Pulmonary arterial hypertension (PAH) is a serious and fatal cardiovascular disorder characterized by increased pulmonary vascular resistance and progressive pulmonary vascular remodeling, which eventually leads to right ventricular dysfunction and heart failure associated with premature death. Current available therapies for PAH are limited, mostly either designed to depress vascular contractility or reduce pulmonary arterial resistance, such as calcium channel inhibitors, phosphodiesterase-5 inhibitors, prostacyclin analogues, soluble guanylate cyclase agonists, and inhaled nitric oxide. However, these drugs fail to reverse the progression of PAH and improve the poor prognosis. Thus, more effective pharmacological interventions are urgently needed for the treatment of PAH.

The underlying pathological mechanisms of PAH are multi-factorial and multi-cellular. Pulmonary vascular remodeling, which usually affects all vessel layers, represents one the most important pathological features of PAH. The characteristic histologic changes of pulmonary vascular remodeling include endothelial cell injury, neointima formation, muscularization of peripheral arteries, collagen deposition, and adventitial thickening. Oxidative stress and inflammation are considered two key mechanisms responsible for the remodeling of pathological vessels. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase derived superoxide anion \( \left( \text{O}_2^- \right) \) has been demonstrated to play an important role in pulmonary vascular remodeling, and perivascular inflammation response also has been reported to contribute to vascular remodeling. Therefore, suppressing oxidative stress and inflammation response may be beneficial to re-

**Key words:** Seaweed polysaccharides, Pulmonary vascular remodeling, TGF-β1, Oxidative stress, Inflammatory cytokine
verse pulmonary vascular remodeling and prevent the progression of PAH.

Alginic oligosaccharide (AOS), which is produced by depolymerizing alginic, has a relatively low molecular weight and is a non-immunogenic, nontoxic, biocompatible, and biodegradable polymer. AOS shows better pharmacological activities and beneficial effects than alginic, including anti-oxidative, anti-inflammatory, anti-apoptotic and anti-proliferative effects. AOS has been shown to have cardiac and neuroprotective effects. However, the ability of AOS to protect against PAH was not shown in previous studies.

In previous studies, monocrotaline (MCT) has been suggested to induce pulmonary hypertension in rats. After a single injection of MCT, rats develop severe pulmonary hypertension. Specifically, MCT initiates endothelial damage and triggers obstructive vascular remodeling with the presence of oxidative stress and inflammatory response. In this study, we used the MCT-induced pulmonary hypertension rat model to investigate whether AOS could prevent pulmonary hypertension and pulmonary vascular remodeling. The potential mechanisms underlying the protective effects were also explored.

Methods

Animals: Male 8-week-old Sprague-Dawley rats (200-250 g body weight, n = 36) were obtained from Jinan Pengyue Experimental Animal Breeding Co., LTD (Jinan, China). All rats were fed with normal diet and housed at the animal care facility of the Medical Department of Qingdao University under specific pathogen-free conditions. The environmental temperature was maintained at 22°C and the rats were subjected to 12-hour light/12-hour dark photoperiods. All animal experimental protocols complied with the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health and Public Health Service Policy on Humane Care and Use of Laboratory Animals. We took all steps to minimize the pain and suffering of the animals. The study procedures were approved by the Committee on the Ethics of Animal Experiments of Qingdao University.

