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Original Article

Fluoxetine inhibited extracellular matrix of pulmonary artery and inflammation of lungs in monocrotaline-treated rats

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Aim: To investigate the effects of the selective serotonin reuptake inhibitor (SSRI) fluoxetine on extracellular matrix (ECM) remodeling of the pulmonary artery and inflammation of the lungs in pulmonary arterial hypertension (PAH) induced by monocrotaline in rats.
Methods: MCT-induced chronic PAH was established in Wistar rats. After treatment with fluoxetine for 3 weeks, pulmonary hemodynamic measurement and morphological investigation of lung tissues were undertaken. The main components of the ECM, elastin and collagen, were detected using Van Gieson stain and Orcein stain, respectively, or using Victoria-ponceau's double stain. The ECM proteolytic enzymes matrix metalloproteinase (MMP)-2 and MMP-9, and the tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2, were detected by Western blot. Inflammation of lung tissue was assayed using lung morphology and inflammatory cytokine expression.
Results: Fluoxetine (2 and 10 mg/kg) significantly inhibited MCT-induced PAH, attenuated pulmonary arterial muscularization and ECM remodeling, and decreased MMP/TIMP expression. Fluoxetine also suppressed inflammatory responses in lung tissue and inhibited the expression of the inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), monocyte chemotactic protein (MCP-1) and intercellular adhesion molecule-1 (ICAM-1).
Conclusion: Fluoxetine inhibited MCT-induced ECM remodeling of the pulmonary artery and inflammation of lung tissue. These effects were related to its inhibition on MMPs/TIMPs and cytokine productions.

Keywords: extracellular matrix; inflammation; pulmonary arterial hypertension; selective serotonin reuptake inhibitor

Introduction

Pulmonary arterial hypertension (PAH) is characterized by a sustained and progressive increase in pulmonary arterial pressure (PAP) with pathological changes involving vasoconstriction, vascular remodeling and inflammation, which may lead to right-heart failure and ultimately death. Whereas the pathogenesis of PAH is complicated, it is believed that the main processes lead to progressive pulmonary arterial remodeling, including hypertrophy and hyperplasia of pulmonary arterial smooth muscle cells (PASMCs), muscularization of normally nonmuscular peripheral arteries, and deposition of the extracellular matrix (ECM)[1–3].

The ECM, as a biologically active and dynamic composition of vasculature, plays important roles in maintaining the histological structure of the vessel wall and regulating PASMCs

contractility and proliferation[4]. Collagen and elastin are the major structural components of the ECM, which interlace in a complex network and are well adapted to accomplish mechanical tasks[5, 6]. The ECM is regulated by specific and unique proteolytic enzymes, the matrix metalloproteinases (MMPs). Among the MMPs, MMP-2, and MMP-9 degrade collagen more efficiently than the others, and they are involved in the vascular smooth muscle cell activation and neointimal formation that characterize arterial tissue remodeling after injury[7, 8]. MMPs are modulated by the tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2. The imbalance of MMPs/TIMPs induces matrix abnormality and remodeling, which has been found in idiopathic PAH patients[9]. Thus, the integrity and balance of ECM is essential for normal lung function and response to injury[10], and identifying MMP/TIMP changes will help us better understand the pathobiology of PAH.

Serotonin, as a type of vasoconstrictor and mitogen for smooth muscle cells, is an important endogenous vasoactive substance involved in PAH. The internalization of

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serotonin into PASMCs by high-affinity serotonin transporters (SERT) promoted smooth muscle cell hyperplasia and hypertrophy[11, 12]. It was reported that the plasma concentration in serotonin was significantly increased in PAH patients[13]. We have previously reported that serotonin induced PASMCs mitogenesis in vitro, and serotonin selective reuptake inhibitor (SSRI) fluoxetine inhibited serotonin-induced PASMCs proliferation via blocking SERT[14]. We have also found that SSRI fluoxetine and sertraline protected against pulmonary vascular remodeling by inhibiting pulmonary vascular muscularization in monocrotaline (MCT)-induced pulmonary hypertensive rats[15, 16]. However, whether SSRI has a protective effect against ECM remodeling in the pulmonary artery remains unknown.

