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# Fasudil reversed MCT-induced and chronic hypoxia-induced pulmonary hypertension by attenuating oxidative stress and inhibiting the expression of Trx1 and HIF-1α

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ABSTRACT

Antioxidant therapy attenuates pulmonary hypertension (PH). In the present study, we tested the antioxidant effects of fasudil against PH in rats. Monocrotaline (MCT)-induced and chronic hypoxia-induced PH models of rats were established, and the haemodynamic and pathomorphologic results of three different doses of fasudil (10 mg/kg, 30 mg/kg, and 75 mg/kg per day) were subsequently compared with those of bosentan (30 mg/kg per day). Additionally, the protein expressions of thioredoxin-1 (Trx1) and hypoxia inducible factor-1α (HIF-1α), the content of superoxide dismutase (SOD), and the levels of hydrogen peroxide ($H_2O_2$), malonyldialdehyde (MDA), and hydroxy radical (•OH) were investigated. Fasudil effectively reduced the right ventricular systolic pressure (RVSP) and alleviated right ventricle (RV) hypertrophy, as well as the histological changes in the pulmonary arterioles. Moreover, fasudil markedly lessened the expression of Trx1 and HIF-1α, up-regulated the concentration of SOD, and lowered the levels of $H_2O_2$, MDA, and •OH. In conclusion, fasudil is a notably attractive potential therapy for PH.



## 1. Introduction

Pulmonary hypertension (PH) is characterised by a progressive increase in the pulmonary vascular resistance and vascular remodelling, which ultimately leads to right ventricular hypertrophy and right heart failure with high mortality (Farber, 2008; Rich, 2006). Even though the pathogenesis of PH is very complex, oxidative stress has been considered to be involved in the progression of PH, as evidenced by antioxidant intervention studies performed in foetal lambs and in hypoxia- or monocrotaline (MCT)-exposed adult rodents (Bowers et al., 2004; DeMarco et al., 2008; Hoshikawa et al., 2001). Accordingly, antioxidant therapy attenuates the development of PH (Redout et al., 2010; Voelkel et al., 2013).

Thioredoxin (Trx) is a ubiquitously expressed and multifunctional protein. Thioredoxin-1 (Trx1), which is one of the major isoforms of the thioredoxin family and is mainly present in the extracellular milieu, cytoplasm, and nucleus, exhibits chemokine-like activity, scavenges reactive oxygen species (ROS), and activates redox-sensitive transcription factors, such as nuclear factor-κB (NF-κB), P53, and hypoxia inducible factor-1α (HIF-1α) (Powis and Montfort, 2001; Watson et al., 2004). Moreover, the extracellular concentration of Trx1 strikingly increased in the plasma or serum upon exposure to oxidative stress, which has been reported in many pathological conditions (Jikimoto et al., 2002; Koura et al., 2000; Nakamura et al., 2009).

Hypoxia inducible factors-1 (HIF-1), a member of the heterodimeric transcription factor family, is composed of HIF-1α and HIF-1β subunits. HIF-1β is readily found in all cells, whereas the expression of HIF-1α is considered to be tightly regulated by hypoxia (Jaakkola et al., 2001). When cells are exposed to hypoxic conditions, the protein expression and transcriptional activity of the hypoxia inducible factors complex increase, which leads to the transcriptional activation of many downstream genes that regulate energy metabolism, erythropoiesis, vasomotor tone, and

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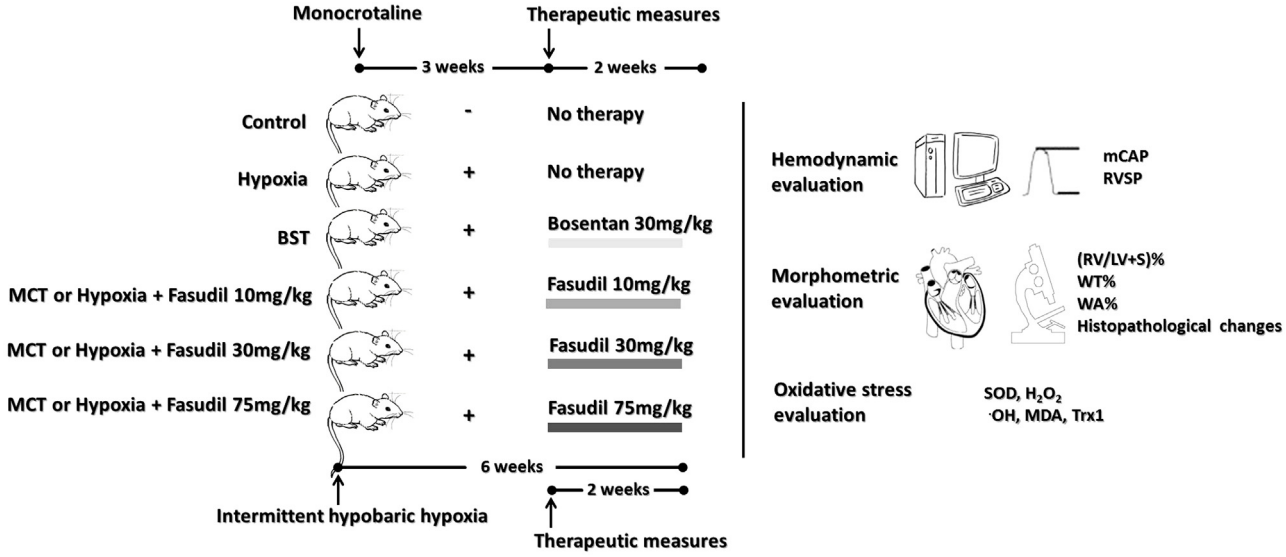


