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Original Article

Protective Effect of Captopril against Doxorubicin-Induced Oxidative Stress in Isolated Rat Liver Mitochondria

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Abstract

Doxorubicin (DOX) is an anthracycline antibiotic that has been used for a long time in therapy of an array of human malignancies either alone or in combination with other cytotoxic agents. The dose-dependent cardiotoxicity of DOX significantly limits its anticancer efficacies. Oxidative stress caused by enhanced production of reactive oxygen species is an important contributor to DOX mitochondrial toxicity. In the present study, DOX produced a significant elevation in TBARS, which is an indicator of lipid peroxidation, and significantly inhibited the activity of superoxide dismutase in rat liver mitochondria. Mitochondrial GSH dramatically decreased while GSSG was increased upon treatment of mitochondria by DOX. Co-treatment with captopril significantly reduced the lipid peroxidation in mitochondria and prevented the inhibition of superoxide dismutase activity induced by DOX. Captopril also significantly increased the level of GSH in DOX-treated mitochondria. These results, therefore, suggest that captopril acts as an antioxidant and can protect the mitochondria against DOX-induced oxidative stress. This effect appears to be due to the sulfhydryl groups of captopril which may act as antioxidant or scavenger of reactive oxygen species.

Key words: Captopril; Doxorubicin; Oxidative stress; Rat liver mitochondria.
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1. Introduction

Doxorubicin (DOX) is a powerful anthracycline antibiotic widely used to treat many human neoplasms, including acute leukemias, lymphomas, stomach, breast and ovarian cancers, Kaposi's sarcoma, and bone tumors [1]. However, among the anticancer drugs, DOX is well-known for its cardiotoxicity, and long-term administration of DOX is associated with cumulative and irreversible cardiomyopathy [2].

Increased oxidative stress caused by enhanced production of reactive oxygen species (ROS) is an important contributor to vascular dysfunction in congestive heart failure (CHF), and endothelial dysfunction caused by increased vascular ROS production is an independent predictor of future cardiovascular

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events [3]. Different enzymatic sources have been proposed to stimulate vascular superoxide production in CHF, including xanthine oxidase and the mitochondria [4, 5]. Some studies reported that DOX would induce cardiac toxicity through free radical generation by the "redox-cycling" of the anthracycline molecule or by the formation of anthracycline-iron complexes [6]. Some of these include involvement of ROS whereas others are oxidative stress-independent. Other investigators suggested that oxidative alteration of mitochondrial calcium regulation may play a critical role in mediating the pathogenesis of DOX cardiomyopathy [7]. There are a number of reports that DOX and its aglycone metabolite interfere with cardiac mitochondrial calcium transport and diminish calcium-loading capacity both *in vitro* [8-11], and *in vivo* [12]. It is widely accepted that DOX-induced cardiac myopathy resides for the most part on oxidative stress and the production of free radicals [13, 14]. Thus the study of mitochondrial DOX toxicity, because of its role in generation of free radicals, is important.

A number of studies have shown that angiotensin converting enzyme inhibitors (ACEIs) can inhibit oxidative stress and fibrosis [15, 16]. These effects of ACEIs occur independently from their ability to reduce arterial blood pressure [17, 18]. Treatment with captopril has been shown to increase antioxidant enzymes and non-enzymatic antioxidant defenses in several mouse tissues [19]. Captopril could also limit superoxide generation, and could modulate reactive oxygen and nitrogen species generation [20]. Some *in vitro* studies indicated that, sulfhydryl containing compounds such as captopril can scavenge free radicals [21-23]. Therefore, in the present study, the inhibitory effect of captopril on the mitochondrial toxicity of DOX was studied in isolated rat liver mitochondria. The results suggest that captopril can protect mitochondria from oxidative stress-induced damage mostly by protecting sulfhydryl groups and preventing lipid peroxidation.

2. Materials and methods

2.1. Chemicals

Doxorubicin hydrochloride, 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT), captopril and other chemicals were purchased from the

