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Salvia Miltiorrhiza Bge.f.alba Ameliorates the Progression of Monocrotaline-Induced Pulmonary Hypertension by Protecting Endothelial Injury in Rats

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Pulmonary hypertension (PH) is a life-threatening disease that is characterized by elevated pulmonary blood pressure, abnormally thickened pulmonary arteries, and right ventricular hypertrophy. Monocrotaline (MCT) has been used to generate an experimental model of PH in rats, with PH initiated from injuries of lung vascular endothelium. Salvia Miltiorrhiza Bge.f.alba is a widely used traditional herb in China, known to exert protective effects on vascular endothelial cell injury in animal experiments. However, the role of Salvia Miltiorrhiza Bge.f.alba in PH remains unclear. Thus, we investigated the effects of the aqueous extract of Salvia Miltiorrhiza Bge.f.alba (AESM) on MCT-induced PH and explored the pertinent mechanism. PH was induced in rats by a single subcutaneous injection of MCT (60 mg/kg body weight). Low or high dose (4.6 g/kg or 14 g/kg body weight) of AESM was then administered orally for 21 days to PH rats. Hemodynamic study showed that AESM reduced mean pulmonary artery pressure and improved right ventricle function. Lung pathological analysis revealed that AESM reduced wall thickness and lumen stenosis of pulmonary vessels. Also AESM ameliorated right ventricular hypertrophy. Measurement of biochemical parameters indicated that AESM decreased endothelin-1 and thromboxane A_2 in plasma and increased nitrogen monoxide and prostacyclin in the plasma and reduced the increase of transforming growth factor β_1 in lung tissue. Our results suggest that AESM may ameliorate the progression of MCT-induced PH in rats, at least in part by its protective effect on endothelial injury. Therefore, Salvia Miltiorrhiza Bge.f.alba could be useful in the treatment of PH.

Keywords: aqueous extract; endothelium; pulmonary hypertension; remodeling; Salvia Miltiorrhiza Bge.f.alba
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Introduction

Pulmonary hypertension (PH), a condition of elevated pulmonary arterial pressure, can lead to right ventricular (RV) hypertrophy and, if untreated, right heart failure. PH has a complex pathogenesis, poor prognosis, and high mortality. PH may have functional or pathological changes including abnormal vasomotor control, endothelium dysfunction, endothelial cell proliferation, thrombotic obliteration of the vascular lumen, and chronic remodeling of the vascular wall (Yen et al. 2010). Pulmonary vascular remodeling is a characteristic pathological change in PH occurring mainly in small vessels (< 500 μm diameter). Cell growth and proliferation of vascular cells, such as smooth muscle cells and endothelial cells promote the aberrant vascular remodeling. Specially, endothelial cells contribute to vascular wall thickening by proliferation and regulation of the synthesis of a variety of vasoactive factors (Yamanaka et al. 2010). Thus, targeting endothelial cell function and reversing pulmonary vascular remodeling may provide a new direction for treating PH.

Monocrotaline (MCT), a pyrrolizidine alkaloid, has no

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intrinsic activity. MCT is transformed to bioactive monocrotaline pyrrole by monooxygenase in the liver, which selectively injures the vascular endothelium of lung vessels. Progressive pulmonary vasculitis leads to increased vascular resistance and gradual arterial pressure increases beginning approximately one week after a single dose of MCT (Handoko et al. 2008). MCT-induced PH in rats mimics several aspects of primary or secondary human PH, including endothelial cell dysfunction, vascular remodeling, and upregulation of inflammatory cytokines, and is therefore used as an appropriate in vivo PH model (Alencar et al. 2014).

