Original Paper

Oxymatrine Ameliorates Doxorubicin-Induced Cardiotoxicity in Rats

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Key Words

Oxymatrine • Doxorubicin • Cardiotoxicity • Apoptosis • Oxidative stress.

Abstract

Background/Aims: Doxorubicin-induced cardiac toxicity has been a major concern of oncologists and is considered the main restriction on its clinical application. Oxymatrine has shown potent anti-cancer, anti-fibrosis, and anti-oxidative effects. Recently, it has been reported that oxymatrine is protective against some cardiovascular diseases. In this study, we aimed to investigate the effects of oxymatrine on doxorubicin-induced cardiotoxicity in rat hearts and H9c2 cells. **Methods:** Creatine Kinase - MB (CK-MB) and Lactate Dehydrogenase (LDH) levels were determined using commercial kits. Biochemical indices reflecting oxidative stress, such as catalase (CAT), malonyldialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) were also analyzed with commercial kits. Mitochondrial reactive oxygen species (ROS) 2',7'-dichlorofluorescein diacetate (DCFH-DA) was measured by fluorescence microscopy. Histological analyses were conducted to observe morphological changes, and apoptosis was measured using a commercial kit. Western blots were used to detect the level of expression of cleaved caspase-3. **Results:** Doxorubicin treatment significantly increased oxidative stress levels, as indicated by catalase, malonyldialdehyde, superoxide dismutase, glutathione peroxidase and reactive oxygen species. Doxorubicin also increased pathological damage in myocardial tissue, myocardial ROS levels, and malonyldialdehyde levels, and induced apoptosis in myocardial tissues and H9c2 cells. All of these doxorubicin-induced effects were attenuated by oxymatrine. **Conclusion:** These *in vitro* and *in vivo* findings indicate that oxymatrine may be a promising cardioprotective agent against doxorubicin-induced cardiotoxicity, at least in part mediated through oxymatrine's inhibition of cardiac apoptosis and oxidative stress.

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Introduction

Doxorubicin is a potent chemotherapeutic agent that is highly effective in treating patients with various types of cancers, such as lung, liver, breast, ovarian and many solid

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tumors [1-4]. However, oncologists are most concerned with its cumulative and dose-dependent cardiotoxicity, which eventually leads to severe heart failure. Intensive study of doxorubicin-related cardiotoxicity has been ongoing [5, 6]. Evidence on the pathogenesis of doxorubicin-induced cardiotoxicity has included putative mechanisms, but its precise mechanism has not been entirely elucidated. A number of studies favor reactive oxygen species (ROS) as one of the main factors responsible for doxorubicin-induced cardiotoxicity [7]. In addition, the administration of antioxidants has been shown to protect cardiac tissue from doxorubicin-induced oxidative stress injury.

Oxymatrine, a quinolizidine alkaloid derived from the traditional Chinese herb *Sophora flavescens* Aiton, exhibits a wide range of pharmacological activities. Its anti-oxidative activity has been useful in the treatment of liver fibrosis, viral hepatitis, skin diseases, and autoimmune disease [8]. Recently, oxymatrine has been extensively studied for its protective effects against heart injuries induced by isoproterenol [9], myocardial ischemia [10, 11], hypertension [12], aldosterone [13], septic shock [14], and arrhythmias [15]. However, the effects of oxymatrine on doxorubicin-induced cardiotoxicity have not yet been explored. Therefore, the present study investigates the potential benefit of oxymatrine in doxorubicin-induced cardiotoxicity in rats and H9c2 cells, and whether the underlying mechanism for any such protection involves its anti-oxidative activity.

Materials and Methods

Materials and antibodies

Doxorubicin and 3-(4, 5-dimethylthiazol-2-yl)-5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma (St. Louis, MO, USA). Antibody against cleaved caspase-3 was purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies against β -actin, anti-rabbit secondary antibody, and anti-mouse secondary antibody were purchased from Wuhan Boster Biotechnology Limited Company (Wuhan, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Carlsbad, CA, USA). LDH, CK-MB, and oxidative stress-related enzymes (CAT, MDA, SOD and GSH-Px) were purchased from NanJing JianCheng Bioengineering Institute. The Hoechst 33258 test kit and 2', 7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Beyotime Institute of Biotechnology. All other chemicals and reagents were purchased from local agencies.

