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Protective effects of telmisartan against acute doxorubicin-induced cardiotoxicity in rats

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Abstract:
The therapeutic usefulness of doxorubicin (DXR), an anthracycline antibiotic, is limited by its cardiotoxicity. The present study investigated the effects of telmisartan, an angiotensin II receptor (AT1) antagonist against doxorubicin-induced cardiotoxicity in rats using biochemical and histopathological approaches. Doxorubicin (20 mg/kg) was injected intraperitoneally (*ip*) as a single dose and telmisartan (10 mg/kg/day) was administered orally for 7 days. Rats treated with DXR showed cardiotoxicity as evidenced by elevation of serum lactate dehydrogenase (LDH) activity, tissue malondialdehyde (MDA) level, catalase activity and a decrease in the level of glutathione (GSH). Pre- and post-treatment with telmisartan elicited a significant decrease in the activities of LDH and catalase in comparison with DXR-treated group. Furthermore, pretreatment with telmisartan also decreased lipid peroxidation (MDA level) and increased the GSH content in comparison with DXR group. However, the difference in lipid peroxidation and GSH content were not statistically significant in post-treated group. Histopathological studies showed disruption of cardiac tissues in DXR groups. Pre- and post-treatment with telmisartan reduced the damage of cardiac tissue in rats. These results suggest that telmisartan treatment provides a significant protective effect against acute-doxorubicin induced cardiotoxicity in rats.

Key words:
doxorubicin, telmisartan, cardiotoxicity, angiotensin II, oxidative stress

Introduction

Doxorubicin (DXR) is a broad-spectrum anticancer drug [5, 8]. Despite its broad therapeutic effectiveness, the clinical use of DXR is limited by a dose-dependent and cumulative cardiotoxicity [19, 41]. The adverse effects can vary from transient electrocardiography abnormalities to cardiomyopathy and heart failure. The exact mechanism of DXR-induced cardiotoxicity remains unclear, but most studies support the view that oxidative stress plays an important role in the pathogenesis of DXR cardiotoxicity [22, 32, 35].

Cellular damage induced by doxorubicin is mediated by the formation of an iron-anthracycline complex that generates free radicals, which then cause severe damage to the plasma membrane and interfere with the cytoskeleton structure [4]. Due to the presence of less developed antioxidant defense mechanisms, heart is particularly vulnerable to injury by anthracycline-induced reactive oxygen species. Because liberation of free radicals is central to the mechanism of DXR-induced damage to the myocardium [34], considerable efforts have been made to use antioxidants and iron chelators to protect the heart against DXR-toxicity.

Several compounds with antioxidant properties have been investigated *in vitro* with some degree of success [13, 27]. However, the rate of success when performing *in vivo* studies has been less gratifying. Molecules with antioxidant characteristics *in vitro* such as selenium [45] or nimesulide [21] were found to be of limited value in counteracting DXR cardiotoxicity *in vivo*. Additionally, traditional antioxidants, like N-acetylcysteine and tocopherol are not very successful in the prevention of DXR-induced cardiotoxicity [25, 28]. Dexrazoxane, an iron chelator with potent antioxidant properties is the only drug approved by the US Food and Drug Administration (FDA) to prevent DXR-induced cardiotoxicity. However, it has been suggested that dexrazoxane decreases the effect of DXR on leukemia cells [37] and also due to high incidence of dexrazoxane-induced myelosuppression, its use has been limited to some advanced stages of malignant disorders [40].

Many lines of evidence have suggested that the renin-angiotensin system (RAS) plays an important role in the development of cardiac hypertrophy, failure and reperfusion injury [2]. Suppression of the RAS ameliorates the remodeling process of heart and prolongs long-term survival in animal models and humans with cardiac hypertrophy, failure and reperfusion injury [15, 18, 46]. Toko et al. [44] reported the non-toxic effect of doxorubicin on cardiac muscle of angiotensin II type1_a receptor (AT1) knockout mice, indicating that AT1 mediated angiotensin II (Ang II) signaling pathway plays an important role in the doxorubicin-induced cardiac impairment. Telmisartan is a non-peptide AT1 receptor antagonist which selectively and insurmountably inhibits AT1 receptor subtype [24] without affecting other systems involved in cardiovascular regulation [47]. The aim of the present study was to investigate the possible effects of telmisartan against DXR-induced cardiotoxicity in rats using biochemical markers of oxidative stress and cellular damage.