Salvia Miltiorrhiza Bge.f.alba, a white flower variant of Salvia Miltiorrhiza Bge (Danshen), is different from genuine Danshen that has a purple corolla, and has been recognized as a new variety. Salvia Miltiorrhiza Bge.f.alba is an endemic species mainly distributed on Mount Taishan and its surrounding area, belonging to a rare and endangered medical plant (Hang et al. 2008). Our previous studies showed that Salvia Miltiorrhiza Bge.f.alba can protect vascular endothelial cells against hydrogen peroxide-induced injury (Jiao et al. 2007; Yu et al. 2010), and has a protective effect on diseases related to endothelial dysfunction (Li et al. 2013) or vascular endothelial injury, such as focal cerebral infarction (Wang et al. 2005; Cai et al. 2006) and cerebral ischemia (Cai et al. 2007). In addition, Salvia Miltiorrhiza Bge.f.alba has been shown to possess unique efficacy in treating thromboangiitis obliterans (Zhang 1979). Based on these observations, we hypothesized that Salvia Miltiorrhiza Bge.f.alba may have therapeutic effects against PH through its protective role on vascular endothelial cells.

Therefore, the aim of this study was to observe the effects of Salvia Miltiorrhiza Bge.f.alba on PH in regard to pulmonary hemodynamics, RV hypertrophy, and pulmonary vascular remodeling, and to explore its mechanism of action. In Chinese traditional herbal medicine, hot water extraction of the Danshen root for human consumption is the most commonly used extraction method; therefore, we used aqueous extracts of Salvia Miltiorrhiza Bge.f.alba (AESM) in this study.

Materials and Methods

Reagents

MCT was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Nitrogen monoxide (NO) assay reagents were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Transforming growth factor β_1 (TGF-β_1) enzyme-linked immunosorbent assay (ELISA) kit was purchased from USCN Life Science & Technology Company (Wuhan, China). An iodine [^{125}I] endothelin (^{125}I-ET-1) radioimmunoassay kit, iodine [^{125}I] 6-Keto-prostaglandin $F_1\alpha$ (^{125}I-6-Keto-PGF$_1\alpha$) radioimmunoassay kit, and iodine [^{125}I] thromboxane B_2 (^{125}I-TXB$_2$) radioimmunoassay kit were obtained from the RIA of Technology Development Centre of the PLA General Hospital (Beijing, China).

Preparation of AESM

Salvia Miltiorrhiza Bge.f.alba root, identified by professor Zuo-Li Xia, was collected in 2012 near Mount Taishan (Shandong, China), and was washed and allowed to dry. The dried root was cut into small pieces and soaked for 2 h, then boiled for 1 h under a medium-heat fire after first being boiled under a high-heat fire. After being filtered, the residue was boiled again using the same method. All filtrate was collected, combined and then concentrated at 60°C to a final concentration of 0.3 g/mL.

Animals and experimental plant

Male Sprague-Dawley rats, weighing 200-220 g, were provided by the Experimental Animal Center of Shandong University of Traditional Chinese Medicine (Shandong, China). Rats were housed with free access to food and water under a natural 12 h day/ 12 h night cycle. Rats were acclimated for seven days before start of experimental procedures. All rats received humane care according to the Guide for the Care and Use of Laboratory Animals by the Chinese Academy of Sciences.

Rats were randomly divided into the following 4 groups of 10 rats each: control group (Control), in which rats received an equal volume of vehicle; MCT-treated group (MCT), in which rats received a single subcutaneous injection of MCT (dissolved in 1 N HCL buffered to pH 7.0 with 1 N NaOH) at a dose of 60 mg/kg body weight (Gary-Bobo et al. 2010); low-dose AESM group (MCT+LD), in which rats received AESM at a dose of 4.6 g/kg body weight daily in the drinking water from day 1 to day 21 after MCT injection; and high-dose AESM group (MCT+HD), in which rats received AESM at a dose of 14 g/kg body weight daily in the drinking water from day 1 to day 21 after MCT injection. Rats in Control and MCT groups were administered equal volume of distilled water from day 1 to day 21. During the treatment period, rats in AESM groups were provided with water following AESM administration. All rats were caged individually. All the procedures were approved by the Ethics Committee of Taishan Medical University (Approval No. 2013-004, Taian, China).