Inflammatory mechanisms play an important role in the development of PAH. It has been demonstrated that lymphocytes and macrophages were present in the vicinity of remodeled pulmonary vessels and that cytokines such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) were increased in PAH patients[17-20]. We have also reported previously that chronic lung inflammation existed in MCT-induced PAH rats[21]. However, several studies have shown that fluoxetine had an anti-inflammatory effect by decreasing cytokine production from peripheral blood in patients suffering from major depressive disorder[22] and by attenuating carrageenan-induced inflammation response in rats[23]. Yet, whether fluoxetine inhibits an inflammatory response in PAH is not clear.

Therefore, the present study is to investigate the effects of fluoxetine on the extracellular matrix of the pulmonary artery and on the inflammation of lung tissue in MCT-induced PAH rats.

Materials and methods
Animal models
Male Wistar rats (167±18 g) from Animal Resource Center, China Medical University (Certification No: Liaoning 034) were divided into four groups, *ie*, control, MCT, MCT plus fluoxetine 2 mg·kg^{-1}·d^{-1} (MCT+F2) and MCT plus fluoxetine 10 mg·kg^{-1}·d^{-1} (MCT+F10). Rats in the MCT, MCT+F2, and MCT+F10 groups were treated with a single intraperitoneal injection of 60 mg/kg MCT (Sigma, St Louis, USA), and rats in the control group were treated with an equivalent amount of vehicle. Rats in the MCT+F2 and MCT+F10 groups also received fluoxetine (Eli Lilly, Indianapolis, USA) at doses of 2 mg·kg^{-1}·d^{-1} and 10 mg·kg^{-1}·d^{-1} by gavage, respectively. Meanwhile, rats in the control and MCT groups received vehicle only. Rats were fed with solid food and water *ad lib* in an alternating 12 h light/dark cycle under controlled temperature (18–22 °C) and humidity (50%–65%) for 3 weeks.

Hemodynamic measurement
After 3 weeks, rats were anaesthetized with 3% sodium pentobarbital (40 mg/kg). A polyethylene catheter (PE-50) was inserted into the right carotid artery to measure systemic arterial pressure (SAP). A PV-1 catheter was inserted into the pulmonary artery through the right jugular vein via the right atrium and ventricle for measurement of pulmonary arterial pressure (PAP). Hemodynamic variables were measured with a pressure transducer and recorded on a polygraph system (RM6000, Kohden, Tokyo, Japan).

Lung morphology
The lower lobe of right lungs and pulmonary arteries were fixed with formalin solution. After paraffin embedding, 5 μm sections were stained with hematoxylin and eosin for investigation of inflammation and the thickness of the pulmonary arterial wall by light microscopy. The external and internal diameters of 7–10 intra-acinar pulmonary arteries per rat were measured in 5 rats of each group. The ratio of the medial thickness of the pulmonary artery was calculated by the equation shown as follows[24]:

$$\text{The thickness of pulmonary arterial wall} = \frac{\text{External diameter–internal diameter}}{\text{External diameter}} \times 100\%$$

Collagen and elastin staining
Serial paraffin sections were stained with Van Gieson stain, Orcein stain, or Victoria-ponceau's double stain to localize collagen and elastin in lungs and pulmonary arteries.

