Oxymatrine protects against the effects of cardiopulmonary resuscitation via modulation of the TGF-β1/Smad3 signaling pathway

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Abstract. Previous studies have demonstrated that oxymatrine may inhibit ventricular remodeling and serves an important role in the treatment of cardiovascular disease. The present study investigated whether oxymatrine treatment protects against the effects of cardiopulmonary resuscitation (CPR) via regulation of the transforming growth factor-β1 (TGF-β1)/mothers against decapentaplegic (Smad) signaling pathway. A CPR model was established in Sprague-Dawley (SD) rats by asphyxiation, and rats were subsequently anaesthetized by intraperitoneal injection of chloral hydrate. SD rats were then administered 25 or 50 mg/kg oxymatrine once a day for 4 weeks. Oxymatrine treatment significantly improved troponin I levels, the ejection fraction, hydroxyproline content and the myocardial performance index in model rats. However, treatment with oxymatrine significantly reduced arterial oxygen tension, arterial lactate levels and oxygen extraction. Treatment with oxymatrine following CPR significantly inhibited the protein expression levels of TGF-β1, TGF-β1 receptor type 1 and Smad homolog 3 (Smad3) in model rats. The results of this research indicated that oxymatrine treatment may protect against the effects of CPR via regulation of the TGF-β1/Smad3 signaling pathway and may be a novel drug for CPR in a clinical setting.

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Introduction

Complete global brain ischemia and reperfusion injury triggered by cardiac arrest (CA) are fundamental causes of the reduced survival rates for patients with anabiosis following CA (1). As brain tissues have a low tolerance to hypoxic ischemia and its adverse reactions to ischemia reperfusion, cerebral protection following cardiopulmonary resuscitation (CPR) is difficult to treat (2). Complex mechanisms are responsible for the cerebral lesions caused by CA/CPR, including free radical formation, calcium overload, integrated enzyme reactions and the activation of death signaling pathways (3). This subsequently leads to apoptosis and necrosis of neurons. Previous clinical research and trials have demonstrated that therapeutic hypothermia greatly increases defibrillation success rates, ameliorates neural function and survival, and improves overall prognosis (4). However, the optimal opportunity and temperature range in which to conduct this therapy is difficult to obtain; it is generally understood that therapeutic hypothermia should be induced rapidly and at a low temperature during CPR following sudden cardiac arrest (5).

A previous study have indicated that the expression levels of transforming growth factor-β1 (TGF-β1) are increased following cerebral ischemia, suggesting that TGF-β1 is associated with the restoration and survival of neurons (6). However, certain exogenous intravenous and local intracerebral injections of TGF-β1 may facilitate the development of neurological impairment syndromes following cerebral ischemia, and reduce infarction volumes and encephaledema. It is understood that TGF-β1 is a multi-functional cytokine that is involved in various pathological and physiological procedures, stimulates secretion of the extra-cellular matrix and promotes angiogenesis (7). Furthermore, it exhibits anti-inflammatory, chemotaxis, movement-associated and anti-proliferative properties, and serves an essential role in repairing injured vessels (8).

A previous study indicated that, following activation of the TGF-β1 receptor, signal transduction is primarily mediated by phosphorylation of plasmosin; a member of the mothers against decapentaplegic (Smad) protein family (9). Once

combined with Smad4, activated and phosphorylated Smad2 and 3 proteins facilitate the transport of TGF-β1 to the cell nucleus, thus promoting its biological effects (9).

As an alkaloid extracted from the kuh-seng compound, oxymatrine demonstrates anti-inflammatory and anti-oxidative stress properties, and has become an essential drug for the treatment of tumors and hepatic fibrosis (10,11). Studies involving diabetic rats have demonstrated that oxymatrine improves cognitive impairment, reduces oxidative stress levels and increases the expression levels of antioxidative factors, such as superoxide dismutase (SOD) and glutathione (11). In addition, oxymatrine may significantly reduce the expression levels of nuclear factor (NF)-κB, tumor necrosis factor-α, interleukin (IL)-1β and caspase-3 (12). Following administration of oxymatrine, the expression levels of melanoma differentiation-associated protein, alanine transaminase, arginine succinyltransferase, thyroglobulin, IL-6 and NF-κB were markedly decreased, while the expression levels of SOD were increased (13). The aim of the present study was to examine the protective effects of oxymatrine against CPR, as well as the potential underlying mechanisms of oxymatrine in cardiac fibrosis.