Oxymatrine suspension

Oxymatrine with a purity of 98% was purchased from Aladdin Industrial Corporation (Shanghai, China). A stock suspension at 50 mg/ml concentration was prepared by dilution with distilled water and stored at 4°C.

Ethics statement

All animal experiments were performed according to institutional ethical committee guidelines and were approved by the Animal Ethics Committee of Guizhou Medical University. Experiments were in accordance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals." All efforts were made to minimize suffering.

Animals and pharmacological treatment in vivo

A total of 50 male Sprague-Dawley (SD) rats weighing 220-250 g and 25 weeks old were used in the experiments. The animals were housed in polypropylene cages and maintained in 12-h light/12-h dark cycle, 50% humidity and 25 ± 2°C. The animals had free access to a standard pellet diet (M/S. Pranav AgroIndustries Ltd., Bangalore, India) and water *ad libitum*.

Animals were randomly assigned to the following groups: control (saline), doxorubicin (3 mg/kg), and doxorubicin+oxymatrine (12.5, 25, and 50 mg/kg). The rats were intragastrically administered saline or oxymatrine for 15 days and then intraperitoneally injected with normal saline or a single dose of doxorubicin every other day for a total of three injections. Fourteen days after the first administration of doxorubicin, the rats were euthanized for morphological and cellular studies.

Determination of enzymatic indices of tissue cardiotoxicity

Tissue samples were assayed for cardiac enzymes (LDH, CK-MB) and oxidative stress related enzymes (CAT, MDA, SOD and GSH-Px) using kits with an automatic chemical analyzer (Hitachi, Tokyo, Japan) according to previously described methods [16-18].

Heart histopathological examination

The heart was isolated immediately after sacrificing the animal and washed with ice-cold normal saline, trimmed, fixed in 4% paraformaldehyde, and embedded in paraffin blocks. The heart apex was sectioned and stained with hematoxylin and eosin (H&E). The structure was then examined under a light microscope by a pathologist blinded to the groups under study.

Pathomorphological examination of myocardium

For transmission electron microscopic examination, samples of the myocardium were collected and cut into fragments of 1 mm diameter as previously described [19]. They were then fixed in 2.5% glutaraldehyde, post-fixed with 2% osmium tetroxide, dehydrated using a graded sequence of ethanol washes, passed through propylene oxide, and then embedded in diallylphthalate (PDAP). Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using a Zeiss Libra 120 transmission electron microscope (Zeiss AG, Oberkochen, Germany).

H9c2 cell culture

H9c2 cells derived from rat myocardium were obtained from American Type Culture Collection (ATCC, Rockville, MD). These cells were cultured in DMEM supplemented with 10% FBS, 100 µ/ml penicillin, and 100 µM/ml streptomycin, and were maintained in a humidified atmosphere of 5% CO₂ at 37°C. The medium was changed every 2 days. All assays contained appropriated controls, were performed in triplicate, and were repeated on three separately initiated cultures.

Determination of apoptosis by Hoechst 33258 staining

To detect apoptosis, Hoechst 33258 staining was performed as previously described [20]. After treatment with doxorubicin, the slices of cardiac tissue and H9c2 cells were placed in fixing solution for 5 min. They were then washed three times in PBS, incubated in Hoechst 33258 solution (0.5 ml) for 5 min, then washed three times in PBS again. For each treatment group, the number of cells undergoing apoptosis per unit area was calculated using fluorescence microscopy by randomly selecting 5 fields and counting the number of apoptotic cells.

Assessment of H9c2 cells viability by MTT Assay

Cell viability was determined using the MTT assay (Beyotime, Beijing, China). The cells were seeded at 1×10⁴ cells/well in 96-well plates. After treatment with control (saline), doxorubicin (1 µM), or doxorubicin+oxymatrine for 24h, 20 µl of 5 mg/ml MTT solution was added to each well, and incubated for 4 h. The supernatants were aspirated, and the formazan crystals in each well were dissolved in 150 µl of dimethyl sulfoxide. The absorbance was measured at 570 nm using a micro plate reader (Spectrafluor, TECAN, sunrise, Austria).

Measurement of Mitochondrial ROS

Measurement of mitochondrial ROS was performed as previously described [21, 22]. H9c2 cells seeded in a petri dish were loaded with 50 µM 2', 7'-dichlorofluorescein diacetate (DCFH-DA) in modified Krebs solution (135 mM NaCl, 5.9 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgCl₂, 11.5 mM glucose, 11.6 mM HEPES, pH 7.4) supplemented with 0.1% bovine serum albumin and 0.02% Pluronic F127 for 15 minutes in the dark at room temperature. After 1 hour, the cells were washed three times and incubated for an additional 45 minutes in fresh Krebs solution. ROS generation was recorded using fluorescence microscopy at excitation wavelength 488 nm and emission wavelength 525 nm.