Materials and Methods

Animals

Laboratory bred Wistar albino rats of both sexes (10–12 weeks old) having body weight range of 180–250 g were used for this study. They were kept in the animal house (Faculty of Pharmacy, Jamia Hamdard, New Delhi, India) for one week for proper acclimatization before starting the experiment under controlled conditions of illumination (12 h light/12 h darkness) and temperature 20–25°C. They were housed under the above laboratory conditions, maintained on standard pellet diet (Lipton rat feed, Ltd; Pune) and water *ad libitum* throughout the experimental period. The study was approved by the Institutional Animal Ethics Committee of Jamia Hamdard, New Delhi, and both the international guidelines for the welfare of the animals and the compatible local regulations for experimenting with laboratory animals were fully respected during the study.

Drugs and chemicals

Doxorubicin and telmisartan were obtained as gift samples from Dabur Research Foundation (India) and Glenmark Pharmaceuticals Limited (India), respectively. Lactate dehydrogenase (LDH) assay kit was purchased from Span Diagnostics Ltd. All the other chemicals used were of analytical grade.

Experimental protocol

The rats were randomly assigned into six groups consisting of three animals of each sex per group (n = 6), at random to body weight variation and hereditary deviation.

Group A received physiological saline (5 ml/kg, *po*), daily for seven days and served as the *control*.

Group B received a single dose of DXR (20 mg/kg, *ip*) only, at 10 a.m. and was sacrificed after 48 h which served as *toxic control for pretreatment*.

Group C received telmisartan (10 mg/kg, *po*), daily for seven days, and on day five, two hours after drug administration, a single dose of DXR (20 mg/kg, *ip*) which served as *pretreatment*.

Group D received a single dose of DXR (20 mg/kg, *ip*) only, at 10 a.m. on day one, and was sacrificed on day seven which served as *toxic control for post-treatment.*

Group E received telmisartan (10 mg/kg, *po*), daily for seven days, and on day one, two hours after drug administration, a single dose of DXR (20 mg/kg, *ip*) which served as *post-treatment.*

Group F received telmisartan (10 mg/kg, *po*) only, daily for seven days.

On day seven two hours after drug treatment, the blood samples were collected and later the animals

were sacrificed and heart was removed, cleaned and washed in ice-cold physiological saline for biochemical studies.

Determination of lactate dehydrogenase (LDH) activity

LDH activity was estimated in serum by commercially available kit using an UV-visible spectrophotometer (Shimadzu).

Determination of lipid peroxide level

Lipid peroxide level was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA), a product formed due to the peroxidation of membrane lipids [31]. Heart tissues were homogenized in 0.15 M KCl using motor driven Teflon pestle followed by centrifugation of tissue homogenate (10% w/v) at 10,500 × g. Aliquot of 1 ml of the suspension medium was taken from the supernatant obtained after centrifugation and 0.5 ml of 30% trichloroacetic acid (TCA) followed by 0.5 ml of 0.8% TBA was added to it. The tubes were kept in shaking water bath for 30 min at 80°C. After 30 min of incubation, the tubes were taken out and kept in ice-cold water for 10 min. These were then centrifuged at 800 × g for 15 min. The absorbance of supernatant was read at 540 nm against an appropriate blank. The concentration of MDA was measured from the standard calibration curve prepared by using tetraethoxypropane. Protein was estimated by Lowry et al. [23] method. Lipid peroxidation was expressed as nmoles of MDA per milligram of protein.

Determination of glutathione (GSH) content

GSH content was estimated by Sedlac et al. [39] method. The heart tissues were homogenized in 0.02 M ethylenediaminetetraacetic acid (EDTA). Aliquots of 5 ml of the homogenates were mixed with 4 ml of cold distilled water and 1 ml of 50% TCA in test tubes. The tubes were shaken for 10 min using a vortex mixer and then centrifuged at 1,200 × g for 15 min. Following centrifugation 2 ml of the supernatant was mixed with 4 ml of 0.4 M tris buffer (pH 8.9). The whole solution was mixed and 0.1 ml of 0.01 M DTNB [5,5'-dithiobis (2-nitrobenzoic acid)] was added to it. The absorbance was read within 5 min of addition of DTNB at 412 nm using UV-spectrophotometer (Shimadzu, UV-1601, Japan) against a reagent blank with no homogenate.