Materials and methods

Animal experiments. Animal experiments were approved by the University Laboratory Animal Research Committee of The First Hospital of Jilin University (Changchun, China). A total of 32 adult Sprague-Dawley (SD) rats (weight, 250±30 g) were purchased from the Qinghai Experimental Animal Center (Zhejiang, China) and maintained in a specific pathogen-free environment (22-24°C, 55-60% humidity, 12 h light/dark cycle and free access to food and water). Rats were anaesthetized by intraperitoneal injection of chloral hydrate (300 mg/kg, intraperitoneal, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and a CPR model was established by asphyxiation. To do this, the tracheal intubation was closed at the end of expiration for 5 min. SD rats were randomly divided into sham-operated (n=6), model (n=10), 25 mg/kg oxymatrine-treated (n=8) and 50 mg/kg oxymatrine-treated (n=8) groups. In the 25 and 50 mg/kg oxymatrine groups, SD rats were administered with 25 and 50 mg/kg oxymatrine (Intragastrically) once a day for 4 weeks, respectively. In sham-operated and model groups, rats were anaesthetized by intraperitoneal injection of chloral hydrate without the CPR model, and administered with normal saline once a day for 4 weeks (Intragastrically). Oxymatrine (purity, >95%) was purchased from Shanghai Leiyunshang Green Valley Pharmaceutical Co., Ltd. (Xi'an, China). Its structural formula is presented in Fig. 1.

Enzyme-linked immunosorbent assay (ELISA) analysis of troponin I concentration and ejection fraction. After treatment with oxymatrine, rat was anaesthetized and peripheral blood was acquired from caudal vein. Serum was separated after centrifugation at 1,000 x g for 10 min at 4°C to measure troponin I concentrations using an ELISA kit (cat. no. 2010-2-HSP; Life Diagnostics, Inc., West Chester, PA, USA) according to the manufacturer's protocol. The absorbance value was detected at a wavelength of 450 nm.
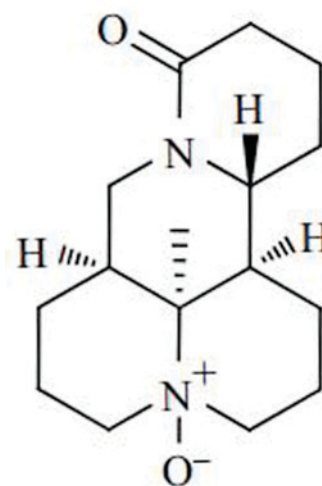

Figure 1. The chemical structure of oxymatrine.

The percentage ejection fraction (EF) was calculated using the same area-length method described previously (14). Lactate levels (cat no. M002; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were determined from 0.3-ml samples at baseline. A partial pressure of oxygen (PaO_2) value of <5 mmHg was considered to indicate hypoxia, and a PaO_2 value of between 100 and 300 mmHg was considered to indicate mild hyperoxia. The myocardial performance index (MPI) was calculated as (a-b)/b, where a=mitral valve closure-to-opening interval and b=left ventricular ejection time. Oxygen extraction was calculated as the difference between the arterial (Ca) and venous concentrations (Cv) of oxygen (CaO_2-CvO_2). Therefore, CaO_2 = 1.38 x hemoglobin (HB) x arterial oxygen saturation + PaO_2 x 0.0031; CvO_2 = 1.38 x HB x mixed venous oxygen saturation + mixed venous oxygen tension (PvO_2) x 0.0031.

