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ORIGINAL PAPER

Aspirin Attenuates Pulmonary Arterial Hypertension in Rats by Reducing Plasma 5-Hydroxytryptamine Levels

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Abstract Pulmonary arterial hypertension (PAH) is characterized by increasing pulmonary pressure, right ventricular failure, and death. The typical pathological changes include medial hypertrophy, intimal fibrosis and in situ thrombosis. Serotonin (5-HT) and other factors contribute to the development of pathologic lesions. Aspirin (ASA), a platelet aggregation inhibitor, inhibits 5-HT release from platelets. The aim of this study was to determine the efficacy of ASA in preventing or attenuating PAH. Sprague–Dawley rats injected with monocrotaline (MCT) developed severe PAH within 31 days. One hundred forty rats were randomized to receive either vehicle or ASA (0.5, 1, 2, or 4 mg/kg/day). The pre-ASA group was treated with ASA (1 mg/kg/day) for 30 days before the MCT injection. Thirty-one days after the injection (day 61 for the pre-ASA group), pulmonary arterial pressure (PAP), right ventricular hypertrophy and pulmonary arteriole thickness were measured. Plasma 5-HT was measured by high-performance liquid chromatography. Aspirin suppressed PAH and increased the survival rate compared with the control group (84 vs. 60%, $P < 0.05$). Aspirin treatment also reduced right ventricular hypertrophy and pulmonary arteriole proliferation in ASA-treated PAH model. In addition, plasma 5-HT was decreased in our ASA-treated PAH model. The degree of 5-HT reduction was associated with systolic PAP, right ventricular hypertrophy and wall thickness of pulmonary arterioles in rats. These results showed that ASA treatment effectively attenuated MCT-induced pulmonary hypertension, right ventricular hypertrophy, and occlusion of the pulmonary arteries. The effects of ASA was associated with a reduction of 5-HT.

Keywords Pulmonary arterial hypertension · Aspirin · Monocrotaline · 5-HT

Abbreviations

ASA	Aspirin
IPAH	Idiopathic pulmonary arterial hypertension
HPLC	High-performance liquid chromatography
LV	Left ventricle
MCT	Monocrotaline
mPAP	Mean pulmonary arterial pressure
mSAP	Mean systemic arterial pressure
PAH	Pulmonary arterial hypertension
PASMC	Pulmonary arterial smooth muscle cell
PDE-5	Phosphodiesterase-5
RV	Right ventricle
RVHI	Right ventricular hypertrophy index
RVSP	Right ventricular systolic pressure
S	Septum
SERT	Serotonin reuptake transporter
sPAP	Systolic pulmonary arterial pressure
WA %	Percent wall area

Introduction

Pulmonary arterial hypertension (PAH) is characterized pathophysiologically by sustained elevation in PAH and pulmonary vascular resistance, which ultimately lead to

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right-sided heart failure and death. The structural pathological findings of PAH include medial hypertrophy, intimal fibrosis, and in situ thrombosis in the small muscular pulmonary arteries [1]. A number of molecules are involved in these structural pathological changes, such as serotonin (5-HT), endothelin-1, prostacyclins, and vascular endothelial growth factor. The molecular pathways involved in the pathological changes represent an imbalance between vasodilation and vasoconstriction, growth inhibitors and growth promoting factors, and prothrombotic and antithrombotic molecules [2]. Studies of these molecules are fundamental to pharmacological management, such as prostacyclin therapy, endothelin receptor inhibitors and phosphodiesterase-5 inhibitors.

The 5-HT pathway has been associated with PAH since the first appetite depressant-induced epidemic of pulmonary hypertension occurred in Europe [3]. 5-HT stimulates pulmonary arterial smooth muscle cell (PASMC) proliferation, pulmonary arterial vasoconstriction, and local microthrombosis [4] through interactions with 5-HT receptors and serotonin reuptake transporters (SERTs) [5]. Both experimentally and clinically, elevated circulating peripheral 5-HT is related to the development of PAH [6, 7]. Using a rat model of monocrotaline (MCT)-induced pulmonary hypertension, studies have shown that inhibition of 5-HT receptors and SERTs can inhibit the development of PAH [8, 9]. Aspirin (ASA), which inhibits 5-HT release from platelets, has not been studied for its biomedical effects on idiopathic pulmonary arterial hypertension.

Aspirin is a well-known cyclooxygenase inhibitor that controls platelet release of 5-HT by restraining platelet activation and interaction [10]. Because 99% of whole-blood 5-HT is contained in platelets [11], the plasma 5-HT level is dependent upon 5-HT released from platelets. Although previous studies have investigated the efficacy of ASA on hypoxic pulmonary hypertension [12] and pulmonary hypertension secondary to pulmonary embolism [13], the results were not definitive. Moreover, very few studies have investigated the effects of ASA on PAH. Therefore, the purpose of the present study was to directly evaluate the efficacy of ASA in MCT-induced PAH rats, which models PAH in humans.