MCT-induced pulmonary hypertension and drug administration: The rats were randomly assigned into six groups (n = 6 each group) as follows: control, MCT, MCT + Alprostadil — prostaglandin E1, PGE1 — (MCT + PGE1), MCT + low-dose AOS (MCT + AOS-5), MCT + middle-dose AOS (MCT + AOS-10), and MCT + high-dose AOS (MCT + AOS-20) groups. Pulmonary hypertension was induced by a single intraperitoneal injection of MCT (60 mg/kg, Sigma-Aldrich, St. Louis, MO, USA), which was dissolved in 1% dimethyl sulfoxide (DMSO; Sigma). The control rats received a single intraperitoneal injection of the equivalent amount of 1% DMSO. Five weeks after the injection of MCT, the MCT + PGE1, MCT + AOS-5, MCT + AOS-10, and MCT + AOS-20 groups were injected intraperitoneally with Alprostadil (5 μg · kg⁻¹ · d⁻¹) or AOS (5, 10, and 20 mg · kg⁻¹ · d⁻¹) for three weeks, respectively. AOS was purchased from Qingdao BZ Oligo Biotech Co., Ltd. (Qingdao, China), and Alprostadil was purchased from Beijing Tide Pharmaceutical Co., Ltd. (Beijing, China). The control group and MCT group rats received a daily injection of the equivalent amount of saline.

Hemodynamic measurements: Transthoracic echocardiography was performed using a Vevo2100 (VisualSonics, Toronto, Canada) at the end of the experiment. Rats were anesthetized with 2% isoflurane and their thoraxes were shaved. Then, the animals were placed in the supine position on a heating pad to keep their body temperature at 37°C. Pulse-wave Doppler of pulmonary outflow was recorded in the parasternal short-axis view at the level of the aortic valve. Two-dimensional echocardiography and pulsed Doppler were collected. Pulmonary artery acceleration time (PAT), pulmonary arterial ejection time (ET), and pulmonary arterial diameter (PAD) were measured as previously described. Transthoracic echocardiography was performed by an experienced echocardiographer and all parameters were measured in three consecutive cardiac cycles to obtain the mean value.

Right ventricular hypertrophy measurements: At the end of the experiment, all rats were sacrificed and hearts were collected. Each ventricle was carefully isolated and weighed. The ratio of right ventricle (RV) weight to the left ventricle (LV) plus the interventricular septum weight, i.e., RV/(LV + S), was used as an index of right ventricular hypertrophy (RVH).

Histological analysis: The lung samples were fixed with 10% formalin for 24 hours and then embedded in paraffin. Four-micrometer-thick sections were cut and stained with hematoxylin and eosin (H&E) and elastic van Gieson for histological analysis. The slices were examined with an inverted microscope (TE 2000; Nikon, Japan) equipped with digital imaging, and the terminal arterioles (external diameter of 50-150 μm) were analyzed. The external diameter (ED) and medial thickness (MT) of the terminal arterioles were measured and then the percentage of MT in ED was calculated as 2MT/ED × 100. Moreover, Mason’s trichrome staining was used to investigate perivascular fibrosis, and the slices were visualized by microscope. The lung sections were labeled with anti-CD68 antibody (Elabscience, Wuhan, China) at 4°C overnight. The CD68-positive cells represented the infiltration of macrophages. Corresponding data was calculated and analyzed as previously described by investigators who were blinded to the experimental group assignment.

Oxidant status measurement: After the rats were sacrificed, blood was drawn from the abdominal aorta to heparinized tubes and centrifuged at 1000 × g for 15 minutes. Then, the supernatant was transferred into centrifuge tubes and stored at ~80°C. The concentration of malondialdehyde (MDA) in the serum was detected by spectrophotometry according to the kits’ instructions. The MDA assay kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China).

Western blot analysis: Western Blot Analysis was performed as previously described. The rabbit polyclonal antibodies against p67-phox and gp91-phox and the mouse monoclonal antibody against TGF-β1 were obtained from Abcam Inc. (Cambridge, UK). The mouse monoclonal antibodies against p47-phox, TNF-α, IL-1β, and IL-10 were obtained from Santa Cruz Biotechnology,
Figure 1. Effects of AOS on the Development of Pulmonary Hypertension and Right Ventricular Hypertrophy. Transthoracic echocardiography was performed to detect the hemodynamic state of the rats. PAT (A), PAT/ET (B), and PAD (C) were used as echocardiographic indicators of pulmonary artery pressure and pulmonary vascular resistance measured invasively. RVHI (D) was used as an index of right ventricular hypertrophy. The values shown are the mean ± SEM from each group of rats, respectively. **P < 0.01 versus control rats, #P < 0.05 versus MCT rats, ##P < 0.01 versus MCT rats. MCT indicates monocrotaline; PGE1, Alprostadil, prostaglandin E1; AOS, alginate oligosaccharide; PAT, pulmonary artery acceleration time; ET, ejection time; PAD, pulmonary arterial diameter; and RVHI, index of right ventricular hypertrophy.
prevented the development of MCT-induced pulmonary hypertension and right ventricular hypertrophy.