Hydroxyproline content assessment. The hydroxyproline content of heart tissues was assessed following a previously described method (13) using ELISA kits (cat no. A030-2; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. The absorbance value was detected at a wavelength of 450 nm.

Western blot analysis. Heart tissue samples were homogenized using 10 μg/ml radioimmunoprecipitation buffer and protease inhibitors (EMD Millipore, Billerica, MA, USA). The supernatant was collected and total protein was quantified using a bicinchoninic acid assay (Beyotime Institute of Biotechnology, Shanghai, China). Total protein (50 μg) was separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. Following blocking with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20, membranes were incubated with the following primary antibodies at 4°C overnight: Anti-TGF-β1 (cat no. sc-7892, dilution, 1:4,000; Santa Cruz Biotechnology, Inc), anti-TGF-β receptor type 1 (TβR1; cat no. 3712, dilution, 1:3,000; Cell Signaling Technology, Inc.), anti-Smad3 (cat no. sc-8332, dilution, 1:4,000; Santa Cruz Biotechnology, Inc) and anti-β-actin (cat no. sc-7210, dilution, 1:2,000; Santa Cruz Biotechnology, Inc). This was followed by incubation with secondary antibodies (dilution, 1:5,000, cat
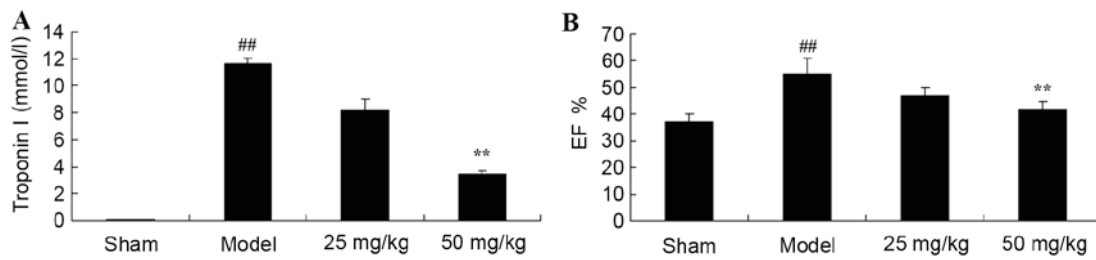
Figure 2. Oxymatrine treatment protects against elevated troponin I levels and increased EF in a rat model of CPR. (A) Troponin I levels as determined by enzyme-linked immunosorbant assay analysis, and (B) EF. Data are expressed as the mean ± standard error. ##P<0.01 vs. sham; **P<0.01 vs. model. Sham, sham-operated group; model, CPR model group; 25, 25 mg/kg oxymatrine-treated group; 50, 50 mg/kg oxymatrine-treated group; EF, ejection fraction; CPR, cardiopulmonary resuscitation.

no. sc-2030; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Densitometry was detected using Beyo enhanced chemiluminescence Plus (Beyotime Institute of Biotechnology) and performed using ImageJ software version 1.47 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are expressed as the mean ± standard error and SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA) was used. Student's t-test were used to analyze the differences between groups. P<0.05 was considered to indicate a statistically significance difference.

Results

Oxymatrine treatment reduces troponin I levels and EF in a rat model of CPR. The present study investigated whether oxymatrine may protect against an increase in troponin I levels and EF in a rat model of CPR. The levels of troponin I and the percentage EF of rats in the model group were significantly increased when compared with the sham-operated group (Fig. 2). However, treatment with 50 mg/kg oxymatrine significantly reduced troponin I levels and the EF percentage when compared with the model group (Fig. 2).

Oxymatrine treatment protects against increases in heart hydroxyproline content in a rat model of CPR. At 4 weeks following induction of the rat CPR model, hydroxyproline content was significantly increased in the model group when compared with the sham group (Fig. 3). Following administration of 50 mg/kg oxymatrine, hydroxyproline content was significantly ameliorated when compared with the model group (Fig. 3).