Western blot
The left lungs were immediately removed to liquid nitrogen for measurement of protein expression. Lung samples were homogenized in lysis buffer. Total protein from each sample was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membranes were blocked by TBS-0.05% Tween-20 (TBS-T) with 5% nonfat dry milk for 60 min and were then incubated with mouse anti-rat MMP-2 (1:600, Santa Cruz, California, USA) and TIMP-2 (1:400, Santa Cruz, California, USA); goat anti-rat MMP-9 (1:600, Santa Cruz, California, USA), TIMP-1 (1:400, Santa Cruz, California, USA) and TNF-α (1:1000, Santa Cruz, California, USA); rabbit anti-rat IL-1β (1:400, USCN, Missouri, USA), ICAM-1 (1:800, Santa Cruz, California, USA), MCP-1 (1:400, Boster, Wuhan, China) and β-actin (1:2000, Santa Cruz, California, USA) antibodies in TBS-T with 5% BSA overnight at 4 °C, respectively. After a corresponding secondary antibody treatment, the membranes were exposed to a mixture of enhanced chemiluminescence reagent (Applygen Technologies Inc., Beijing, China), and the resulting chemiluminescent reaction was detected by Fuji X-ray film. Then the film was scanned, and the intensity of immunoblot bands was quantified by densitometry using imaging software.

Statistical methods
All data are expressed as the mean±SD. Statistical comparisons were made by one-way analysis of variance, and statistical differences between two groups were established using the least significant difference test.

Results

Effect of fluoxetine on hemodynamics and the thickness of the pulmonary arterial wall

The mean PAP was elevated in the MCT group compared with the control group ($P<0.01$). Mean PAPs in the MCT+F2 and MCT+F10 groups were both decreased significantly ($P<0.05$, *vs* MCT). However, the SAPs in the four groups were not significantly different. The muscularization of lung tissue from the right lower lobe was investigated under light microscope. The thickness of pulmonary arterial walls in the MCT group was increased ($P<0.01$ *vs* control). Also, fluoxetine decreased the thickness ratio in the MCT+F2 and MCT+F10 groups compared with the MCT group in a dose-related manner ($P<0.01$, Table 1).