Materials and Methods

Study Sample

This study was approved by the Shanghai Jiaotong University Panel on Laboratory Animal Care, and all animals received humane care. One hundred forty pathogen-free male Sprague–Dawley rats (body weight, 250–300 g) were randomized into seven groups. The experimental protocol is summarized in Fig. 1.

MCT Administration

MCT (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in phosphate-buffered saline and 0.1 N HCl. The pH was adjusted to 7.4, and the volume was increased to achieve a final concentration of 20 mg/ml. The MCT solution was sterilized by filtration through a 0.45-μm syringe filter, aliquoted, and stored at 20°C. On day 0, all rats except

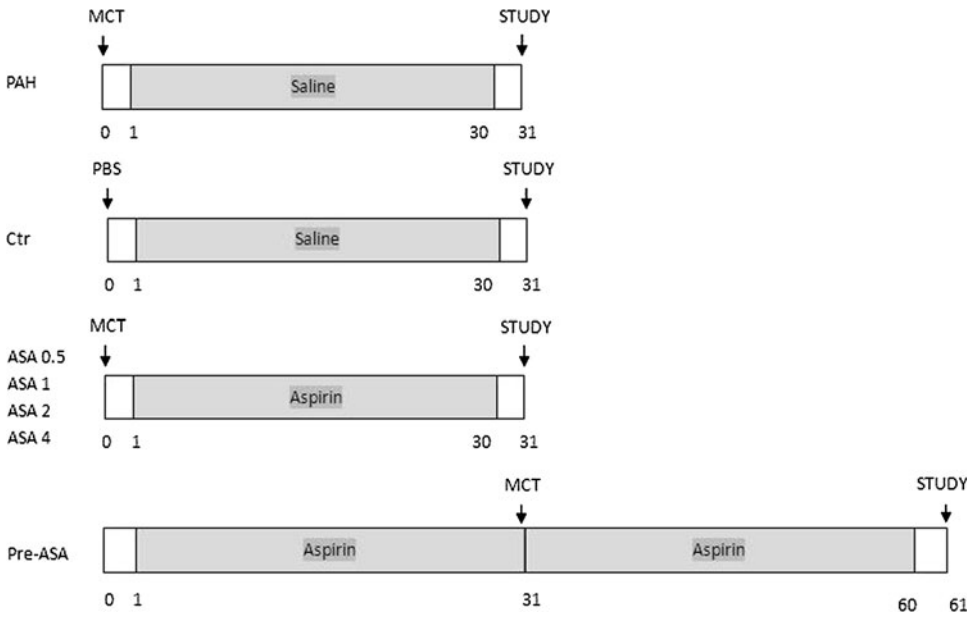

Fig. 1 The timeline represents the experimental protocol for the different groups

those rats in the control and pre-ASA groups were injected intraperitoneally with MCT (50 mg/kg). The control group received an injection of saline. After pretreatment with ASA for 30 days, the pre-ASA group received an MCT injection on day 31.

Treatment Groups

Rats were randomly divided into seven groups to receive different dosages of ASA or vehicle (normal saline) by daily gavage: Group control, Group PAH, 0.5, 1, 2, and 4 mg/kg/day ASA groups (respectively, Group ASA 0.5, Group ASA 1, Group ASA 2 and Group ASA 4) and the Group pre-ASA ($n = 20$ per group). All of these ASA treated groups received ASA from day 1 to day 30, and the pre-ASA group continued to receive ASA (1 mg/kg/day) after the MCT injection on day 31 (from day 31 to 60). The control group (without MCT injection) and the PAH group (MCT 50 mg/kg) received a vehicle (saline) solution from day 1 to 30 ($n = 20$ per group).

Survival Analysis

We examined the effect of ASA on the survival rate of MCT-injected rats. The day of MCT injection was defined as day 0. This survival analysis covered the experimental period from day 0 to 30. Because the experimental period for the Group pre-ASA was 60 days (compared with 30 days for the other groups), rats from this group were excluded from the survival analysis.

Hemodynamic Studies

On day 31 (day 61 for Group pre-ASA), the rats were anesthetized with chloral hydrate (0.4 g/kg), and a specially designed J-shaped catheter (1-mm o.d., being filled with heparinized saline) was maneuvered into the right ventricle (via the right external jugular vein and right atrium) and ultimately positioned in the pulmonary artery. The catheter position was confirmed by the shape of the pressure trace curve. The ascending aorta was cannulated via the right carotid artery. After catheter placement, we waited 20 min to reach a steady state, with respect to systemic and pulmonary pressure, before beginning the recordings. Heart rate, systolic pulmonary arterial pressure (sPAP), mean pulmonary arterial pressure (mPAP), right ventricular systolic pressure (RVSP) and mean systemic arterial pressure (mSAP) were measured using an ADInstrument ML110 pressure transducer and recorded on an ADInstrument ML866 physiological recorder. All of the data were recorded on a computer, and the results were analyzed offline.

Right Ventricular (RV) Hypertrophy Determination

After exsanguination, the right ventricle (RV) was dissected from the left ventricle (LV) and the septum (S). All of the regions were weighed to determine the RV hypertrophy index (RVHI): RV/(LV + S).