Oxymatrine treatment protects against increased PaO2 levels and decreased lactate levels in a rat model of CPR. Following induction of the CPR model, PaO2 levels were significantly decreased, while lactate levels were significantly decreased in the CPR model group when compared with the sham group (Fig. 4). Following treatment with 50 mg/kg oxymatrine, PaO2 levels decreased and lactate levels increased when compared with the model group (Fig. 4).

Oxymatrine treatment protects against increased oxygen extraction in a rat model of CPR. In order to evaluate whether oxymatrine protects against increased oxygen extraction in a rat model of CPR, the level of oxygen extraction was compared among all experimental groups. A significant increase in oxygen
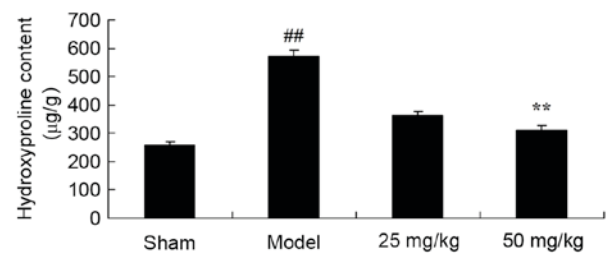

Figure 3. Oxymatrine treatment protects against increases in heart hydroxyproline content in a rat model of CPR. Data are expressed as the mean ± standard error. ##P<0.01 vs. sham; **P<0.01 vs. model. Sham, sham-operated group; model, CPR model group; 25, 25 mg/kg oxymatrine-treated group; 50, 50 mg/kg oxymatrine-treated group; CPR, cardiopulmonary resuscitation.

extraction was observed in the model group when compared with the sham group (Fig. 5). By contrast, 50 mg/kg oxymatrine administration significantly reduced oxygen extraction when compared with the model group (Fig. 5).

Oxymatrine treatment protects against increased MPI in a rat model of CPR. The present study investigated whether oxymatrine may protect against increases in MPI in the rat model of CPR. The MPI of model rats was significantly increased when compared with the sham group (Fig. 6). By contrast, continuous administration of 50 mg/kg oxymatrine for 4 weeks, was associated with a significant reduction in MPI when compared with the model group (Fig. 6).

Oxymatrine treatment reduces the protein expression levels of TGF-β1 in a rat model of CPR. A total of 4 weeks following CPR model induction, TGF-β1 protein expression levels were increased significantly in the model group when compared with the sham group (Fig. 7). By contrast, following 4 weeks of treatment with 50 mg/kg oxymatrine, TGF-β1 protein expression levels were significantly suppressed when compared with the model group (Fig. 7).

Oxymatrine treatment reduces the protein expression levels of TβR1 in a rat model of CPR. Representative western blot images are presented in Fig. 8A. Compared with the sham group, CPR significantly induced TβR1 protein expression levels (Fig. 8). By contrast, treatment with 50 mg/kg oxymatrine significantly reduced TβR1 protein expression levels when compared with the model group (Fig. 8).
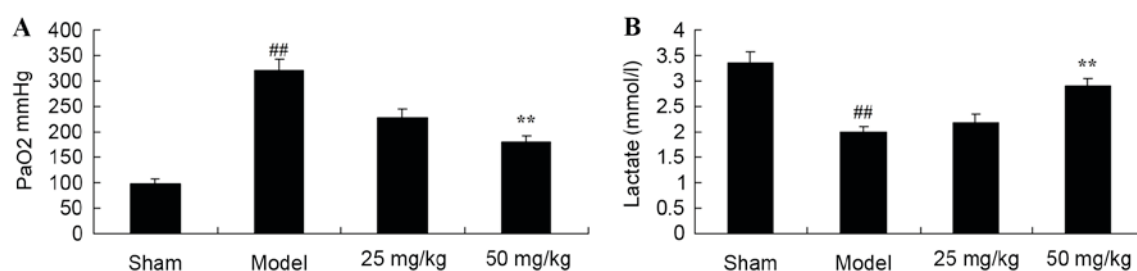
Figure 4. Oxymatrine treatment protects against increases in (A) PaO_2 levels and decreases in (B) lactate levels in a rat model of CPR. Data are expressed as the mean ± standard error. [##]P<0.01 vs. sham; [**]P<0.01 vs. model. Sham, sham-operated group; model, CPR model group; 25, 25 mg/kg oxymatrine-treated group; 50, 50 mg/kg oxymatrine-treated group; CPR, cardiopulmonary resuscitation; PaO_2, partial pressure of oxygen.
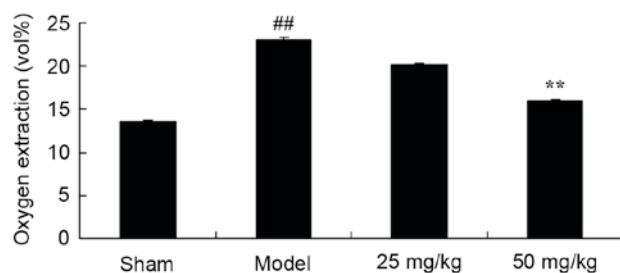