Evaluation of elastin and collagen

Figure 1A shows collagen staining of lung tissue, and demonstrates that collagen in the MCT group was significantly increased and was diffused all around the lungs and pulmonary arterioles; fluoxetine markedly decreased collagen deposition and ameliorated structural destruction of lungs in a dose-related manner. Figure 1B shows elastin staining of the main pulmonary arteries and demonstrates that the elastic fibers in the MCT group were significantly increased and disrupted; fluoxetine at doses of 10 mg/kg decreased elastic fiber hyperplasia and kept the integrity of the arterial structure. Similar results were also found in Figure 1C and 1D indicating that fluoxetine markedly decreased remodeling and destruction of elastin and collagen induced by MCT treatment.
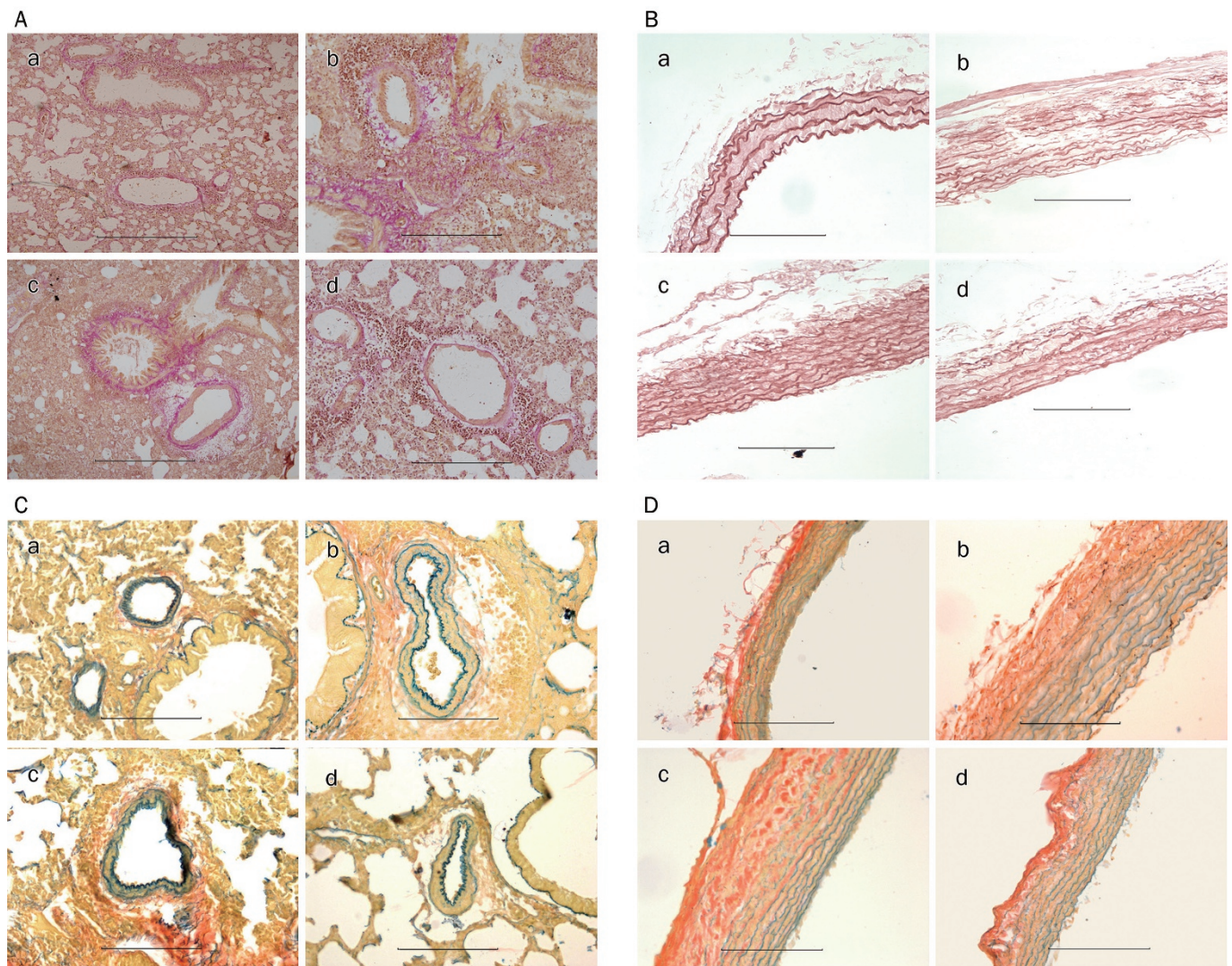

Figure 1. Comparison of elastin and collagen deposition in different groups. (A) collagen deposition (staining red) in lungs. a, b, c, and d represent control, MCT, MCT+F2, and MCT+F10 group, respectively (bars, 100 μm). (B) elastin in main pulmonary arteries. a, b, c, and d represent control, MCT, MCT+F2, and MCT+F10 group, respectively (bars, 100 μm). (C) Double staining in elastin and collagen in lungs. a, b, c and d represent elastin(staining bluish green) and collagen (staining red) in control, MCT, MCT+F2, and MCT+F10 group, respectively (bars, 100 μm). (D) Double staining in elastin and collagen in main pulmonary arteries. a, b, c, and d represent elastin (staining bluish green) and collagen (staining red) in control, MCT, MCT+F2, and MCT+F10 group (bars, 100 μm).

Table 1. Comparison of hemodynamic measurement and the thickness of pulmonary arteries in different groups. Data were expressed as mean±SD. [c]$P<0.01$ *vs* control. [e]$P<0.05$, [f]$P<0.01$ *vs* MCT group.