Rationale for AESM doses

The drug dose was equivalent to the amount of crude drug. The rationale for the doses of AESM was as follows: in China-Pharmacopeia, the high dose of Salvia Miltiorrhiza used on human is 15 g/60 kg body weight daily (that is 0.25 g/kg body weight daily). This dose multiplied by 6.17 (human and rat drug dose conversion coefficient) equals 1.5 g/kg body weight daily. In our preliminary experiments, effects of three dosages (1.5, 4.6, and 14 g/kg body weight daily) of AESM were investigated. We observed that only the dosages (4.6 and 14 g/kg body weight daily) of AESM were effective. Thus, the final doses used in this study were determined as 4.6 and 14 g/kg body weight daily. The length of drug administration was 21 days.

Hemodynamic studies

On day 22, rats were anesthetized with 1% pentobarbital sodium (40 mg/kg i.p.) and placed in a supine position. The MP150 system (BIOPAC, USA) was applied in our experiments, as previously described (Wang et al. 2011). Briefly, a polyethylene catheter was introduced into the right ventricle through the jugular vein to measure right ventricular systolic pressure (RVSP). Peak rates of RV pressure rise (+dp/dt max) and fall (−dp/dt max) were measured as well. Then the catheter was advanced to the pulmonary artery to

measure mean pulmonary artery pressure (mPAP). After hemodynamic measurements, blood was collected from the pulmonary artery and plasma was prepared according to the kits instructions. Lung and hearts were processed for histological evaluation or frozen in liquid nitrogen for further analysis.

Evaluation of RV hypertrophy and remodeling of pulmonary arteries

Elevated pulmonary arterial pressure leads to RV hypertrophy. To assess the hypertrophy degree of the RV, the ratio of RV weight to left ventricle and septum (LV+S) weights (Zhang et al. 2014) was calculated for the different animal groups.

For histopathological observations, specimens of the right lower lung were harvested, rinsed with normal saline, fixed in 4% paraformaldehyde for 24 h, and embedded in paraffin. Sections of 4 μm were stained with hematoxylin-eosin (H&E). BX51 microscopy (Olympus, Japan) and Image-Pro Plus (IPP) for Windows (version 4.0) were used for analysis. The pulmonary artery with a diameter ranging from 100 to 200 μm was selected. Wall thickness, the ratio of the internal diameter to external diameter (ID/ED), and the ratio of the lumen area to vascular cross-sectional area (LA/CSA) were measured.

Measurement of NO, endothelin-1, prostacyclin and thromboxane A_2 levels in plasma

The levels of NO in plasma were measured by a nitrate reductase test according to the manufacturer's instructions. Plasma levels of endothelin-1 (ET-1), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2) were detected using a radioimmunoassay technique, of which PGI_2 and TXA_2 levels were represented as their metabolites 6-Keto-$PGF_1\alpha$ and TXB_2 respectively.

Measurement of TGF-β_1 levels in lung tissue

Lung tissue was rinsed with 1 × PBS to remove excess blood, homogenized in 1 × PBS (1 g lung tissue in 6 mL 1 × PBS) and stored overnight at −20°C. After two freeze-thaw cycles were performed to lyse cell membranes, the homogenates were centrifuged for 5 min at 5,000 *g*. The supernatant was removed and assayed immediately by ELISA according to the manufacturer's instructions.

Statistical analysis

Results are expressed as mean ± SD. All data were statistically analyzed with SPSS software, version 11.5 (SPSS Inc., Chicago, IL, USA). Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls's post hoc test. A p value less than 0.05 was considered to be statistically significant.

Results

AESM improved hemodynamic indices of MCT-induced PH rats

As shown in Fig. 1A-D, mPAP, RVSP, RV +dp/dt max, and −dp/dt max of the rats in the MCT group increased significantly ($p < 0.01$), compared to the controls, and indicated that these rats developed severe PH. However, after low or high dose of AESM treatment, mPAP, RVSP, RV +dp/dt max, and −dp/dt max decreased significantly com-