Morphometric Analysis of Pulmonary Arteries

After exsanguination, lung tissue was prepared for morphometric analysis by H&E staining. All arteries of 100–200 μm in diameter were evaluated for wall thickness at a magnification of 400×. For each artery, the wall thickness was calculated based on the following equation: percent wall area (WA%) = [wall area/overall area] × 100.

High-Performance Liquid Chromatographic (HPLC) Analysis of Plasma 5-HT

Blood samples were collected from the catheter to measure the plasma 5-HT level during the operation. Eight to ten blood samples were randomly chosen from each group for the HPLC analysis of plasma 5-HT.

Statistical Analysis

Data are presented as mean ± SD. The data from Group control were compared with the Group PAH (the disease model) by Student's t test (statistical significance was indicated by the value of $P < 0.05$). The Group control, Group PAH, the Group pre-ASA, and the Group ASA 0.5, Group ASA 1, Group ASA 2, Group ASA 4 were analyzed by two-way ANOVA. Correlations between 5-HT and sPAP, RVHI, and WA% were evaluated by Pearson's correlation coefficient. A value of $P < 0.05$ was considered statistically significant for the correlations and the comparisons.

Results

Aspirin Attenuates PAH

Body Weights

During the 30-d growth period, rats in the Group control showed a 43% increase in body weight. In contrast, rats in the Group PAH, which developed severe PAH after the MCT injection and vehicle treatment, only showed a 29.4% increase in body weight. Rats treated with ASA gained less weight than the Group control but much more weight than the Group PAH. Normal rats treated with aspirin for 30 days before MCT injection (Group pre-ASA) was weighted on day 31. There were no significant differences between the

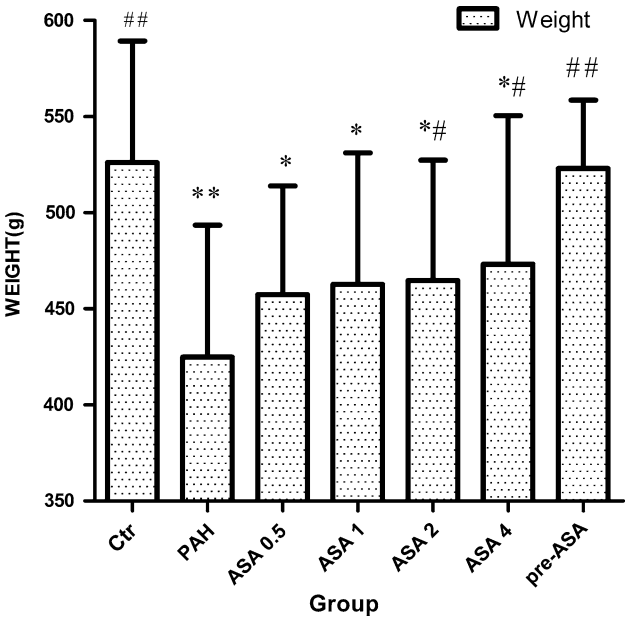

Fig. 2 Aspirin increased the body weight of MCT-treated rats. This figure shows the mean body weight of each group on day 31 (dead rats were weighed on the day of death) ($n = 20$ per group). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the Group control, and $^{\#\#}P < 0.01$ and $^{\#}P<0.05$ compared with the Group PAH

Group pre-ASA and the Group control on day 31, which suggests that ASA did not affect weight gain (Fig. 2).

Survival Analysis

Pulmonary arterial hypertension developed after the MCT injection and continued to deteriorate until death. By day 31, rats that received vehicle for 30 days after the MCT injection lost weight and scarcely moved before death ($n = 20$, 60% survival; Fig. 3, triangles). In contrast, control rats showed no mortality by day 31 ($n = 20$, 100% survival; Fig. 3, circles). Interestingly, ASA treatment conferred an 84% survival advantage ($n = 80$, Fig. 3, squares) compared with rats in the PAH group that received vehicle ($P < 0.05$).

Hemodynamic Parameters

Hemodynamic measurements in the Group control showed mSAP and sPAP values of 116 ± 9 mmHg, and 22.6 ± 2.3 mmHg, respectively, which are typical of healthy adult rats [14].

sPAP

Monocrotaline (50 mg/kg, i.p.) resulted in a dramatic increase in sPAP (PAH: 40.4 ± 5.0 mmHg versus control: 22.6 ± 2.4 mmHg), which was attenuated by ASA