Figure 5. Oxymatrine treatment protects against increased oxygen extraction in a rat model of CPR. Data are expressed as the mean ± standard error. [##]P<0.01 vs. sham; [**]P<0.01 vs. model. Sham, sham-operated group; model, CPR model group; 25, 25 mg/kg oxymatrine-treated group; 50, 50 mg/kg oxymatrine-treated group; CPR, cardiopulmonary resuscitation.

Figure 6. Oxymatrine treatment reduces the MPI in a rat model of CPR. Data are expressed as the mean ± standard error. [##]P<0.01 vs. sham; [**]P<0.01 vs. model. Sham, sham-operated group; model, CPR model group; 25, 25 mg/kg oxymatrine-treated group; 50, 50 mg/kg oxymatrine-treated group; CPR, cardiopulmonary resuscitation; MPI, myocardial performance index.
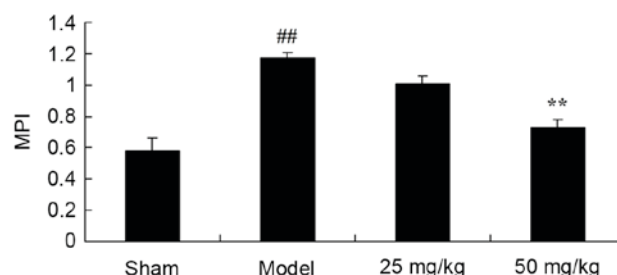
Oxymatrine treatment reduces the protein expression levels of Smad3 in a rat model of CPR. Representative western blot images are presented in Fig. 9A. Compared with the sham group, CPR significantly induced Smad3 protein expression levels (Fig. 9). However, treatment with 50 mg/kg oxymatrine significantly reduced Smad3 protein levels when compared with the model group (Fig. 9).

Discussion

CA is a primary cause of mortality and severe disability worldwide. Recovery of cerebral function is a complex issue during CPR (15). Reperfusion following cerebral ischemia may trigger serious cerebral anoxia, resulting in dysneuria (5). The present study demonstrated that oxymatrine significantly inhibited troponin I levels, EF and hydroxyproline content in a rat model of CPR.

CPR may lead to severe hypoxic-ischemic states. Once circulation has halted for 4-6 min, irreversible nerve injury may occur (16). Even if resuscitation is successful, numerous oxygen radicals may induce oxidative stress, thus provoking ischemia reperfusion injuries (16). A previous study revealed that oxidative stress injury is a key underlying mechanism of cerebral injury following CPR, and oxygen extraction assessment demonstrates the oxygen-intake capability of brain tissues and is a key indicator of hypoxic-ischemic encephalopathy (17). The present study demonstrated that oxymatrine significantly reduced PaO_2, increased lactate levels, decreased oxygen extraction and reduced the MPI of CPR model rats. Shen *et al* (13) indicated that oxymatrine protects against myocardial fibrosis via the TGF-β1-Smad signaling pathway.