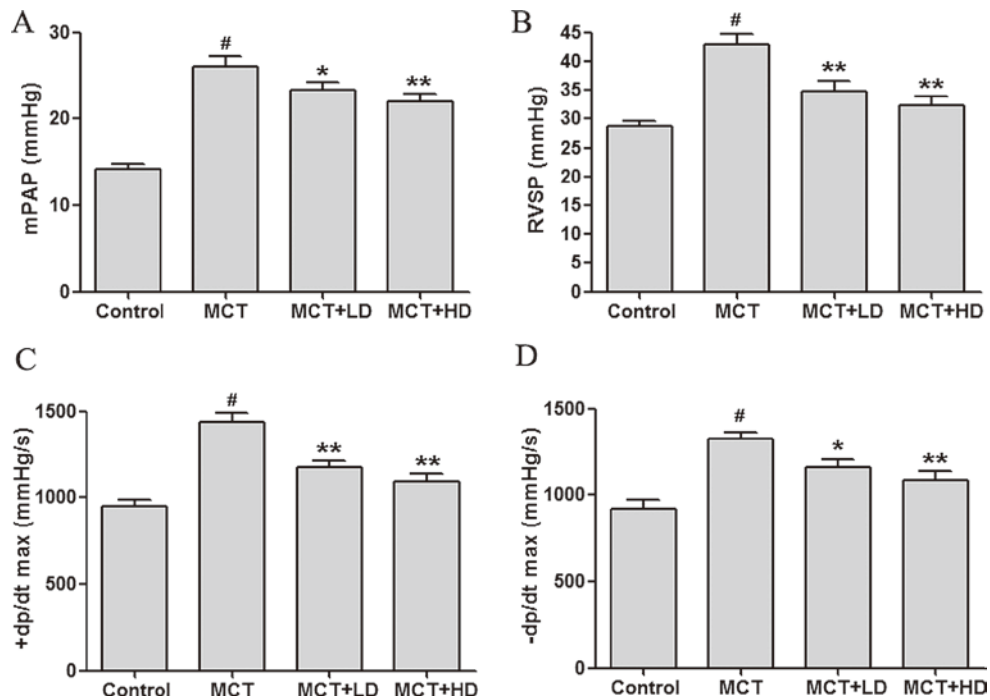

Fig. 1. AESM improved the hemodynamics of PH rats.
(A)-(D) represent mPAP, RVSP, +dp/dt max and −dp/dt max respectively. Control, control group; MCT, MCT-treated group; MCT+LD, MCT rats received low dose AESM (4.6 g/kg body weight) group; MCT+HD, MCT rats received high dose AESM (14 g/kg body weight) group; AESM, aqueous extract of Salvia Miltiorrhiza Bge.f.alba; mPAP, mean pulmonary artery pressure; RVSP, right ventricular systolic pressure; +dp/dt max, peak rates of right ventricle pressure rise; −dp/dt max, peak rates of right ventricle pressure fall. Data are presented as mean ± SD (n = 10 in each group). $^{\#}p < 0.01$ *vs.* control group; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* MCT group.