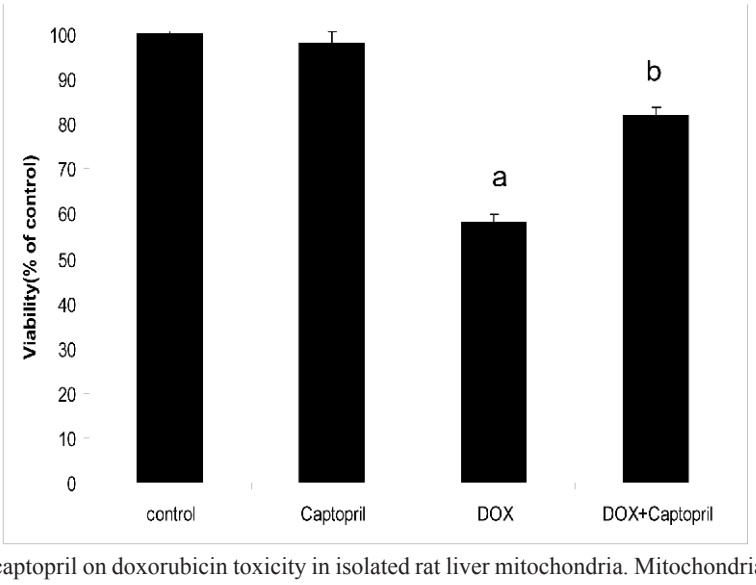

Figure 1. Effect of captopril on doxorubicin toxicity in isolated rat liver mitochondria. Mitochondria were treated with 50 μM DOX and 0.08 mM captopril and viability was assessed by MTT assay. Data are Mean±SEM of 6 different experiments. [a]Significantly different from control (p<0.001). [b]Significantly different from DOX-treated mitochondria (p<0.05).

Table 1. Effect of captopril on alleviation of GSH/GSSG content of mitochondria by DOX.

	Mean±SEM	
Group	**GSH (nmol/mg protein)**	**GSSG (nmol/mg protein)**
Control	5.09±0.02	1.03±0.02
Captopril 80 µM	6.31±0.26	0.79±0.01
DOX 50 µM	2.57±0.11[a]	1.75±0.01[a]
DOX 50 µM+Captopril 80 µM	5.196±0.01[b]	1.044±0.021[b]

Note: GSH and GSSG were measured as explained under materials and methods.

All data are given as Mean±SEM of 6 different experiments. [a]significantly different from untreated group (p<0.001). [b]significantly different from doxorubicin-treated group (p<0.001).

Sigma Chemical Co. (Germany). Other chemicals were of highest grade available in the market.

2.2. Animals

Male Sprague-Dawley rats (200-250 g) were obtained form the Laboratory Animals Research Center of Shiraz University of Medical Sciences. The rats were maintained under controlled temperature, 12 hours light/12 hours dark conditions for one week before the start of the experiments. They were allowed to feed standard laboratory chaw and tap water *ad libitum*. The animals were treated according to the guideline of the Ethics Committee of Shiraz University of Medical Sciences.

2.3. Preparation of mitochondria

Rats were anaesthetized by injection of 60 mg/kg thiopental and the liver was removed and minced in a cold manitol solution containing 0.225 M D-manitol, 75 mM sucrose and 0.2 mM ethylenediaminetetraacetic acid (EDTA). Approximately 30 g of the minced liver was gently homogenized in a glass homogenizer with a Teflon pestle and then centrifuged at 700×g for 10 min. at 4 °C to remove nuclei, unbroken cells and other non-subcellular tissues. The supernatant was centrifuged at 7000×g for 20 min. The second supernatant was pooled as the crude microsomal fraction and the pale loose upper layer, which was rich in swollen or broken mitochondria and lysosomes was washed away. The dark packed lower layer (heavy mitochondrial fraction) was resuspended in the manitol solution and recentrifuged twice at 7000×g for 20 min. The heavy mitochondrial sediments were suspended in Tris solution containing 0.05 M Tris-HCl buffer (pH 7.4) 0.25 M sucrose, 20 mM KCl, 2.0 mM $MgCl_2$ and 1.0 mM Na_2HPO_4 at 4 °C before assay.

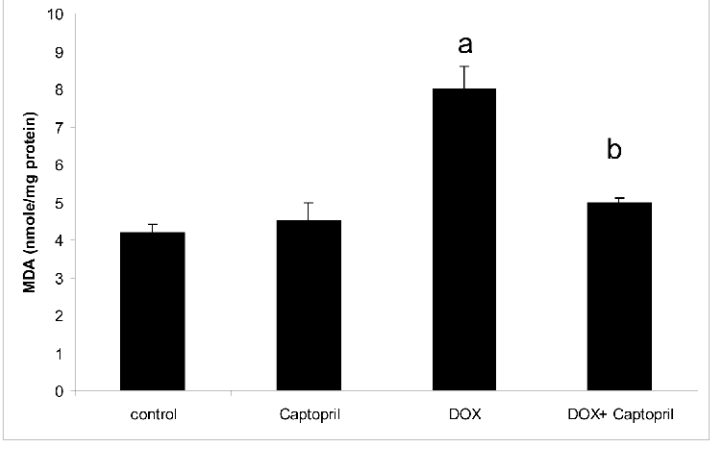

Figure 2. Effect of captopril on doxorubicin toxicity in isolated rat liver mitochondria. Mitochondria were treated with 50 µM DOX and 0.08 mM captopril and lipid peroxidation was assessed by measuring TBARS. All data are given as Mean±SEM of 6 different experiments. [a]Significantly different from control (p<0.001). [b]Significantly different from DOX-treated group (p<0.001).