Western Blot Analysis

H9c2 cells from each group were lysed on ice with a tissue or cell protein extraction reagent containing a 0.1mM dithiothreitol and proteinase inhibitor cocktail. The protein concentration was determined using a

BCA kit (Pierce Corporation, Rockford, USA). Equal amounts of protein fractions were separated with 12% SDS-PAGE and then transferred onto nitrocellulose membranes (Millipore Corporation, USA) in Tris-glycine buffer at 100V for 55min. The membranes were blocked with 5% (w/v) nonfat milk powder in Tris-buffer that contains 0.05% (v/v) Tween-20 (TBST) at room temperature for 2 h. After incubation overnight at 4°C with appropriate primary antibodies, the membrane was washed three times with TBST, incubated with secondary antibodies for 2 h at room temperature, and then washed again three times with TBST. Protein blots were developed using an enhanced chemiluminescence solution. The protein expression levels were visualized with Image Lab Software (Bio-Rad, USA).

Statistical Analysis

All assays were independently repeated at least three times. All data were expressed as mean $\pm$ SEM. Statistical comparisons between different groups were determined with a one-way ANOVA followed by Duncan's multiple range test using SPSS 18.0 Software (SAS, NC). Statistical significance was considered at $p < 0.05$.

Results

Effects of oxymatrine on doxorubicin-induced morphological and ultrastructural changes in rat myocardium

To microscopically investigate the protective effects of oxymatrine on doxorubicin-induced changes in rat myocardium, we used H&E staining and transmission electron microscopic examination as described above. Hearts from control rats showed normal architectures on H&E staining. Compared with controls, doxorubicin produced massive changes in rat myocardium, consisting of necrosis, intracellular edema, myofibrillar derangements and rupture, swollen and damaged mitochondria, and wavy degeneration of cardiac muscle fibers. Pre-treatment with oxymatrine prevented these types of injury (Fig. 1, HE). Transmission electron microscopy revealed that control hearts had an intact myocyte ultrastructure, without significant abnormalities in the extracellular space. Treatment with doxorubicin resulted in serious heterogeneous subcellular abnormalities of both cardiomyocytes and the extracellular space. Pre-treatment with oxymatrine attenuated the severity of several of these deleterious subcellular alterations (Fig. 2, EM).

Fig. 1. Effects of oxymatrine on doxorubicin-induced morphological and ultrastructural changes in rat myocardium. HE: HE staining. EM: Electron micrograph. Dox: doxorubicin, OMT: oxymatrine.

Fig. 2. Effects of oxymatrine on doxorubicin-induced cardiac apoptosis and enzyme alterations. A: Hoechst staining. B: Percentage of apoptotic cells. C, D: Expression of Lactic Dehydrogenase (LDH) and Creatine Kinase-MB (CK-MB) in heart. Data are represented as means $\pm$ SEM. # $p < 0.05$ vs the control group, * $p < 0.05$ vs the DOX group.

Fig. 3. Effects of oxymatrine on doxorubicin-induced oxidative stress in rat hearts. A, B, C, D: Expression of catalase (CAT), malonyldialdehyde (MDA), superoxide dismutase (SOD), and Glutathione peroxidase (GSH-Px) in hearts. Data are represented as the means $\pm$ SEM. # $p < 0.05$ vs the control group, * $p < 0.05$ vs the DOX group.

Fig. 4. Effects of oxymatrine on doxorubicin-induced H9c2 cell apoptosis and oxidative stress. A: cell viability test using MTS. B: Hoechst staining. C: Expression of cleaved caspase-3. D: Expression of LDH. E: ROS generation.

Effects of oxymatrine on doxorubicin-induced cardiac apoptosis and enzyme alterations

To quantify myocardial apoptosis, we examined slices of myocardial tissue with Hoechst 33258 staining. As shown in Figures 2A and 2B, compared with the control group, treatment with doxorubicin led to marked morphological changes typical of cells undergoing apoptosis, such as chromatin condensation, nuclear fragmentation, and apoptotic bodies. Hearts from those rats which were pre-treated with oxymatrine had significantly less evidence of apoptosis. These findings suggest that oxymatrine exerts its protective effect on doxorubicin-induced cardiotoxicity primarily by inhibiting the apoptotic pathway.