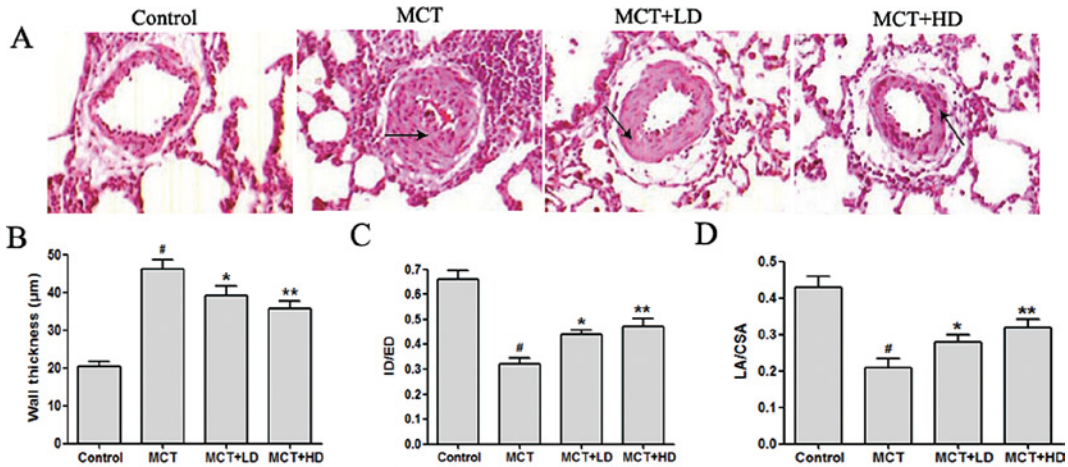

Fig. 2. AESM ameliorated pulmonary artery remodeling.
Representative photomicrographs of lung arteries are shown in (A). Rats from the MCT group showed markedly thickened arterial walls with hypertrophic media indicated by the arrow, while sections of the lung from control rats were essentially normal. AESM treated rats at low or high doses demonstrated reduction of wall thickness of pulmonary arteries, as indicated by the arrow, compared with MCT treatment alone. Quantitative analysis showed a thickened pulmonary artery wall and decreased artery lumen after MCT injection, but these changes were significantly inhibited in the AESM treatment groups. Sections were stained with H&E (200×). (B) Wall thickness of a small pulmonary artery. (C) and (D) changes in the artery lumen. Control, control group; MCT, MCT-treated group; MCT+LD, MCT rats received low dose AESM (4.6 g/kg body weight) group; MCT+HD, MCT rats received high dose AESM (14 g/kg body weight) group; AESM, aqueous extract of Salvia Miltiorrhiza Bge.f.alba; RV, right ventricle; ID/ED, the ratio of internal diameter to external diameter; LA/CSA, the ratio of lumen area to vascular cross-sectional area. Data are presented as mean ± SD (n = 10 in each group). $^{\#}p < 0.01$ *vs.* control group; $*p < 0.05$, $**p < 0.01$ *vs.* MCT group.

pared with the MCT group ($p < 0.05$), suggesting that AESM attenuated the effects of MCT.

AESM ameliorated pulmonary artery remodeling and RV hypertrophy of MCT-induced PH rats

The pulmonary artery with a diameter ranging from 100 to 200 μm in H&E stained sections of lung tissue from each group was selected to evaluate the extent of artery remodeling. Representative photomicrographs are shown in Fig. 2A. Image analysis showed that compared to the control group, the wall thickness (Fig. 2B) in the MCT group was increased significantly, and ID/ED (Fig. 2C) and LA/VCSA (Fig. 2D) were clearly diminished, which suggested that the lumen of the pulmonary artery narrowed and remodeling had occurred. Whereas, after low or high dose of AESM treatment, wall thickness was decreased, and ID/ED and LA/CSA were markedly increased compared with the MCT group. These results suggested that AESM partly reversed remodeling of the pulmonary artery.

Regarding hypertrophy degree of RV, results in Fig. 3 showed that the ratio of RV weight to LV+S weights in the MCT group were elevated significantly compared with the control group, indicating that RV hypertrophy developed as a consequence of increased pulmonary pressure. After low or high dose of AESM treatment, the ratio of RV weight to LV+S weights fell significantly. These data suggested that AESM can reverse MCT-induced RV hypertrophy to a certain extent.

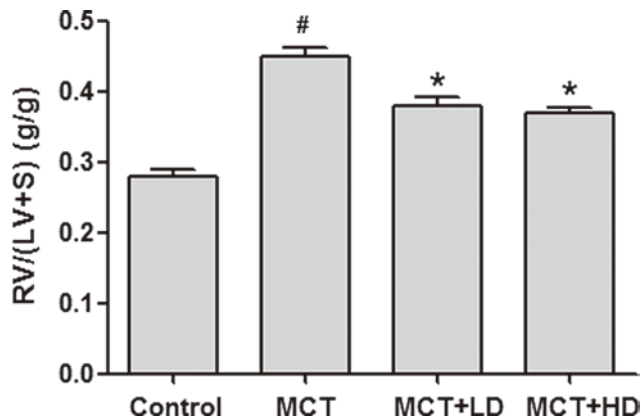

Fig. 3. Effect of AESM on RV hypertrophy.
The RV hypertrophy degree was reflected by the ratio of RV weight to LV+S weights. Control, control group; MCT, MCT-treated group; MCT+LD, MCT rats received low dose AESM (4.6 g/kg body weight) group; MCT+HD, MCT rats received high dose AESM (14 g/kg body weight) group; AESM, aqueous extract of Salvia Miltiorrhiza Bge.f.alba. Data are presented as mean ± SD (n = 10 in each group). $^{\#}p < 0.01$ *vs.* control group; $*p < 0.05$ *vs.* MCT group.