	Control (*n*=15)	MCT (*n*=10)	MCT+F2 (*n*=9)	MCT+F10 (*n*=9)
Body weight (g)	260±21	236±29	226±36	241±20
SAP (mmHg)	120.2±10.7	108.3±16.3	110.3±9.3	110.3±13.4
PAP (mmHg)	17.4±1.7	29.8±7.5[c]	26.9±5.7[e]	24.5±3.3[e]
Ratio of medial thickness of pulmonary artery (%)	36.1±10.3	53.5±8.8[c]	44.7±9.7[f]	38.5±10.1[f]

Evaluation of lung inflammation

As shown in Figure 2, marked perivascular and peribronchiolar inflammatory cell infiltration and angiogenesis of lung tissues were found in the MCT group. Fluoxetine predominantly attenuated MCT-induced inflammation and angiogenesis of lung tissues.
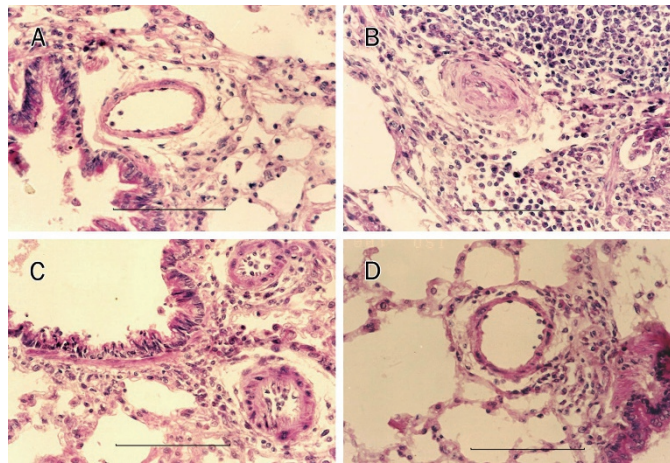
Figure 2. Comparison of lung inflammatory changes illustrated by representative photomicrographs. A, B, C, and D represent control, MCT, MCT+F2, and MCT+F10 group, respectively (bars, 100 μm).

Figure 3. Comparison of MMP-2, MMP-9, TIMP-1, and TIMP-2 protein expression in lungs from different groups 1, 2, 3, and 4 represent control, MCT, MCT+F2, and MCT+F10 group, respectively. Data expressed as mean±SD (*n*=4). [b]$P<0.05$, [c]$P<0.01$ compared with control. [e]$P<0.05$, [f]$P<0.01$ compared with MCT.

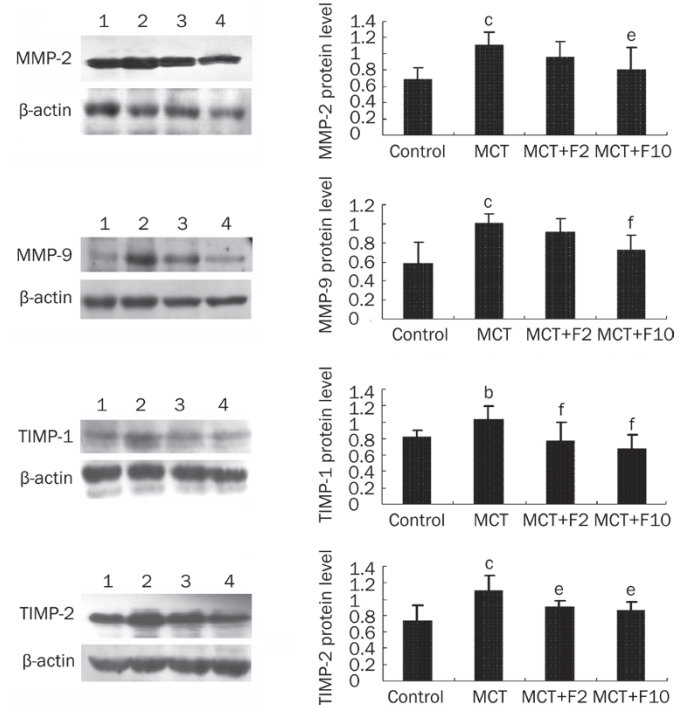### MMP-2, MMP-9, TIMP-1, and TIMP-2 protein expressions

As shown in Figure 3, the levels of MMP-2, MMP-9, TIMP-1, and TIMP-2 in the MCT group were significantly increased compared with control. Fluoxetine inhibited MCT-induced increase of MMPs and TIMPs in a dose-dependent manner.