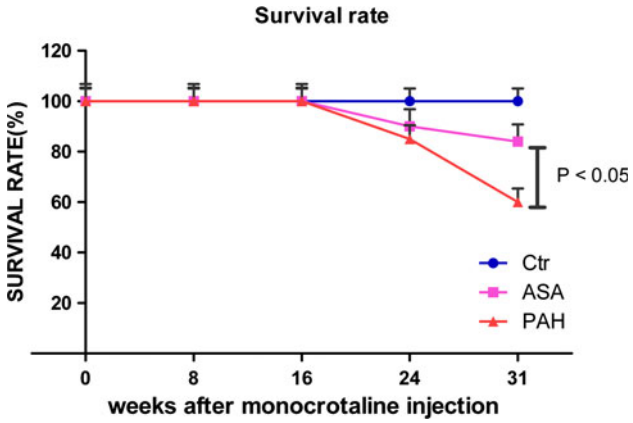

Fig. 3 The survival rates at day 31 were 100% for the Group control ($n = 20$, *circles*), 84% for all ASA-treated rats ($n = 80$: Group ASA 0.5 + Group ASA 1 + Group ASA 2 + Group ASA 4, *squares*) and 60% for the Group PAH ($n = 20$, *triangles*). The overall survival rate of the ASA-treated rats was greater than the Group PAH that received vehicle ($P < 0.05$)

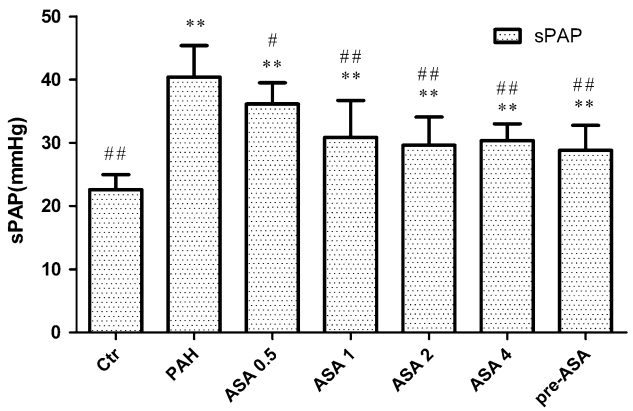

Fig. 4 Aspirin reduced systolic pulmonary arterial pressure in MCT-injected rats. This figure shows the sPAP measurements for each group. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the Group control, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the Group PAH

treatment. Rats in ASA 1, Group ASA 2, Group ASA 4, and Group pre-ASA all had significantly lower sPAP than the Group PAH ($P < 0.01$); however, there were no significant differences between these doses of ASA (Fig. 4). In addition, RVSP showed the same pattern as sPAP (Fig. 5).

mPAP/mSAP

To exclude possible interference from mSAP on mPAP, we examined the mPAP/mSAP ratio. Rats in the Group PAH had the highest mPAP/mSAP ratio (45.77 ± 1.63%), and rats in the control group had the lowest mPAP/mSAP ratio (27.36 ± 3.26%). Except for the Group ASA 1, the other ASA-treated groups had significantly lower mPAP/mSAP ratios than the PAH group (Fig. 6). Two-way ANOVA and multiple comparisons analysis revealed that the Group

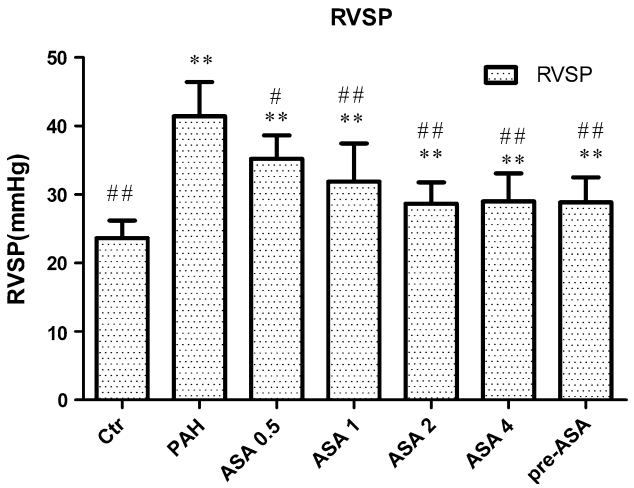

Fig. 5 Aspirin reduced RVSP in MCT-injected rats. This figure shows the RVSP measurements for each group. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the Group control, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the Group PAH

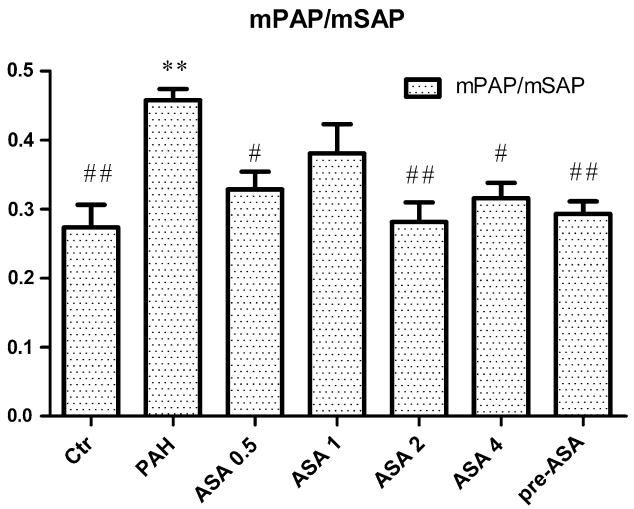

Fig. 6 Except for the Group ASA 1, MCT-injected rats treated with ASA had significantly lower mPAP/mSAP values than the Group PAH. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the Group control, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the Group PAH

ASA 2 showed the most dramatic decrease in mPAP/mSAP ratio ($P < 0.01$).