To assess membrane leakage and cellular damage, the integrity of plasma membranes is commonly measured by monitoring the activity of cytoplasmic enzymes such as LDH and CK-MB in serum or tissue. As shown in Figures 2C and 2D, compared with the control group, exposure to doxorubicin led to elevated levels of LDH and CK-MB in cardiac myocytes. Pre-treatment with oxymatrine significantly reduced the release of LDH and CK-MB in a dose-dependent manner. These results suggest that oxymatrine's prevention of doxorubicin-induced cardiotoxicity is partly mediated by the stabilization of cell membranes.

Effects of oxymatrine on doxorubicin-induced oxidative stress in rat hearts

CAT, MDA, SOD and GSH-Px are important markers of oxidative stress and are widely regarded as indicators of oxidative injury [23, 24]. We measured levels of these markers to test whether pre-treatment with oxymatrine improved doxorubicin-induced metabolic

derangements in rat myocardium. Compared with controls, in doxorubicin-treated rats, the activities of CAT, SOD and GSH-Px decreased, while MDA levels increased (Fig. 3). Compared to the doxorubicin-treated group, pre-treatment with oxymatrine led to increased cardiac CAT, SOD and GSH-Px activities, with a dose-dependent decrease in the levels of MDA. These results suggest that oxymatrine might prevent doxorubicin-induced oxidative stress.

Effects of oxymatrine on doxorubicin-induced apoptosis of H9c2 cells

To analyze the effects of oxymatrine on doxorubicin-induced cytotoxicity in H9c2 cells, cell viability, apoptosis, and LDH leakage were measured after exposure to doxorubicin (1 μ M) with oxymatrine used either concurrently (for measures of viability), or as a pre-treatment (to measure apoptosis and LDH leakage). As shown in Fig. 4A, three concentrations of oxymatrine (10, 50, and 100 μ g/mL) were used to treat the cells. The most potent treatment concentration was 50 μ g/mL; therefore, 50 μ g/mL was chosen for the remainder of the experiments on H9c2 cells. Doxorubicin-induced increases in both the number of Hoechst positive cells and the amount of LDH leakage was attenuated by oxymatrine as shown in Figures 4B and 4D.

Caspase-3 is one of the major contributors to the apoptotic pathway and is therefore a marker of apoptosis. To determine the effect of oxymatrine on apoptosis, levels of cleaved caspase-3 were measured using western blot analysis as shown in Fig. 4C. The levels of cleaved caspase-3 were elevated in H9c2 cells treated with doxorubicin for 24 h, but this increase was significantly attenuated by pretreatment with oxymatrine for 1h. These results suggest that pre-treatment with oxymatrine inhibits doxorubicin-induced apoptosis in H9c2 cells.

Effects of oxymatrine on doxorubicin-induced oxidative stress in H9c2 cells

Doxorubicin-induced ROS generation in the mitochondrial respiratory chain is thought to be one of the major contributors to H9c2 cell death [25]. To determine whether pretreatment with oxymatrine mitigated doxorubicin-induced oxidative stress, cellular ROS were measured by incubating the control, doxorubicin, or doxorubicin+oxymatrine groups of H9c2 cells with 10 μ M DCF fluorescence-AM. As shown in Fig. 4E, exposure to doxorubicin without oxymatrine significantly increased fluorescence, indicating doxorubicin-induced oxidative stress in H9c2 cells. Pretreatment with oxymatrine significantly attenuated the increase in intracellular concentrations of ROS compared with the doxorubicin-treated cells.

Discussion

In this study, we investigated the protective effects of oxymatrine on doxorubicin-induced cardiotoxicity, and the underlying mechanisms of this protection. We found that pre-treatment with oxymatrine attenuates doxorubicin-induced oxidative stress, decreases cardiac apoptosis, and tempers doxorubicin-induced cardiotoxicity. These results suggest that oxymatrine could be used as a potential therapeutic tool to help prevent doxorubicin-induced cardiotoxicity.