AOS treatment alleviated pulmonary vascular remodeling in MCT-induced PH rats: In order to clarify the development of the pulmonary vascular remodeling, H&E, elastic van Gieson, and Masson’s trichrome staining were used to investigate the morphological changes, medial thickness, and perivascular fibrosis of pulmonary vascular, respectively. H&E staining showed that hypertrophy of the pulmonary vessel wall was significantly increased in the MCT group. However, vessel-wall hypertrophy was markedly reduced in the PGE1 and AOS groups (Figure 2A). We also observed that the relative medial thickness of pulmonary arterioles was significantly increased in the MCT group compared with the control group. AOS treatment, like the positive control drug PGE1, significantly decreased the relative medial thickness (%) in a dose-dependent manner (Figures 2B, 2D). As shown in Figures 2C, 2E, Masson’s trichrome staining results revealed that the percent collagen area was also significantly increased in the MCT group compared with the control group. AOS treatment significantly decreased the percent collagen area of pulmonary arterioles in a dose-dependent manner compared with the MCT treatment. This data suggested that AOS treatment prevented the progression of MCT-induced pulmonary vascular remodeling.

AOS inhibited the activation of TGF-β1/Smads signaling pathway in MCT-induced PH rats: TGF-β1 is an important regulator of fibrogenesis, and elevated TGF-β1 signaling has been implicated in MCT-induced PAH rats. The expression of TGF-β1/p-Smad2 signaling in pulmonary arteries was determined by Western blot. MCT injection induced significant increases in TGF-β1 and p-Smad2 protein expression (Figures 3A-D). Similar to the positive control drug PGE1, AOS treatment significantly reversed the increase in TGF-β1 and p-Smad2 protein expression. These results showed that AOS treatment prevented the activation of MCT-induced TGF-β1/p-Smad2 signaling pathway.

AOS attenuated the oxidative stress in MCT-induced PH rats: MDA is an important indicator of lipid peroxidation. As shown in Figure 4A, the level of MDA was significantly increased in the serum of MCT-induced PH rats, and similar to the positive control drug PGE1, the administration of AOS markedly downregulated the MDA level in a dose-dependent manner. In addition, we also investigated the activity of NADPH oxidase in the pulmonary artery tissues. Our results showed that the expressions of p47-phox, p67-phox, and gp91-phox, subunits of NADPH oxidase, were significantly increased in MCT-induced PH rats (Figures 4B, 4C). Treatment with AOS significantly inhibited MCT-induced activation of NADPH oxidase in a dose-dependent manner. These results showed that AOS treatment attenuated the oxidative stress in MCT-induced PH rats.

AOS suppressed the inflammatory response in MCT-induced PH rats: Inflammatory cells secrete pro-inflammatory cytokines, which play an important role in the development of MCT-induced PH. As shown in Figures 5A, 5B, we observed a significant upregulation of the pro-inflammatory IL-1β and TNF-α proteins and a significant downregulation of the anti-inflammatory IL-10 protein in MCT-exposed rats. Administration of AOS, similar to the positive control drug PGE1, induced a significant downregulation of the IL-1β and TNF-α protein expressions and upregulation of the IL-10 protein expression in a dose-dependent manner. In addition, we used CD68 immunostaining to assess the infiltration of macrophages in lung sections. Our result showed that there was more macrophage recruitment in the MCT-induced PH rats, and that this infiltration was significantly suppressed by PGE1 and AOS treatments (Figures 5C, 5D). These results showed that AOS treatment suppressed the inflammatory response in MCT-induced PH rats.