Heart Rate

To exclude interference from heart rate on mPAP, heart rates were measured and ranged from 330 to 380 beats per minute. Student's *t* tests showed no significant differences in heart rate between the groups.

Right Ventricular Hypertrophy

The development of chronic PAH results in a compensatory hypertrophy of the right ventricle (increased ratio of

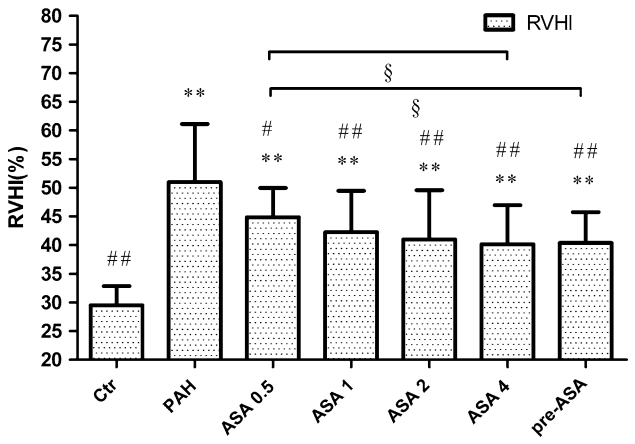

Fig. 7 Aspirin reduced the development of right ventricular hypertrophy in MCT-injected rats. This figure shows the ratios of right ventricular mass to left ventricle plus septal mass (RV/(LV + S)) in each of the groups. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the Group control, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the Group PAH, and $^{\S}P < 0.05$ compared with the Group ASA 0.5

RV/(LV + S)). Indeed, the RV/(LV + S) ratio was 0.29 ± 0.03 in control rats, but it increased to 0.51 ± 0.10 in the Group PAH. Rats treated with ASA had lower RV/(LV + S) values than the Group PAH, and the Group ASA 4 had the lowest value (0.40 ± 0.07) (Fig. 7). Two-way ANOVA and multiple comparisons analysis revealed that the main effect occurred in the Group ASA 4 ($P < 0.01$).

Morphometric Analysis of Pulmonary Arteries (WA%)

Representative photomicrographs showed that the wall thickening in the pulmonary artery was markedly inhibited in the ASA-treated groups (Fig. 8 1, 2). Quantitative analysis of pulmonary arteries (100–200 μm in diameter) demonstrated that MCT induced a significant increase in wall thickness. Interestingly, the development of MCT-induced wall thickening in pulmonary arteries was significantly inhibited in the Group ASA 1, Group ASA 2, Group ASA 4 as well as Group pre-ASA (Fig. 9).

Plasma 5-HT

We measured plasma 5-HT levels by HPLC. Rats in the Group PAH showed high plasma 5-HT levels (44.94 ± 3.74 μg/ml). Aspirin-treated groups had lower plasma 5-HT values than the Group PAH, and the level of 5-HT correlated with the ASA dose. Indeed, of all the groups, the Group ASA 4 showed the lowest plasma 5-HT level (25.35 ± 1.92 μg/ml) (Fig. 10). Pearson's correlations showed that the 5-HT level was positively correlated with sPAP, RVHI, and WA% (r = 0.76, 0.78, and 0.69, respectively, $P < 0.05$; Figs. 11, 12, 13).