Tris buffer, 0.4 M, pH 8.9, was prepared by dissolving 24.23 g Tris in 100 ml of redistilled water, adding 50 ml of 0.2 M EDTA and bringing the volume up to 1,000 ml with distilled water. The pH was adjusted to 8.9 with 6N HCL.

Determination of catalase (CAT) activity

Heart tissues were homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing potassium chloride (1.17%) using a Potter Elvehjem homogenizer. The homogenate was centrifuged at 800 × g for 5 min at 4°C in a refrigerated centrifuge to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 × g for 20 min at 4°C to get the post-mitochondrial supernatant (PMS) which was used to assay CAT activity. CAT activity was estimated using the method of Claiborne [10]. The reaction mixture consisted of 1.95 ml of phosphate buffer (0.1 M, pH 7.4), 1.0 ml of hydrogen peroxide (0.019 M) and 0.05 ml of PMS (10% w/v) in a final volume of 3 ml. Changes in absorbance were recorded at 240 nm. The enzyme activity was calculated as nmoles of H_2O_2 consumed/min/mg protein.

Histopathological studies

The left ventricular portion of heart tissues was fixed in 10% neutral buffered formalin. Sections of 3–5 μm thickness were stained with hematoxylin and eosin (H&E) for histological examination. A histomorphological evaluation of all the heart sections was carried out in a blinded fashion by a pathologist who was unaware of the treatment groups.

Statistical analysis

Data are expressed as the mean ± standard error (SE) of the means. For statistical analysis, distribution of the groups was analyzed by a one-sample Kolmogrov-Smirnov test. All groups showed normal distributions, so a parametric statistical method; one-way analysis of variance (ANOVA) was used to compare the means. The Tukey-Kramer *post-hoc* test was applied to identify significance among groups. The value of $p < 0.05$ was considered to be statistically significant. GraphPad software, Inc. (version 3.06) was used for statistical analysis.

Results

The results of both pre- and post-treatment schedule are summarized in Table 1 and Figure 1 and 2.

In the pretreatment schedule, an increase ($p < 0.001$) in the activity of serum LDH was observed in the DXR group as compared with the physiological saline group. Similar elevation ($p < 0.001$) was also evident in the tissue MDA levels and CAT activity in the DXR group compared to physiological saline group. Pretreatment with telmisartan significantly decreased the activities of LDH, CAT and the MDA levels compared to the DXR control ($p < 0.001$). The decrease in GSH levels following DXR treatment ($p < 0.001$) was prevented by pretreatment with telmisartan ($p < 0.05$).

In the post-treatment schedule, there was an increase ($p < 0.001$) in the activities of LDH and CAT in the DXR group compared to the physiological saline. Similar increase was also seen in MDA levels ($p < 0.05$). Post-treatment with telmisartan significantly reduced the activities of LDH ($p < 0.05$) and CAT ($p< 0.001$) compared to DXR control. Compared to physiological saline, there was a significant ($p < 0.001$) decrease in the content of tissue GSH in the DXR group. Post-treatment with telmisartan could not alter the MDA and GSH levels significantly compared to DXR control.

Tab. 1. Effect of telmisartan (10 mg/kg/day) on DXR-induced changes in MDA and GSH levels

	MDA (nmoles/mg protein)		GSH (nmoles/mg protein)	
	Pretreatment	Post-treatment	Pretreatment	Post-treatment
Control	0.149 ± 0.011		8.62 ± 0.32	
DXR	0.254 ± 0.010***	0.380 ± 0.019*	5.58 ± 0.38***	5.09 ± 0.47***
DXR + TEL	0.173 ± 0.002###	0.225 ± 0.081	7.45 ± 0.12#	4.88 ± 0.32
TEL	0.158 ± 0.007		8.64 ± 0.57	