Discussion

Alginates are natural polysaccharides extracted from marine brown algae. AOS is composed of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. Previous studies showed that AOS exerted protective effects in neurodegenerative diseases, acute doxorubicin cardiotoxicity, and myocardial ischemia/reperfusion injury. Nevertheless, the ability of AOS to protect against pulmonary hypertension is not yet clear. In the current study, we sought to examine the effects and mechanisms of AOS in rats with MCT-induced pulmonary hypertension. Our results showed that AOS treatment effectively decreased the pulmonary artery pressure, pulmonary vascular resistance, and right ventricular hypertrophy. The protective effects of AOS were also manifested through the inhibition of the pulmonary vascular remodeling through the regulation TGF-β1/p-Smad2 signaling pathway. In addition, we found that the anti-oxidative and anti-inflammatory effects of AOS contributed to blocking the development of pulmonary hypertension and pathological pulmonary vascular remodeling induced by MCT.

Pulmonary vascular remodeling is a key pathological manifestation of the development of pulmonary hypertension. TGF-β1-mediated signaling performs essential functions in the physiologic homeostasis of the vascular structure. Enhanced TGF-β1 signaling is implicated in the pathogenesis of pulmonary vascular remodeling.22) TGF-β1/Smads signaling pathway has been demonstrated to induce hypertrophy, fibrosis, and inflammation in vascular remodeling.23) Previous studies reported that elevated TGF-β1 and increased Smad2 activity were found in humans and rodents pulmonary hypertension.24) In this study, we demonstrated that MCT injection activated the TGF-β1/p-Smad2 signaling pathway, and AOS treatment downregulated TGF-β1 and p-Smad2. These changes in expression of TGF-β1/p-Smad2 signaling pathway may contribute to the protective effect of AOS therapy on pathologic changes of MCT-induced pulmonary vascular remodeling.

Oxidative stress seems to be critical in the pathogenesis of pulmonary vascular remodeling. The imbalance between enhanced reactive oxygen species (ROS) generation and reduced antioxidant response leads to oxidative stress. Fessel et al. emphasized the physiological role of ROS and NADPH oxidase on the modulation of the function of pulmonary arterial endothelial cells and pulmonary arterial smooth muscle cells.25) Previous studies also re-
Figure 2. Effects of AOS on pulmonary vascular remodeling. A: H&E staining was used to investigate the morphological changes of pulmonary vascular. B: van Gieson staining was used to distinguish between collagen fibers and muscle fibers. C: Masson’s trichrome staining was used to investigate the perivascular fibrosis of pulmonary vascular. D: The corresponding quantitative data of MT (%). E: The corresponding quantitative data of collagen area (%). The values shown are the mean ± SEM from each group of rats, respectively. **P < 0.01 versus control rats, ##P < 0.01 versus MCT rats; n = 10 random microscope fields of view.
**Figure 3.** Effects of AOS on the TGF-β1/p-Smad2 signaling pathway. **A:** The expression of TGF-β1 in pulmonary arterial tissue was determined by Western blot. **B:** The corresponding quantitative data of **A.** **C:** The expression of p-Smad2 and Smad2 in pulmonary arterial tissue was determined by Western blot. **D:** The corresponding quantitative data of **C.** The values shown are the mean ± SEM from each group of rats, respectively. **P < 0.01** versus control rats, **## P < 0.01** versus MCT rats, n = 3.