A previous study indicated that expression levels of Smad3 increase during the early stages of cerebral ischemia (18). Smad3 is involved in a series of pathophysiological alterations, including the increase of cerebral microvessel permeability, aseptic inflammation, blood-brain barrier damage and encephaledema (19). Synthetic Smad3 inhibitors may inhibit these alterations to some extent (20). The present study demonstrated that oxymatrine significantly reduced Smad3 protein levels when compared with the model group.

The TGF subfamily includes TGF-β1, activin and bone morphogenetic protein, which possess various functions associated with cell growth and differentiation (6). TGF-β1 is a multi-functional cytokine (6). Almost all cell types, including epithelial, endothelial and nerve cells, as well as connective tissues, may interact with TGF-β1 and express the corresponding receptors (21). TGF-β1 is involved in cell proliferation and differentiation, embryonic development, extracellular matrix synthesis and angiogenesis (8). It serves an essential role in pathological processes involved in fibrosis, DNA damage repair and tumor growth. TGF-β1 possesses neuroprotective functions in various cerebral injuries, including hypoxia-ischemia, glutamate excitotoxicity, amyloid-β protein accumulation, oxidative stress and human immunodeficiency virus infection (22). Accordingly, the results of the present study indicated that oxymatrine significantly suppressed Smad3 protein expression levels in a rat model of CPR. Fan *et al* (23) reported that oxymatrine inhibits collagen synthesis via Smad3-mediated induction of the TGF-β1 signaling pathway.

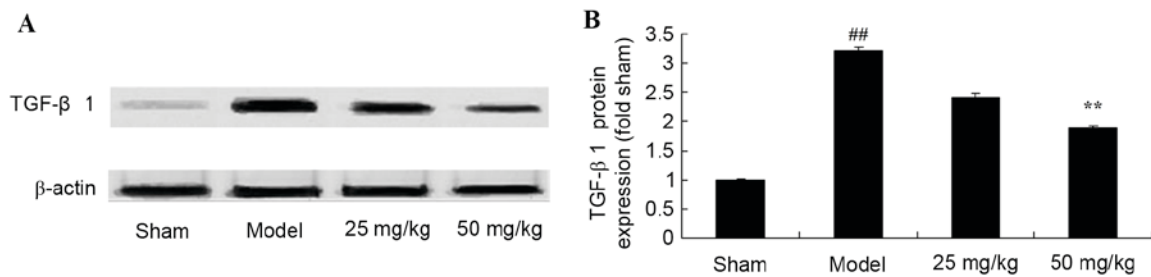The TGF-β1/TβR1 signaling pathway is pleiotropic and serves fundamental roles in tissue repair, the accumulation

Figure 7. Oxymatrine treatment reduces the protein expression levels of TGF-β1 in a rat model of CPR. (A) Representative western blot images and (B) quantification of TGF-β1 protein expression levels. Values are normalized against the sham group. Data are expressed as the mean ± standard error. ##P<0.01 vs. sham; **P<0.01 vs. model. Sham, sham-operated group; model, CPR model group; 25, 25 mg/kg oxymatrine-treated group; 50, 50 mg/kg oxymatrine-treated group; CPR, cardiopulmonary resuscitation; TGF-β1, transforming growth factor-β1.
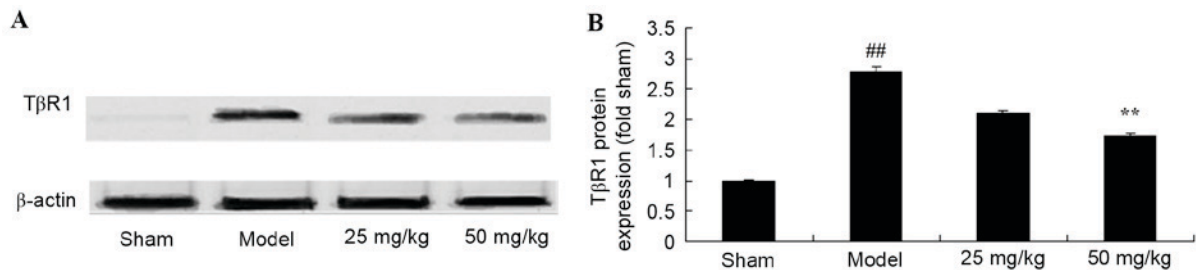