DXR – doxorubicin, TEL – telmisartan. Values are expressed as the mean ± SEM (n = 6). One-way ANOVA with *post-hoc* analysis. Significant differences are indicated by *** $p < 0.001$, * $p < 0.05$ *vs.* control (group A), ### $p < 0.001$, # $p < 0.05$ *vs.* pretreatment DXR (group B)

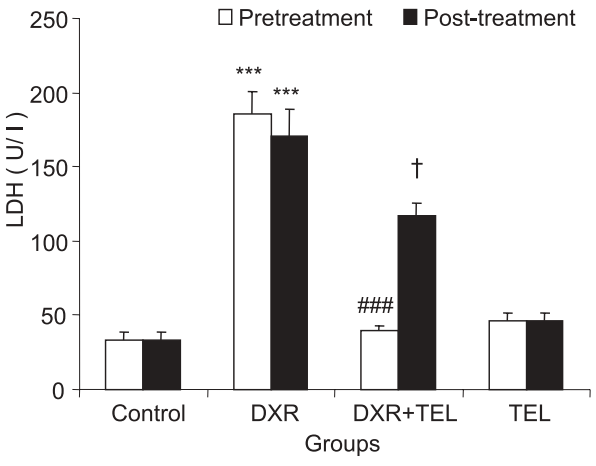

Fig. 1. Effects of telmisartan on lactate dehydrogenase (LDH) activity in DXR-treated rats. Rats were treated with DXR (20 mg/kg) and telmisartan (10 mg/kg) according to the protocol given in Materials and Methods section. DXR – doxorubicin, TEL – telmisartan. Values are expressed as the mean ± SEM (n = 6). One-way ANOVA with *post-hoc* analysis, *** $p < 0.001$ *vs.* control (group A); ### $p < 0.001$ *vs.* pretreatment DXR (group B); †$p < 0.05$ *vs.* post-treatment DXR (group D)

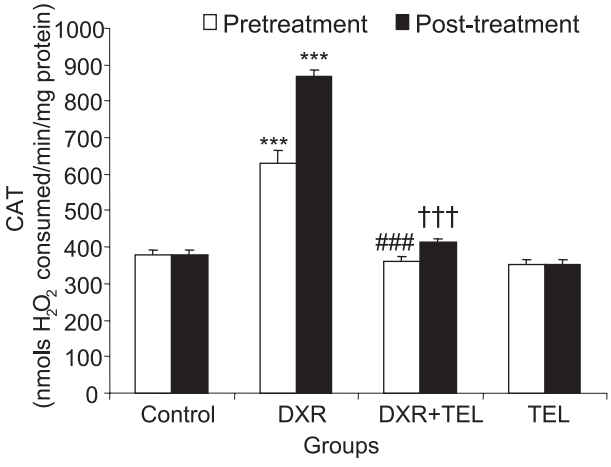

Fig. 2. Effects of telmisartan on catalase (CAT) activity in DXR-treated rats. Rats were treated with DXR (20 mg/kg) and telmisartan (10 mg/kg) according to the protocol given in Materials and Methods section. DXR – doxorubicin, TEL – telmisartan. Values are expressed as the mean ± SEM (n = 6). One-way ANOVA with *post-hoc* analysis, *** $p < 0.001$ *vs.* control (group A); ### $p < 0.001$ pretreatment DXR (group B); ††† $p < 0.05$ *vs.* post-treatment DXR (group D)