Doxorubicin-induced cardiac toxicity is a major concern of oncologists and is considered the main restriction on its clinical application. Recently, accumulating evidence has shown that oxymatrine may have protective effects on the heart. The role of oxymatrine in protection against heart failure has been confirmed in models of isoproterenol-induced and chronic heart failure [9, 26], in which oxymatrine ameliorates the hypertrophy and dysfunction of heart ventricles. Oxymatrine has also been shown to attenuate the myocardial injury induced by ischemia [10, 11] and ventricular arrhythmia [15]. In spontaneously hypertensive rats, oxymatrine prevents ventricular remodeling, myocardial hypertrophy, and cardiac fibrosis [12]. Oxymatrine also exhibits anti-inflammatory activity, which protects against myocardial injury in septic shock [14]. The protective effect of oxymatrine on aldosterone-induced myocardial injury has been shown to be similar to the effect of spironolactone, a competitive aldosterone inhibitor [13].

In the present study, administration of oxymatrine attenuated doxorubicin-induced changes in myocardial ultrastructure in rats in a dose-dependent fashion. It has previously been demonstrated that treatment with doxorubicin induces increased release of LDH and CK-MB from myocardial tissues [27-30]. These enzymes have proven valuable in the diagnosis of doxorubicin-induced myocardial cardiotoxicity [27, 28]. The present study found that the tissue levels of LDH and CK-MB were increased in doxorubicin-treated rats, and that pre-treatment with oxymatrine decreased these levels in a dose-dependent manner. These findings suggest that oxymatrine protects against doxorubicin-induced cardiotoxicity in rats.

Although the underlying mechanisms responsible for doxorubicin-induced cardiotoxicity have not yet been completely elucidated, accumulating evidence supports cardiac apoptosis as a primary mechanism [29]. In the present study, we confirmed an increase in apoptosis in doxorubicin-treated rats and H9c2 cells, and found that apoptosis was decreased by pre-treatment with oxymatrine. These results support the hypothesis that the protection given against doxorubicin-induced cardiotoxicity by pre-treatment with oxymatrine may involve inhibition of cardiac apoptosis.

The increased generation of reactive free radicals plays a vital role in doxorubicin-induced cardiotoxicity. Compared to other mammalian organs, the heart possesses a lower level of those enzymes which attenuate ROS toxicity and protect against oxidative stress, such as SOD, CAT and GSH-Px. This may be the reason why doxorubicin is most toxic to the heart [30]. After doxorubicin enters the cardiomyocytes, mitochondrial enzymes reduce it to the semiquinone form. This reaction generates high concentrations of ROS, such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) [31, 32]. In addition to producing damaging free radicals, doxorubicin also decreases endogenous antioxidant levels; these effects can lead to cardiomyocyte apoptosis [33]. Our findings confirm previous work that has shown that doxorubicin decreases the levels of SOD, CAT and GSH-Px and increases cardiac apoptosis in rat hearts.

Considering the important role of oxidative stress in doxorubicin-induced cardiotoxicity, administering antioxidants in combination with doxorubicin may attenuate its cardiotoxicity. Oxymatrine's antioxidant activity has been implicated in its neuroprotective effects against intracerebral hemorrhage, excitotoxicity, and diabetes-associated cognitive deficits; and its effects against melanoma, renal ischemia-reperfusion injury and pulmonary hypertension. In the present study, pre-treatment with oxymatrine decreased the levels of SOD, CAT and GSH-Px in the hearts of doxorubicin-treated rats in a dose-dependent manner, and attenuated the doxorubicin-induced ROS increase in H9c2 cells. These findings suggest that pre-treatment with oxymatrine protects rat hearts from doxorubicin-induced cardiotoxicity by decreasing oxidative stress.

In conclusion, oxymatrine protected rat hearts from doxorubicin-induced cardiotoxicity *in vivo* and *in vitro* by decreasing oxidative stress, thereby decreasing apoptosis and subsequent changes to myocardial architecture. Our results suggest that oxymatrine has clinical potential for the treatment of doxorubicin-induced cardiotoxicity. The mechanism behind its inhibition of oxidative stress requires further study. Future research is also needed to determine whether administration of oxymatrine in combination with, or prior to, treatment with doxorubicin interferes with doxorubicin's antitumor activity.

Acknowledgements

This study was supported by Grants for Scientific Research from the National Natural Science Foundation of China (No. 31500999) and Hunan Provincial Natural Science Foundation of China (No. 2017JJ2343 and No. 2017JJ3404), Guizhou Province (J2013050) and Grant for Doctoral Fund (201304), Guizhou Medical University.

Disclosure Statement

The authors declare no conflict of interest.

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