Figure 8. Oxymatrine treatment reduces the protein expression levels of TβR1 in a rat model of CPR. (A) Representative western blot images and (B) quantification of TβR1 protein expression levels. Values are normalized against the sham group. Data are expressed as the mean ± standard error. ##P<0.01 vs. sham; **P<0.01 vs. model. Sham, sham-operated group; model, CPR model group; 25, 25 mg/kg oxymatrine-treated group; 50, 50 mg/kg oxymatrine-treated group; CPR, cardiopulmonary resuscitation; TβR1, transforming growth factor β-receptor type 1.
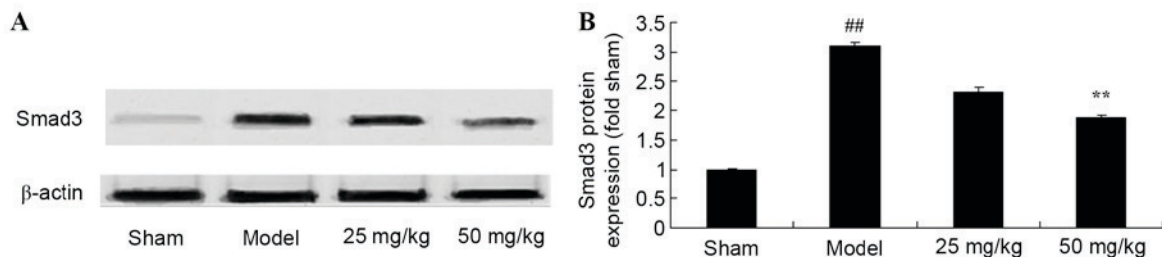

Figure 9. Oxymatrine treatment reduces the protein expression levels of Smad3 in a rat model of CPR. (A) Representative western blot images and (B) quantification of Smad3 protein expression levels. Values are normalized against the sham group. Data are expressed as the mean ± standard error. ##P<0.01 vs. sham; **P<0.01 vs. model. Sham, sham-operated group; model, CPR model group; 25, 25 mg/kg oxymatrine-treated group; 50, 50 mg/kg oxymatrine-treated group; CPR, cardiopulmonary resuscitation; Smad3, mothers against decapentaplegic homolog 3.

of the extracellular matrix, excessive fibrosis and the proliferation and metastasis of tumors (24). TGF-β1 transduces signals via the transmembrane receptor complex together with serine and threonine enzymes (25). As a signaling molecule in cytoplasm, TβR1 directly transduces TGF-β1 signals into the cell nucleus via the cytomembrane. A previous study suggested that TGF-β1/TβR1 may be closely associated with the regulation of inflammation, and activation of the TGF-β1/Smad3 signaling pathway may be pro- or anti-inflammatory (26). In the present study, oxymatrine significantly suppressed TGF-β1/TβR1 protein expression levels in a rat model of CPR. Guo et al (27) indicated that oxymatrine protects against diabetic nephropathy via TGF-β1, and Fan et al (23) reported that oxymatrine inhibits collagen synthesis via the Smad3-induced TGF-β1 signaling pathway.

In conclusion, the results of the present study demonstrated that oxymatrine markedly reduced troponin I levels, EF, hydroxyproline content, PaO$_2$, oxygen extraction and MPI, and increased lactate levels in a rat model of CPR, which may be associated with suppression of reduction the TGF-β1/TβR1/Smad3 signaling pathway. In further study, more in depth signaling pathway analysis and the underlying mechanism of oxymatrine will be explored. The results of this research suggested that oxymatrine treatment may protect against the effects of CPR via regulation of the TGF-β1/Smad3 signaling pathway and may be a novel drug for CPR in a clinical setting.

Acknowledgements

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