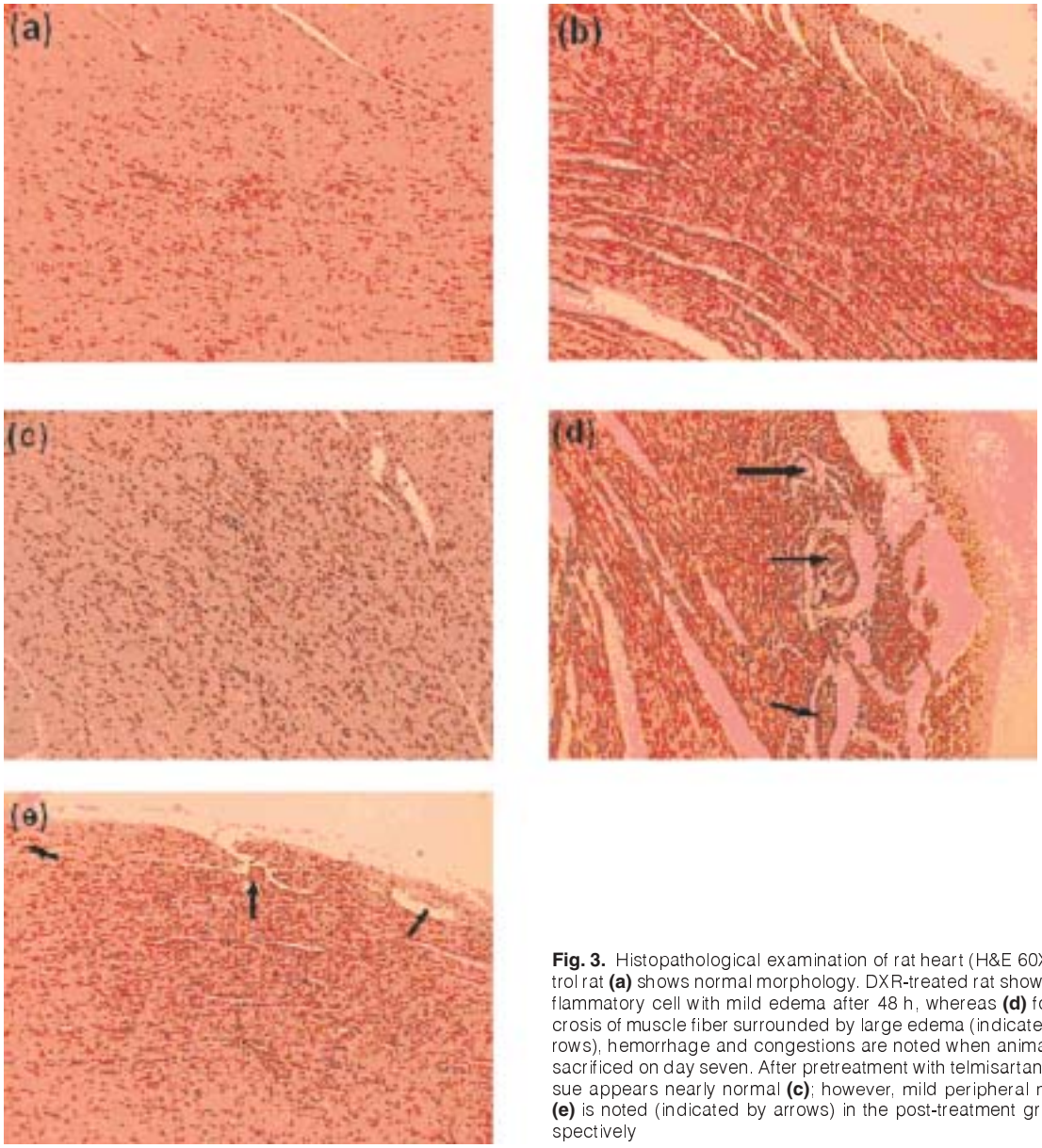

Fig. 3. Histopathological examination of rat heart (H&E 60X). Control rat **(a)** shows normal morphology. DXR-treated rat shows **(b)** inflammatory cell with mild edema after 48 h, whereas **(d)** focal necrosis of muscle fiber surrounded by large edema (indicated by arrows), hemorrhage and congestions are noted when animals were sacrificed on day seven. After pretreatment with telmisartan, the tissue appears nearly normal **(c)**; however, mild peripheral necrosis **(e)** is noted (indicated by arrows) in the post-treatment group, respectively

Histopathological studies

DXR-induced morphological changes in cardiac tissues were observed by light microscopy as shown in Figure 3. Normal morphology of the tissue was seen in the saline group. On the other hand, there were morphological changes in the DXR group. Cross section of cardiac tissue of the rat showed high numbers of inflammatory cells with mild edema when animals were sacrificed 48 h after administration of a single dose of DXR, whereas focal necrosis of muscle fiber with high edema, hemorrhage and congestions were noted when rats were sacrificed on day seven. In contrast, myocardium of rats pretreated with telmisartan appeared nearly normal; however, mild peripheral necrosis was noted in the post-treatment group.