AESM modulated vasoactive factors levels in plasma of MCT-induced PH rats

The endothelium comprises a wide range of vasoactive substances, including vasodilators PGI_2 and NO, and vasoconstrictors ET-1 and TXA_2. Endothelial injury may result in the imbalance of these vasoactive factors (Budhiraja et

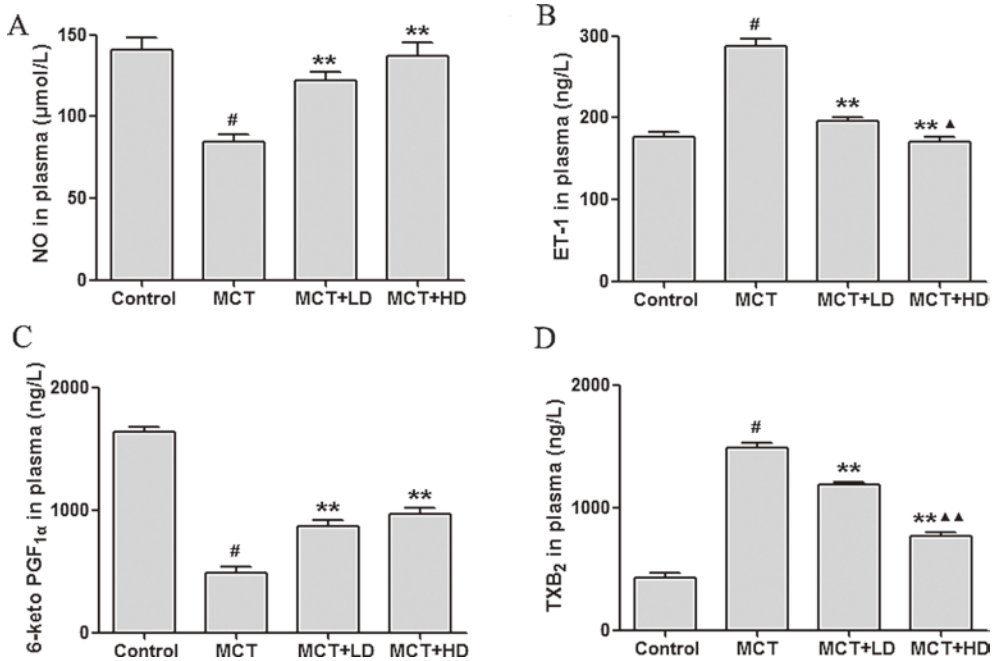

Fig. 4. AESM modulated vasoactive factors in plasma.
Levels of NO (A), ET-1 (B), PGI_2 (C) and TXA_2 (D) were determined, of which PGI_2 and TXA_2 are presented with their metabolites 6-Keto-$PGF_1\alpha$ and TXB_2 respectively. Control, control group; MCT, MCT-treated group; MCT+LD, MCT rats received low dose AESM (4.6 g/kg body weight) group; and MCT+HD, MCT rats received high dose AESM (14 g/kg body weight) group; AESM, aqueous extract of Salvia Miltiorrhiza Bge.f.alba. Data are presented as mean ± SD (n = 10 in each group). $^{\#}p < 0.01$ *vs.* control group; $^{**}p < 0.01$ *vs.* MCT group; $^{\blacktriangle}p < 0.05$ *vs.* (MCT+LD) group; $^{\blacktriangle\blacktriangle}p < 0.01$ *vs.* (MCT+LD) group.