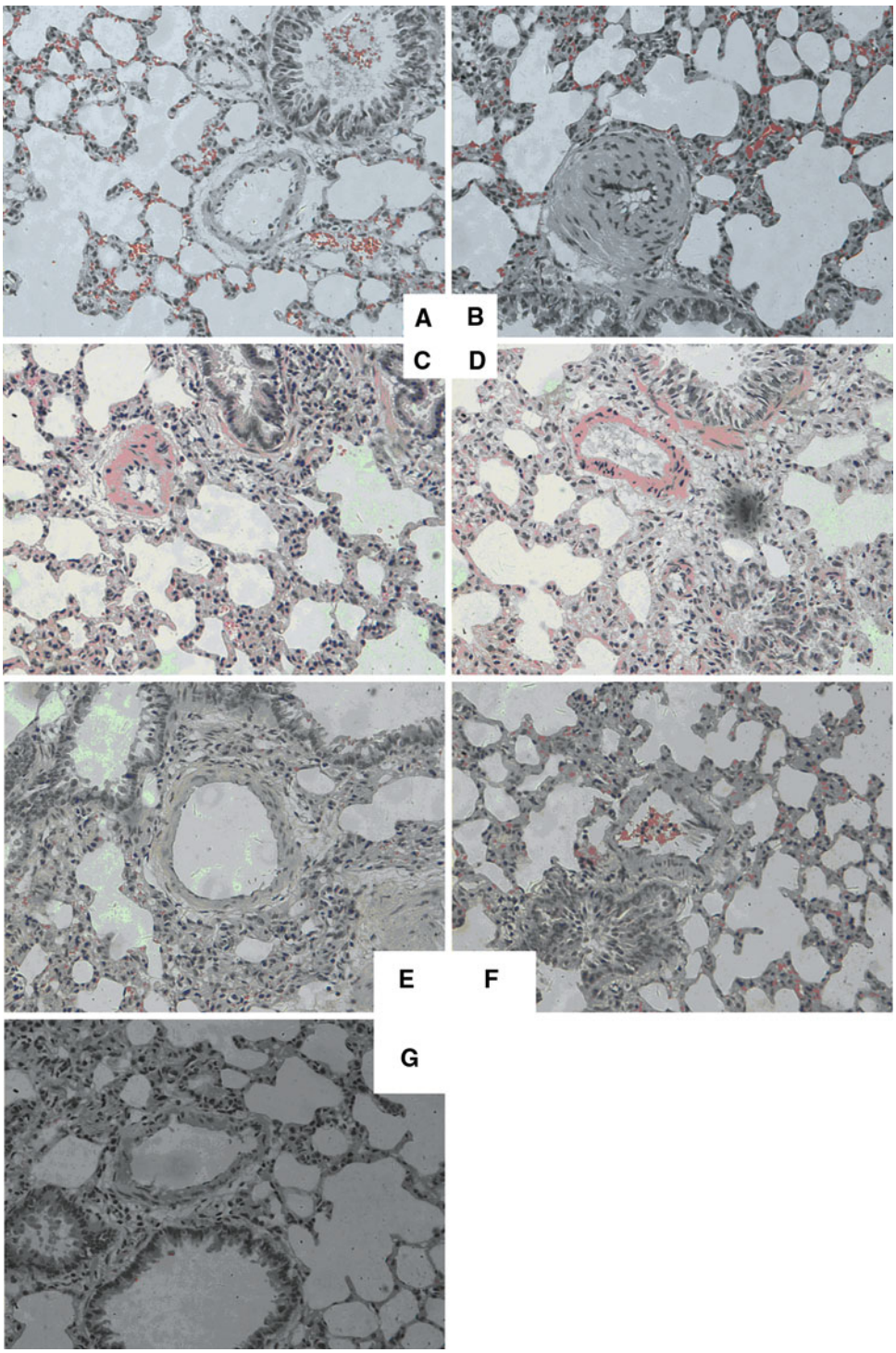

Fig. 8 **1**, **2** Aspirin reduced pulmonary artery wall hypertrophy in muscular pulmonary arteries. **a**) normal muscular pulmonary artery in the Group control. **b** Prominent thickened vascular wall in the Group PAH. **c** Still thickened vascular wall in the Group 0.5. **d** Less thickened vascular wall in the Group 1. **e** Less thickened vascular wall in the Group 2. **f** Less thickened vascular wall in the Group 4. **g** Less thickened vascular wall in the Group pre-ASA

Discussion

During PAH development, 5-HT has been implicated in the pathogenesis of PAH by stimulating PASMC proliferation, pulmonary arterial vasoconstriction, and local microthrombosis [4]. A high level of plasma 5-HT promotes PASMC hyperplasia. In addition, PASMCs from PAH patients grow faster than those PASMCs from control subjects, which is partly due to increased expression of the 5-HT transporter [15]. Overexpressing the 5-HT transporter gene in smooth muscle causes pulmonary hypertension [16]. Combined or separate antagonism of 5-HT receptors (GR55562) and/or SERTs (M100907) inhibits the development of PAH in animal models and could be a novel therapeutic approach to PAH [8, 9, 17]. However, appropriate doses, pharmacodynamics, and toxicology need to be

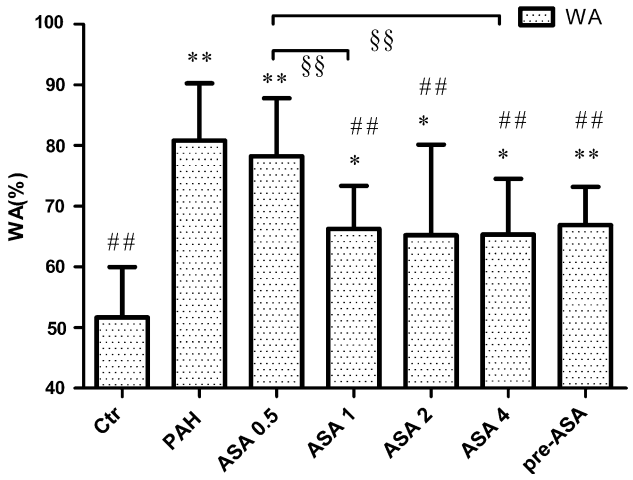

Fig. 9 Quantitative analysis of the wall thickness of the pulmonary artery. Compared with the Group control, the wall thickness of the pulmonary artery was significantly increased in the Group PAH. Compared with the Group PAH and the Group ASA 0.5, the Group ASA 1, Group ASA 2, Group ASA 4, and Group pre-ASA all show marked inhibition of the development of wall thickening. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the Group control, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the Group PAH, and $^{\S}P < 0.05$ compared with the Group ASA 0.5

