MA, Venitz J, Figg WD. 2-Methoxyestradiol, a promising anticancer agent. Pharmacotherapy 2003; 23: 165-172.
9) Parker TA, Kinsella JP, Galan HL, Le Cras TD, Richter GT, Markham NE, Abman SH. Prolonged infusions of estradiol dilate the ovine fetal pulmonary circulation. Pediatr Res 2000; 47: 89-96.
10) Parker TA, Ivy DD, Galan HL, Grover TR, Kinsella JP, Abman SH. Estradiol improves pulmonary hemodynamics and vascular remodeling in perinatal pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 2000; 278: L374-L381.
11) Mair KM, Johansen AK, Wright AF, Wallace E, MacLean MR. Pulmonary arterial hypertension: basis of sex differences in incidence and treatment response. Br J Pharmacol 2014; 171: 567-579.
12) Xiao J, Xu T, Li J, Lv D, Chen P, Zhou Q, Xu J. Exercise-induced physiological hypertrophy initiates activation of cardiac progenitor cells. Int J Clin Exp Pathol 2014; 7: 663-669.
13) Li XW, Hao W, Liu Y, Yang JR. [Effect of sequoyitol on expression of NOX4 and eNOS in aortas of type 2 diabetic rats]. Yao Xue Xue Bao 2014; 49: 329-336.
14) Ventetuolo CE, Ouyang P, Bluemke DA, Tandri H, Barr RG, Bagiella E, Cappola AR, Bristow MR, Johnson C, Kronmal RA, Kizer JR, Lima JA, Kawut SM. Sex hormones are associated with right ventricular structure and function: the MESA-right ventricle study. Am J Respir Crit Care Med 2011; 183: 659-667.
15) Jankov RP, Kantores C, Pan J, Belik J. Contribution of xanthine oxidase-derived superoxide to chronic

hypoxic pulmonary hypertension in neonatal rats. Am J Physiol Lung Cell Mol Physiol 2008; 294: L233-L245.

16) Zhang Y, Dai L, Wu S, Chen P, Zhao S. Atorvastatin attenuates involvement of RhoA/Rho-kinase pathway and NF-kappaB activation in hypoxic pulmonary hypertensive rats. Chin Med J (Engl) 2014; 127: 869-872.

17) Price LC, Caramori G, Perros F, Meng C, Gambaryan N, Dorfmuller P, Montani D, Casolari P, Zhu J, Dimopoulos K, Shao D, Girerd B, Mumby S, Proudfoot A, Griffiths M, Papi A, Humbert M, Adcock IM, Wort SJ. Nuclear factor kappa-B is activated in the pulmonary vessels of patients with end-stage idiopathic pulmonary arterial hypertension. PLoS One 2013; 8: e75415.

18) Ha SK, Moon E, Kim SY. Chrysin suppresses LPS-stimulated proinflammatory responses by blocking NF-kappaB and JNK activations in microglia cells. Neurosci Lett 2010; 485: 143-147.

19) Shao JJ, Zhang AP, Qin W, Zheng L, Zhu YF, Chen X. AMP-activated protein kinase (AMPK) activation is involved in chrysin-induced growth inhibition and apoptosis in cultured A549 lung cancer cells. Biochem Biophys Res Commun 2012; 423: 448-453.