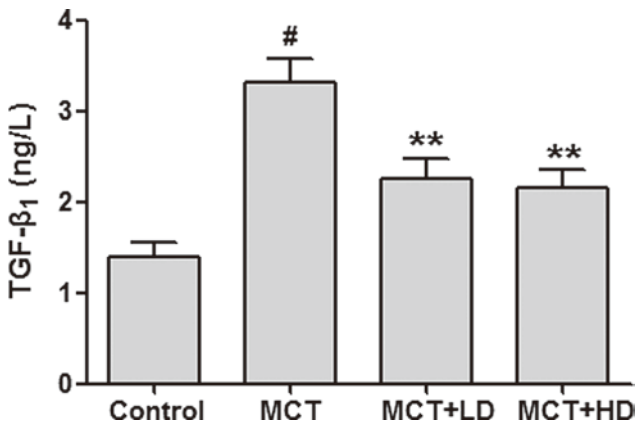

Fig. 5. Effect of AESM on TGF-β_1 levels in lung tissue.
Control, control group; MCT, MCT-treated group; MCT+LD, MCT rats received low dose AESM (4.6 g/kg body weight) group; MCT+HD, MCT rats received high dose AESM (14 g/kg body weight) group; AESM, aqueous extract of Salvia Miltiorrhiza Bge.f.alba; TGF-β_1, transforming growth factor β_1. Data are presented as mean ± SD (n = 10 in each group). $^{\#}p < 0.01$ *vs.* control group; $^{**}p < 0.01$ *vs.* MCT group.

al. 2004). As shown in Fig. 4, low or high dose of AESM treatment reversed the decreases of NO and 6-Keto-$PGF_1\alpha$ levels, and conversely reduced the increases of ET-1 and TXB_2 levels in the plasma that were observed in MCT-induced PH rats.

AESM reduced TGF-β_1 expression in lung tissue

TGF-β_1 plays an important role in vascular remodeling. We determined TGF-β_1 level in lung tissue of different groups using the ELISA method, and found TGF-β_1 increased significantly in the MCT group compared with the control group, while low or high dose of AESM treatment reversed TGF-β_1 levels to those of the control group (Fig. 5).

Discussion

In our study, we found that Salvia Miltiorrhiza Bge. f.alba can decrease pulmonary artery pressure, partly reverse pulmonary vascular remodeling and RV hypertrophy, and improve right heart function. Possible mechanisms may be its protective effect on endothelial injury, through which it balances vasoactive factors and reverses pulmonary vascular remodeling. Additionally, ET-1 and TXA_2 levels of rats treated with the high dose of AESM were reduced significantly compared to those who received the low dose of AESM. There were no significant changes in other indices between the low or high dose of AESM treatment, and thus an obvious dose-response relationship was not observed. Additionally, we replicated the PH model by subcutaneous injection of MCT, in which RVSP and mPAP levels were significantly higher and accompanied by a thicker pulmonary artery wall and narrower lumen after 21 days, which confirmed the success of this experimental model (Lookin et al. 2015).

An improved right heart catheterization method was used to determine the hemodynamic index in our study. The results suggest that AESM can inhibit the increases of RVSP and mPAP. +dp/dt max is commonly used to evaluate myocardial contractility and is influenced by the pre-load and after-load; −dp/dt max is the diastolic parameter that evaluates ventricular relaxation performance (Xu et al. 2002). These two indices of RV were increased significantly in the MCT group, which showed that myocardial systolic and diastolic functions were significantly enhanced, which might be a compensatory increase in response to elevated pulmonary artery pressure. AESM treatment showed a marked decrease of RV +dp/dt max and RV −dp/dt max, suggesting that this drug improved RV function and may relate to lower pulmonary artery pressure or pre-load and after-load. Therefore, RV hypertrophy was also ameliorated in the AESM treatment groups.