**Fig. 1.** Illustration of the experiment protocols of mimetic monocrotaline (MCT)-induced and chronic hypoxia-induced PH rat model.

angiogenesis (Semenza, 2002; Wang et al., 1995; Yu et al., 1998). Many studies have demonstrated a close relationship between HIF-1α and PH. Partial hypoxia in lung tissue led to a remarkable increase in the production of HIF-1α. Subsequently, the excessive production of HIF-1α enhanced mitogenic factors, which in turn promoted the proliferation of pulmonary artery smooth muscle cells and accelerated pulmonary vascular remodelling (Bonnet et al., 2006; Lai and Law, 2004; Wang et al., 2006). In addition to hypoxia, Trx1 overexpression has also been shown to increase HIF-1α in cancer cells, resulting in the increased production of vascular endothelial growth factor (VEGF) and the enhancement of angiogenesis (Welsh et al., 2002). Moreover, Trx1 inhibitors could inhibit HIF-1α transactivation both in vivo and in vitro and reduce the expression of VEGF and nitric oxide synthase 2 (iNOS) (Welsh et al., 2003).

Fasudil, a relatively selective Rho-kinase inhibitor, has emerged as a viable therapeutic target for the treatment of PH via selectively inhibiting the Rho/ROCK pathway and improving the expression of nitric oxide synthase 1 (eNOS) (Abe et al., 2004, 2006; Farber, 2008; Fukumoto et al., 2013; Oka et al., 2008). Furthermore, the Rho/ROCK pathway may reportedly be associated with the enhancement of oxidative stress via the up-regulation of NAD(P)H oxidase (Higashi et al., 2003), and fasudil also exerted antioxidant effects on hypercholesterolemic rats (Ma et al., 2011). However, few studies have focused on the antioxidant effect of fasudil on PH. Therefore, we established monocrotaline (MCT)-induced and chronic hypoxia-induced PH models of rats in this study and then compared the haemodynamic and pathomorphologic results of three different doses of fasudil (10 mg/kg, 30 mg/kg, and 75 mg/kg per day) with those of bosentan (30 mg/kg per day, a endothelin receptor blocker, the most commonly used therapy in PH. The correlation between the antioxidant effects of fasudil against PH and the modulated expression levels of Trx1 and HIF-1α were also investigated.

## 2. Materials and methods

### 2.1. Animals and reagents

Adult male Sprague-Dawley rats (7–8 weeks old, and 180–200 g weight) were obtained from the animal centre (Fourth Military Medical University, Xi'an, and PR China). The rats were housed in a temperature-controlled environment with 12-h light–dark cycles. All experiments were approved by the Animal Care and Use Committee at the Fourth Military Medical University and were in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Fasudil hydrochloride was obtained from Chase Sun Pharmaceutical Co., Ltd. (Tianjin, China), and bosentan was obtained from Actelion Pharmaceuticals Co., Ltd. (Allschwil, Switzerland). Monocrotaline (MCT) was obtained from Sigma Sigma–Aldrich Inc. (St. Louis, MO, USA). Anti-HIF-1α polyclonal and β-actin monoclonal antibodies were obtained from Millipore (Millipore, Bedford, MA, USA). Anti-Trx1 monoclonal antibody was purchased from Epitomics Biotechnology (Burlingame, CA, USA). The kits to determine superoxide dismutase (SOD), hydrogen peroxide ($H_2O_2$), malonyldialdehyde (MDA), and hydroxy radical (•OH) concentrations were obtained from Jiancheng Bioengineering Institute (Nanjing, China). All other reagents were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA).

### 2.2. Duplicate mimetic monocrotaline (MCT)-induced PH rat model

As shown in Fig. 1, a single injection of MCT (60 mg/kg in 0.5 ml saline, subcutaneous) induced severe PH in rats after 3 weeks (Cowan et al., 2000). When severe PH had already been established 21 days after MCT injection, the MCT-treated rats randomly were randomly intragastrically administered bosentan (30 mg/kg per day) and three different doses of fasudil (10 mg/kg, 30 mg/kg, and 75 mg/kg per day) for an additional 2 weeks. Untreated healthy rats served as a control for MCT-induced PH rats.

### 2.3. Duplicate mimetic chronic hypoxia-induced PH rat model

As shown in Fig. 1, the rats were intermittently housed in a hypobaric hypoxia chamber depressurised to 380 mmHg (correspondingly, the $PO_2$ was reduced to approximately 79.6 mmHg) for 8 h. After 8 h of exposure to hypoxia, the rats were placed in room air (21% oxygen) again (Ostadal et al., 1981; Thomas and Wanstall, 2003). The above steps were repeated every day and continued for 42 days. After 28 days of exposure to hypoxia, the chronically hypoxia-treated rats were randomly intragastrically administered bosentan (30 mg/kg per day) and three different