### Inflammatory cytokine IL-1β, TNF-α, MCP-1, and ICAM-1 expressions

Compared with the control group, the levels of IL-1β, TNF-α, MCP-1, and ICAM-1 in the MCT group were significantly increased from 0.74±0.19, 0.58±0.24, 0.64±0.11, and 0.91±0.11 to 1.16±0.22, 1.00±0.22, 0.92±0.12, and 1.04±0.08, respectively. Fluoxetine inhibited MCT-induced increase of these cytokines in a dose dependent manner (Figure 4).

Discussion

The present study shows that MCT-induced PAH is accompanied by an ECM remodeling of pulmonary arteries and an inflammatory response of lung tissue, in which there are marked fragmentation and reconstruction of elastin and collagen, increased expression of MMP-2, 9 and TIMP-1, 2, and increased expression of inflammatory cytokines. ECM remodeling, as an important change in pulmonary arterial reconstruction, results from a complex interplay between synthesis and proteolysis of ECM constituents. Early clinical studies have shown that fragmentation of the internal elastic lamina and an increase of active MMPs exist in the pulmonary arteries of PAH patients[9]. In animal PAH models, the progression of hypoxia-induced PAH is associated with a time-dependent increase in MMP activity that is mainly related to an increase of MMP-2[8]. Higher expressions of MMP-2, 9, TIMP-1 mRNA, and enzymatic activity of MMP-2, 9 in lungs were also found
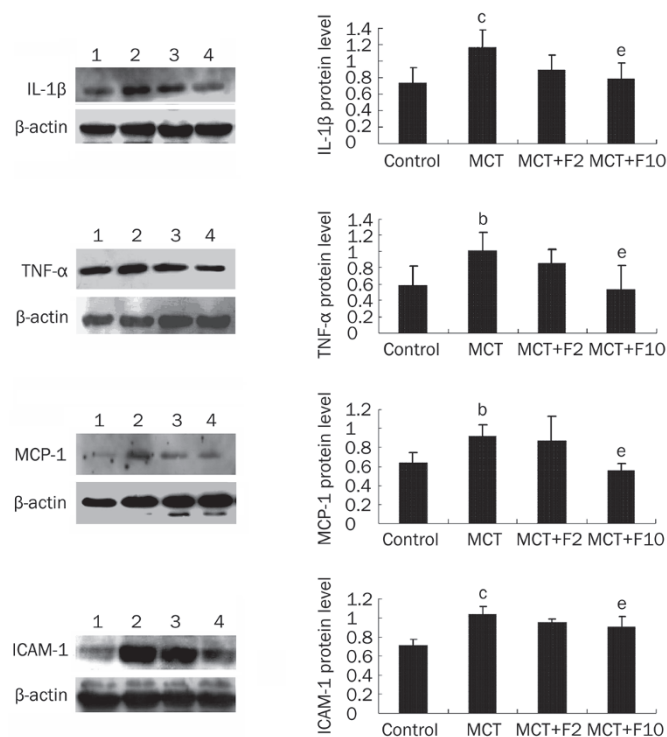
Figure 4. IL-1β, TNF-α, MCP-1, and ICAM-1 protein expression in lung tissues from different groups by Western blot. 1, 2, 3, and 4 represent control, MCT, MCT+F2, and MCT+F10 group, respectively. Data expressed as mean±SD (*n*=4). [b]$P<0.05$, [c]$P<0.01$ compared with control. [e]$P<0.05$, [f]$P<0.01$ compared with MCT.

in MCT-induced PAH rats[25]. It is thought that injection of MCT causes endothelial cell injury in pulmonary arteries and inflammatory response in lungs[21] and induces MMPs to be secreted from injured endothelial cells and inflammatory cells (such as mast cells, monocytes, macrophages)[26, 27]. The activation of MMPs initiates ECM degradation, and TIMPs expression is augmented for keeping a dynamic balance between ECM construction and destruction. The above mentioned evidence indicates that the present findings, in which MCT induced increases of MMP-2, 9/TIMP-1, 2 in lungs, are in accordance with the results from previous studies.