Endothelial injury and subsequent endothelial dysfunction, which is manifested by an increase in the production of vasoconstrictors such as ET-1 and TXA_2 and a reduction in vasodilators such as NO and PGI_2, play an important role in the progression of PH. This imbalance of vasoactive factors leads to smooth muscle cell proliferation and hyperplasia, which eventually causes vascular remodeling and narrowing of the pulmonary artery lumens (Haworth 2006; Crosswhite and Sun 2010). ET-1 is one of the most potent endogenous vasoconstrictors, and is also a vascular smooth muscle mitogen that participates in the regulation of pulmonary vascular tone and pulmonary vascular remodeling (Tokgöz et al. 2010). In PH patients, pulmonary vascular resistance is closely related to increased expression of ET-1 (Droste et al. 2009). As an important vascular relaxing factor, NO adjusts pulmonary arterial pressure through the control of vascular smooth muscle tension. In the lung, NO inhibits vasoconstriction, smooth muscle proliferation, and formation of ET-1 (Kelly et al. 2004). Compared with controls, lower levels of NO have been reported in PH patients (Murdaca et al. 2014). TXA_2, produced by endothelial cells and platelets, has potent pro-aggregatory and vasoconstrictor effects and is also a smooth muscle mitogen, whose metabolites are increased in PH. In addition, its actions are counterbalanced by the effects of PGI_2 released from vessel walls (Takahashi et al. 1989; Budhiraja et al. 2004). Because of their short half-life in the body, the contents of TXA_2 and PGI_2 are usually demonstrated by their two stable metabolites, TXB_2 and 6-Keto-$PGF_1\alpha$ respectively. PGI_2 can improve the balance between ET-1 release and clearance, and prevent vasoconstriction and remodeling caused by various factors in the lung (Sakuma et al. 2008). In PH patients, the metabolic product of TXA_2 is significantly increased, and the metabolite of PGI_2 is markedly reduced (Katugampola and Davenport 2001). Our study showed that levels of NO and PGI_2 were significantly reduced, and ET-1 and TXA_2 were increased in the MCT group compared with the control group, thereby demonstrating that disequilibrium of vasoactive factors existed in PH rats. However, after AESM treatment for 21 days, this imbalance was ameliorated**.** Accordingly, the remodeling of the pulmonary artery was abated in the AESM treatment groups compared to the MCT-induced PH group, as represented by a thinner wall and augmented lumen. According to this date, we suggest that the protective effect of AESM on endothelial injury plays an important role in ameliorating the progression of PH.

TGF-β_1 is a multifunctional cytokine that may participate in body repair, angiogenesis, inflammation and other pathological processes, such as modulating the synthesis and secretion of other cytokines, growth factors, and inflammatory mediators (Goumans et al. 2009). TGF-β_1 also plays an important role in vascular remodeling. Studies have shown that TGF-β_1 stimulates lung microvascular endothelial cells to produce ET-1, which promotes smooth muscle cell proliferation (Star et al. 2008). Furthermore, TGF-β_1 plays an initiation and facilitation role in the process of endothelium-smooth muscle transformation (Masszi et al. 2004). In our study, higher TGF-β_1 levels in the lung tissue of the MCT group may be due to elevated shear force, which can cause increased release of TGF-β_1 from the endothelium (Cucina et al. 1998). Mechanisms for AESM-mediated reduction in the expression of TGF-β_1 may include maintaining the stability of the pulmonary vascular endothelium and reducing mPAP, which further inhibits the formation of ET-1, endothelial-smooth muscle transformation, and final vascular remodeling.

Studies have shown that water-soluble components in roots of purple and white flowers of the Salvia Miltiorrhiza herb were similar, and the latter has higher contents in most components than the former (Hang et al. 2008), which are in accordance with our previous study (Cui et al. 2007). Components mainly include salvianolic acid B, danshensu, rosmarinic acid, protocatechuic aldehyde and protocatechuic acid. Additionly, methylation and glucuronidation are the two main metabolic pathways of the majority of the water-soluble components of Danshen (Zhao et al. 2012). We therefore inferred that AESM would not affect the metabolism of MCT in the liver. Furthermore, we assessed the possible effect of high dose of AESM (14 g/kg body weight) on healthy rats. Results indicated that there were no differences in hemodynamic parameters (mPAP, RVSP, +dp/dt max and −dp/dt max) and organs' indices between healthy rats administrated high dose of AESM or equal volume of distilled water (data not shown). Thus, we concluded that AESM had no obvious toxic effects or alteration of hemodynamics on healthy rats. In order to utilize this endemic species efficiently, future studies are needed to examine (1) which component of AESM has a primary effect on PH and (2) the efficacy of comparison between Salvia Miltiorrhiza Bge.f.alba versus genuine Danshen.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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