**Figure 4.** Effects of AOS on the oxidative response in PAH rats. **A:** The serum MDA levels were evaluated (n = 6). **B:** The expression of p47-phox, p67-phox, and gp91-phox in pulmonary arterial tissue was determined by Western blot. **C:** The corresponding quantitative data of **B** (n = 3). The values shown are the mean ± SEM from each group of rats, respectively. **P < 0.01** versus control rats, **# P < 0.05** versus MCT rats, **## P < 0.01** versus MCT rats.
revealed that excessive ROS generation reduced nitric oxide levels and induced proliferation and hypertrophy of pulmonary arterial smooth muscle cells, and eventually enhanced vasoconstriction and pulmonary vascular remodeling.26,27) Aside from the adverse effects of ROS, the upregulation of vascular NADPH oxidase evoked proliferation and apoptosis-resistance of the pulmonary arterial wall cells.2) In this study, our data showed that the MDA level and NADPH oxidase expression were significantly increased in MCT-induced pulmonary hypertension rats, and AOS reduced the MCT-induced MDA production and NADPH oxidase expression. These results indicated that AOS protected pulmonary hypertension rats via the regulation of redox states.

Inflammation is another main contributor to pulmonary vascular remodeling and pulmonary hypertension. Elevated inflammatory cytokines are observed in patients with PAH, and levels of circulating cytokines are associated with the severity and outcome of the disease.28,29) It is well known that perivascular inflammation drives the process of vascular remodeling. Luan et al. reported that pulmonary vascular remodeling was attenuated by the inhibition of the pulmonary inflammatory response in the MCT-induced models of pulmonary hypertension.30) Previous studies have shown that the recruitment of inflammatory cells leads to vascular lesion and vascular remodeling.31) In addition, inflammatory cells that infiltrate vascular lesions of pulmonary hypertension secrete cytokines and growth factors, which can cause pulmonary vasoconstriction and promote proliferation of vascular cells.32) In addition to the above effects, there is a cross-talk between inflammation and oxidative stress in the development of pulmonary hypertension. Previous studies have shown that NADPH oxidase can be activated by several growth factors and inflammatory cytokines such as TGF-β1, TNF-α, and IL-1. In turn, the inflammatory environment can also produce ROS and disrupt the redox homeostasis. So inflammatory response and oxidative stress interact as a positive feedback. In this study, we also analyzed the levels of inflammatory cytokines in the pulmonary arterial tissue and infiltration of macrophages in lung sections. Our results showed a significant upregulation of pro-inflammatory cytokines (IL-1β and TNF-α) and a significant downregulation of an anti-inflammatory cytokine (IL-10) in pulmonary arterial tissue, and more macrophage recruitment in lung sections after MCT injection. By contrast, AOS treatment significantly normalized the expression of inflammatory cytokines and suppressed the infiltration of macrophages. Our group has shown that the p38 MAPK/NF-κB pathway is involved in the anti-inflammatory effects of AOS in MCT-induced pulmonary hypertension rats.33) Therefore, we presume that the anti-inflammatory effect of AOS may be involved in multiple signaling pathways. We also found that some indicators of inflammation and oxidative stress were lower in PGE1- and AOS-treated rats than in control rats, and we speculate that this may be due to the relatively small number of samples tested in each group. We may think of increasing
the sample size in future study.

Taken together, the present study showed that the treatment of AOS prevented the progression of MCT-induced pulmonary hypertension in rats in a dose-dependent manner, with significant attenuation of pulmonary artery pressure, pulmonary vascular resistance, and pulmonary vascular remodeling. These beneficial effects of AOS might be attributed to the restoration of the TGF-β1/p-Smad2 signaling pathway, as well as the actions of the anti-oxidation and anti-inflammation. PGE1 showed better effects than AOS in this study; this might be due to other protective properties of PGE1 such as microvascular vasodilatation and platelet aggregation inhibition. Considering the therapeutic effects of AOS we show in this study and the potentially different therapeutic mechanisms of AOS in contrast to PGE1, it is of great interest to determine whether a combination of PGE1 and AOS could be more beneficial to patients.

Disclosure

Conflicts of interest: The authors declare that they have no competing interests.

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