In the present study, fluoxetine markedly inhibits MCT-induced pulmonary arterial hypertension and attenuated pulmonary artery muscularization, as we have reported previously[15]. Furthermore, we also found that fluoxetine reduced the elastin and collagen deposition and destruction in pulmonary arteries induced by MCT, in which MMP-2, 9/TIMP-1, 2 expressions are obviously suppressed. It is known that serotonin is involved in the pathophysiology of lung fibrosis via increasing proliferation and collagen synthesis by fibroblasts[28] and that it has reciprocal effects on collagens and collagenase (a type of MMP)[29]. Shum JK *et al* reported that serotonin induces MMP production via phospholipase C, protein kinase C, and extracellular signal-regulated kinase (ERK) 1/2 pathway in smooth muscle cells[30]. Our previous study showed that the serotonin-induced mitogenesis of PASMCs

is mediated by SERT, in which the signal transduction for serotonin is dependent on the ERK1/2 pathway[14]. Benekareddy *et al* also reported that fluoxetine regulates MMP-2/MMP-9 and TIMP1-4 in the adult rat hippocampus[31]. Taken this information and the present results together, we believe that fluoxetine-induced regulation of MMP-2, 9/TIMP-1, 2 is closely related to the inhibition of ECM remodeling, in which the serotonin intracellular signal pathway might be involved. In the fluoxetine group, we found that both MMP and TIMP expressions were inhibited. It is known that regulation of MMP expression also affects TIMP expression, and therefore a delicate balance exists between MMP activation and inhibition by TIMPs[32]. It is thought that a reduction of TIMP expression is related to the inhibition of MMPs expression and ECM remodeling by fluoxetine.

We have also shown how fluoxetine remarkably inhibits MCT-induced pulmonary inflammation and how this inhibition is accompanied by the decreased expression of inflammatory cytokines. Some studies report that serotonin is closely linked to inflammatory responses, including the induction of mast cell adhesion and migration, activation of alveolar macrophages, and development and maintenance of arterial remodeling through the release of cytokines[33–35]. It is found that fluoxetine has anti-inflammatory properties and that there is a positive correlation between SERT and cytokine mRNA expression in patients with depression, who are affected by chronic treatment with fluoxetine[22]. Also, the present study found that fluoxetine suppressed inflammatory responses in MCT-induced PAH rats, in which inhibition of inflammatory cytokines is involved. Thus, it is thought that the serotonergic system is likely to be involved in the modulation of peripheral inflammation, and SSRIs may play an important role in working against the inflammatory response in PAH. It is known that MMPs act broadly in physiological and pathological inflammatory processes via regulating inflammatory cytokines activity[7]. MMPs are regulated by many cytokines, notably TNF-α and IL-1β, that induce MMP activity markedly and increase their mRNA levels via action through the promoter region of MMP genes[36]. Kubera *et al* also reported that the anti-inflammatory effects of fluoxetine developed through suppression of the interferon-gamma/interlenkin-10 production ratio in the central nervous system[37]. Therefore, there is a complex interaction between MMPs and inflammatory response which might be involved in the mechanism of fluoxetine protection against PAH.

In conclusion, fluoxetine inhibited MCT-induced ECM remodeling of pulmonary artery and inflammation of lungs, effects which were related to its inhibition on MMPs/TIMPs and cytokine productions.

Author contribution

Xue-qin LI and Huai-liang WANG designed the research and

wrote the paper. Xue-qin LI, Han-ming WANG, Chun-guang YANG, and Dan-dan HAN performed the research. Xin-hua ZHANG offered technical assistance.

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