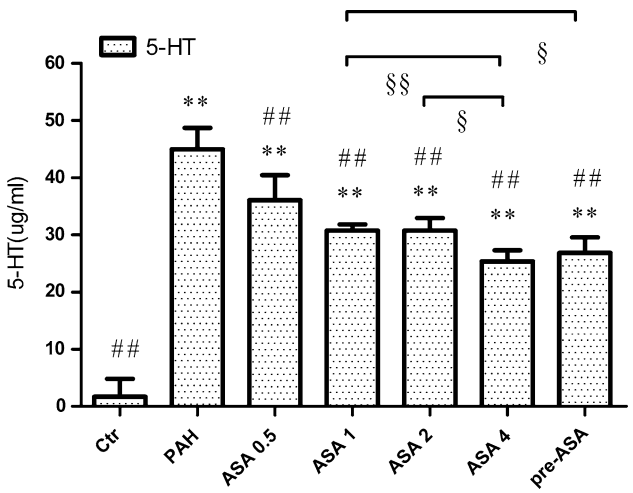

Fig. 10 Aspirin reduced the plasma 5-HT levels in MCT-injected rats. This figure shows the plasma 5-HT levels of each group. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the Group control, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the Group PAH, and $^{\S}P < 0.05$ compared between ASA treatment group

defined before these approaches can be tested clinically. Aspirin is a well-known inhibitor of platelet aggregation, which controls 5-HT release from platelets [10]. Previous studies have investigated the efficacy of ASA on hypoxic pulmonary hypertension [12] and pulmonary hypertension secondary to pulmonary embolism [13] but not on PAH. Because the pharmacodynamics and toxicology of ASA are clearly understood, ASA could be used clinically for PAH if it is proven to be effective in PAH treatment as well.

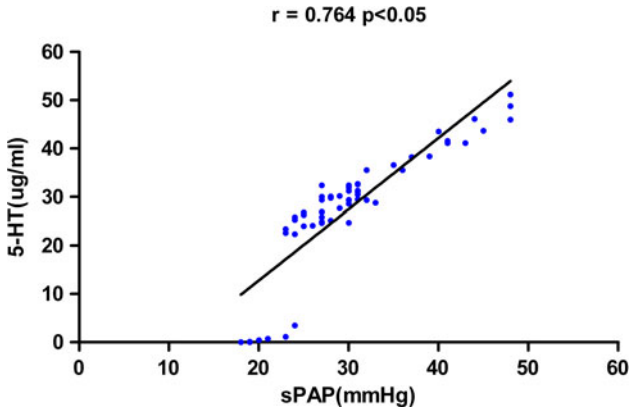

Fig. 11 The correlation between 5-HT levels and sPAP ($r = 0.76$, $P < 0.05$)

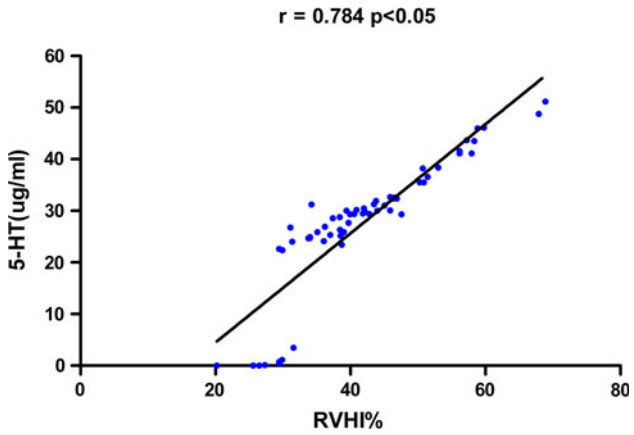

Fig. 12 The correlation between 5-HT levels and RVHI ($r = 0.78$, $P < 0.05$)

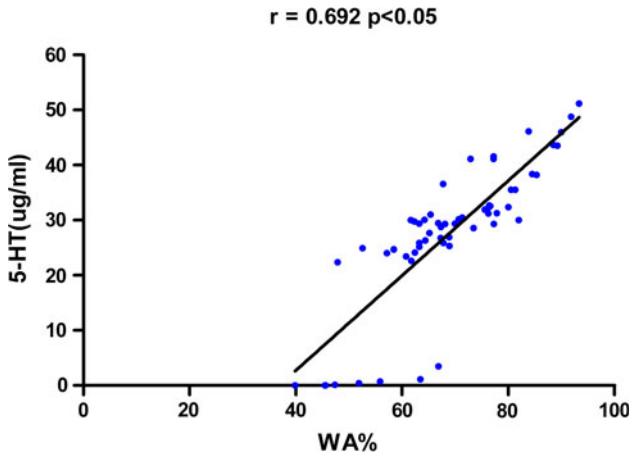

Fig. 13 The correlation between 5-HT levels and WA% ($r = 0.69$, $P < 0.05$)

Therefore, the study focused on the effects of ASA, an inhibitor of platelet 5-HT release [10], to investigate the effects on hemodynamic and pathological changes in an

MCT-induced PAH model, and ASA may be particularly attractive in future PAH treatment [18].