2.4. Experimental design

In order to determine the LC_{50} of DOX, mitochondrial viability was investigated by 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay. Different concentrations of DOX (1 to 100 µM) were used and LC_{50} of DOX was determined.

In the rest of the study, a dose of 50 µM DOX with regards to LC_{50}, and a submaximal dose of captopril (0.08 mM) with regards to pervious studies were selected [21]. Mitochondrial suspensions were divided into 4 groups of six duplicate samples. Group 1 was considered as control, groups 2 to 4 were treated with captopril (0.08 mM), DOX (50 µM) and DOX (50 µM) plus captopril (0.08 mM), respectively. Mitochondrial suspensions were incubated for 1 h at 37 °C.

2.5. MTT assay

This assay is a quantitative colorimetric method for determination of cell viability [24]. In these experiments, MTT assay was modified for rat liver mitochondria suspension in tubes [22].

2.6. Lipid peroxidation

Lipid peroxidation was assessed by measurement of thiobarbituric acid reactive compounds (TBARS). The amount of reactive products formed was calculated by using an extention coefficient of 165 $mM^{-1}.cm^{-1}$ at 530 nm [25].

2.7. Determination of GSH and GSSG

The supernatant was analyzed for reduced glutathione (GSH) by the 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) recycling procedure [26]. GSSG (oxidized glutathione) plus GSH were determined in supernatant after mixing with 1 ml of 5% sodium borohydride ($NaBH_4$), a reducing agent, and the resulting sulfhydryl groups of GSH were assayed as described [27].

2.8. Superoxide dismutase activity

Superoxide dismutase (SOD) activity was determined using a Cayman Chemical SOD assay Kit. SOD catalyzes the dismutation of superoxide anion radical ($O_2^{\bullet-}$) to hydrogen peroxide and oxygen. In the presence of SOD, the superoxide radical $O_2^{\bullet-}$ undergoes a dismutation into O_2 and H_2O_2, which results in formazan formation. Hence, this competing assay yields to the indirect measurement of SOD activity.

2.9. Protein measurment

Mitochondrial protein concentrations were determined using the method developed by Bradford [28].

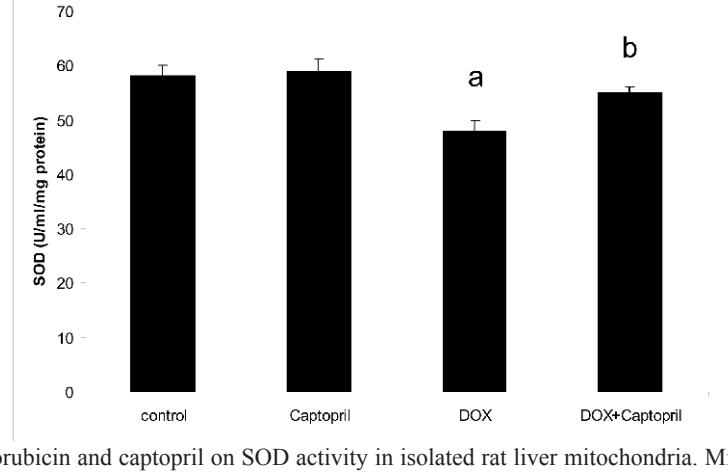

Figure 3. Effect of doxorubicin and captopril on SOD activity in isolated rat liver mitochondria. Mitochondria were treated with 50 µM DOX and 0.08 mM captopril and SOD activity was measured as explained under materials and method. All data are given as Mean±SEM of 6 different experiments. [a]Significantly different from control (p<0.01). [b]Significantly different from DOX-treated group (p<0.05).

2.10. Statistical analysis

All values were expressed as Mean±SEM of 6 experiments. Analysis of variance (ANOVA) followed by student Newmans-Keuls test was used to evaluate the significance of the results obtained. All analyses were performed using SPSS software.

3. Results

3.1. Effect of DOX on the viability of mitochondria

DOX was toxic towards freshly isolated rat mitochondria and incubation of mitochondria with DOX for 1 h resulted in loss of mitochondrial viability with an LC_{50} of about 50.93 (46.32–66.66) µM. Captopril with concentrations used did not affect viability of mitochondria (Figure 1). Simultaneous treatment of mitochondria with captopril (0.08 mM) plus DOX (50 µM) significantly prevented the mitochondrial toxicity induced by DOX (Figure 1).

3.2. Effect of DOX on lipid peroxidation in mitochondria

DOX induced lipid peroxidation in isolated mitochondria and the TBARs concentration was significantly greater in mitochondrial suspension treated with DOX compared to the controls. Captopril prevented lipid peroxidation induced by DOX to a great extent. Captopril alone did not affect lipid peroxidation in isolated mitochondria (Figure 2).

3.3. Effect of DOX on GSH and GSSG content of mitochondria

As shown in Table 1, GSH contents of DOX-treated mitochondria were about half of the levels of untreated controls after one h of incubation. Captopril treatment significantly prevented GSH depletion induced by DOX (Table 1), and restored GSH level to about control levels. GSSG levels of DOX-treated mitochondria were also significantly higher than those of controls, and captopril almost completely prevented increase of GSSG induced by DOX (Table 1). GSSG is expressed as GSH equivalents.

3.4. Effect of DOX on superoxide dismutase activity

DOX treatment induced a small but significant inhibition of superoxide dismutase (SOD) activity, and the administration of captopril provided protection against DOX-induced inhibition of SOD activity (Figure 3).

4. Discussion

Doxorubicin (DOX) is an anthracycline antibiotic that is used as a potent anticancer agent; however, it is exceptionally cytotoxic to the heart. The mechanism of DOX-induced toxicity is not fully understood. Toxicity is believed to occur by DOX-induced mitochondrial dysfunction and subsequent oxidant production [29]. DOX-induced oxidative stress in cardiac tissues as manifested by the alterations observed in cardiac antioxidant defense systems is both enzymatic and non-enzymatic. In this sense, the anthracycline drug reduced significantly the cardiac GSH content, besides it notably lowered the cardiac enzymatic activities of SOD, GST and catalase associated with a marked increase in cardiac lipid peroxidation as manifested by increased malondialdehyde levels [30].

In the present study, we found that DOX induced a significant increase in lipid peroxidation in the isolated rat liver mitochondria, as indicated from the rise of TBARS concentration 1 h after DOX treatment. This is in agreement with several reports *in vitro* in heart [31], liver [32] and the nervous system [33]. The present results also revealed a good amelioration of lipid peroxidation in mitochondria against DOX-induced rise in TBARS level by administration of captopril (Figure 2).

One of the most prevailing hypotheses of cardiac and hepatic damage resulting from

DOX administration is the ability of the drug to produce free radicals and reduce the antioxidant defense mechanism [34]. Free radicals are known to damage several macromolecular and cellular components [35].

H_2O_2 production *in vitro* increases immediately after DOX treatment [36]. Mitochondria themselves are very well equipped with antioxidant defense systems, such as MnSOD, GPX, GR and glutathione.

We found that the activity of SOD in liver mitochondria was inhibited significantly after 1 h of DOX treatment. This effect was prevented significantly by administration of captopril before DOX treatment (Figure 3). It has been reported that DOX decreases the Mn-SOD protein expression level in mitochondria. Mitochondrial Mn-SOD prevents cellular damage because it scavenges toxic superoxide radicals [30].

DOX treatment depleted mitochondrial GSH content and captopril prevented this GSH depletion (Table 1). A large amount of evidence suggests that multiple molecular mechanisms contribute to the GSH protective action. GSH inhibits lipid peroxidation by scavenging free radicals, thereby blocking the lipid chain reaction in a manner similar to vitamin E [37]. The marked increase in GSH maintains the membrane integrity and promotes the non-enzymatic detoxification of hydroxyl radicals [38]. GSH is considered the principal mitochondrial antioxidant and its depletion markedly enhances the sensitivity of the mitochondrial structure to the ROS-mediated injury [39]. Our data demonstrated that treatment with captopril prevented GSH depletion and GSSG formation induced by DOX. Since both the GSH and GSSG represent specific marker of oxidative stress [40], therefore, captopril may be able to protect the glutathione content of mitochondria and the cell by providing thiol groups.

Therefore, clinical experience and *in vitro* studies shows that DOX induces a cumulative and dose-dependent cardiomyopathy that has been attributed to redox-cycling of the drug and production of free radicals on the mitochondrial respiratory chain and induction of oxidative stress. Captopril co-administration may, therefore, provide a protective antioxidant effect on DOX-induced toxicity, and it might serve as a useful combination therapy to limit DOX-induced free radical mediated organ injury.

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