The model of pulmonary hypertension in MCT-injected rats reproduced the neointimal proliferation and vascular occlusion by smooth muscle cells that occurs in human PAH. MCT undergoes hepatic metabolism in rats into monocrotaline pyrrole, which causes endothelial injury in the pulmonary vasculature with subsequent remodeling of the precapillary vessels, progressive pulmonary hypertension, and compensatory right-heart hypertrophy [19]. We successfully created the model by inducing PAH with MCT (50 mg/kg, i.p.). Ramos et al. [20] used 60 mg/kg MCT, but the 60-mg/kg dose in the study resulted in severe PAH and a low survival rate, which limited further research. In addition, 40-mg/kg MCT failed to reliably induce PAH. Therefore, we chose 50-mg/kg MCT for the experiment.

Preliminary experiments showed that rats in the Group ASA 0.5 barely showed any effects, whereas rats in the 4 mg/kg/day ASA group developed gastric bleeding. Thus, these doses were used as the minimal and maximal doses, respectively, and we included the intermediate doses of 1 and 2 mg/kg/day (Fig. 1). Excluding the 0.5-mg/kg/day group, which did not show much of an effect, there were no differences between the different ASA doses in their abilities to attenuate sPAP, mPAP, and RVSP. Moreover, these treatments caused partial attenuation of PAH but not complete reversal. This may be related to the time course and the dosage of ASA because the maximal 4-mg/kg/day ASA treatment was only performed for 30 days in a well-developed PAH rat model. However, the possibility of achieving a greater therapeutic effect with a larger dose of ASA and/or longer treatment cannot be ruled out at this time.

To exclude the possible effects of systemic pressure reduction, the ratio of mPAP/mSAP was calculated. We found that the Group ASA 2 and ASA 4 showed obvious reductions in mPAP/mSAP, and the 2-mg/kg/day treatment was the more effective. Although the Group ASA 1 also reduced the mPAP/mSAP ratio, this group did not differ from the PAH group. The 1-mg/kg/day ASA dose may have reduced pulmonary arterial pressure and systemic pressure. Furthermore, these findings suggested that 2-mg/kg/day ASA may be the best concentration to lower the mPAP with minimal effects on SAP.

After 30 days of ASA therapy, the PAP value was down-regulated, right ventricular hypertrophy was attenuated and pulmonary arteriole wall thickness was decreased, which suggests that ASA could treat PAH. Therefore, determining the pathway responsible for these effects becomes extremely important. Given the existing evidence that PAH patients have elevated plasma 5-HT levels [7], the majority of plasma 5-HT is contained in platelets [11], and ASA inhibits platelet release of 5-HT [10], we proposed that ASA attenuation of PAH involved a reduction in plasma 5-HT. Indeed, this study showed that ASA reduced plasma 5-HT levels, and the degree of reduction had a positive correlation with sPAP, RVHI, and WA%.

This study demonstrated that ASA attenuated the development of PAH and right ventricular hypertrophy in MCT-injected rats. In addition, we showed that ASA could improve the survival rate in MCT-induced PAH rats, and survival rate is one of the most important indices in evaluating therapeutic effects of a compound. To the best of the knowledge, this study was the first to demonstrate the biomedical effects of ASA in MCT-induced PAH rats.

There are two limitations of this study. First, this study did not determine whether ASA could reduce in situ thrombosis in small muscular pulmonary arteries. In situ thrombosis is involved in PAH pathogenesis [1], and the anticoagulant warfarin protects against PAH. In addition, warfarin has been suggested for PAH patients by guidelines [1]. ASA, which is the classical antithrombotic drug [21], is expected to have similar effects as warfarin. In the study, however, the lung tissue was washed with saline for morphometric analysis by H&E staining; thus, in situ thrombosis was not tested. Second, ASA can suppress the production of prostaglandins and thromboxane through irreversible inactivation of the cyclooxygenase enzyme [21]. Pulmonary arterial hypertension is also characterized by abnormal arachidonic acid metabolism, which results in elevated thromboxane A_2 and decreased prostacyclin I_2. The former is a potent vasoconstrictor and smooth muscle mitogen that promotes platelet aggregation, and the latter is a vasodilator that inhibits smooth muscle proliferation and platelet aggregation [22]. We have previously shown that lipo-PGE_1, like PGI_2, can effectively lower pulmonary pressure and improve exercise tolerance in patients with PAH [23]. Interestingly, Robbins et al. [24] showed that ASA reduced thromboxane production without affecting prostaglandin I_2 synthesis. In this study, however, the level of thromboxane production was not studied because the limited amount of blood was only enough for HPLC determination of 5-HT. These two limitations of this study are deserving of further research in the future.

Conclusion

Aspirin treatment (1, 2, or 4 mg/kg/day) and pretreatment with ASA (1 mg/kg/day) inhibited PAH, increased survival rate, and attenuated right ventricular hypertrophy and pulmonary arteriole proliferation in an MCT-induced PAH rat model. The effects of ASA may be related to the reduction of plasma 5-HT. These results suggest that ASA could be a safe, effective, and cheap medication for PAH.

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