Discussion

Acute DXR-induced cardiotoxicity alters the organization of the cardiomyocyte and induces apoptosis, which is a potentially modifiable and preventable form of myocardial tissue loss [1]. This potentially

novel mechanism is transient, but it may be of a key importance to the ensuing heart failure. The results of this study have confirmed that a single dose of DXR (20 mg/kg *ip*) induces acute cardiotoxicity in rats which is in agreement with previous studies [7, 16]. It also demonstrates that toxicity is more prominent and significant in animals those were sacrificed on day seven as compared to after 48 h, which suggests that biochemical abnormalities increase further which might lead to irreversible cardiomyopathy [41].

Existing evidence points to a multitude of molecular mechanisms involved in DXR-induced cardiac dysfunction. An important factor, which can mediate the toxic action of DXR, especially in mitochondria, is high affinity binding of DXR to cardiolipin, an anionic phospholipid in the inner mitochondrial membrane, which is essential in eukaryotic energy metabolism [17, 38]. Cardiolipin-bound DXR would induce dissociation of cardiolipin-associated peripheral proteins from the inner mitochondrial membrane, like e.g. cytochrome c and mitochondrial creatine kinase (MtCK). This could affect electron transport chain and energy channeling, as well as may favor initiation of programmed cell death [43].

Toxicity of mitochondrial, mostly cardiolipin-bound DXR is mediated by oxidative stress, which represents particular threats to cellular energy metabolism in the myocardium and is considered to be the main mediator of DXR cardiotoxic action. Two different mechanisms of free radical formation by DXR have been described. The first implicates the formation of a semiquinone free radical by the action of several NADPH-dependent reductases that produce a one-electron reduction of the DXR to the corresponding DXR semiquinone. In the presence of oxygen, redox cycling of DXR-derived quinine-semiquinone yields superoxide radicals ($O_2^{\bullet-}$). In the second, DXR free radicals are produced by a non-enzymatic mechanism that involves reaction with iron. Iron-DXR complex can reduce oxygen to H_2O_2 and other active oxygen forms [12, 42]. Superoxide anion ($O_2^{\bullet-}$) generated by DXR is transformed to hydrogen peroxide by superoxide dismutase and further detoxified by catalase or glutathione peroxidase. Superoxide can also get converted to peroxynitrite ($ONOO^-$) *via* reaction of $O_2^{\bullet-}$ with nitric oxide. Hydrogen peroxide subsequently leads to the formation of hydroxyl radicals ($OH^{\bullet}$), which is considered to be most damaging [9], greatly enhances the NADH-dependent microsomal lipid peroxidation and thus initiates a lipid radical chain reaction causing oxidative damage to cell membranes. Lipid peroxides which are the breakdown products of lipids are measured in the tissues as an indictor of the lipid peroxidation. In this experiment, MDA levels were significantly elevated after a single dose of DXR and supported the hypothesis that a major role is played by free radicals in DXR cardiotoxicity [26]. The reductions in the levels of MDA in the heart tissue of rats pretreated with telmisartan indicate that telmisartan protects myocardium against DXR-induced lipid peroxidation. Post-treatment with telmisartan reduced the levels of MDA, but it was not statistically significant suggesting that the treatment was less effective when commenced after DXR-induced lipid peroxidation had already occurred.

One of the most important intracellular antioxidant systems is the glutathione redox cycle. GSH is present at high concentrations in all cells of animals. One of its key functions is to serve as the reductant of toxic peroxides, and it also helps in keeping the enzymes in an active state by preventing the oxidation of -SH (sulfydryl) group to -S –S- (disulfide) group. GSH is one of the essential compounds for maintaining cell integrity because of its reducing property, and deficiency or depletion of this peptide causes damage to macromolecules or to membrane lipids. DXR causes depletion of cardiac GSH [14, 48]. This may reflect the consistent formation of oxygen free radicals [48]. In the present study, the deficiency of GSH caused by DXR might be due to its interaction with bio-membrane and subsequent peroxidizing action. The attenuation of GSH depleting effects of telmisartan-pretreated group reveals that AT1 receptor antagonist prevents DXR interaction with biomembrane and subsequent peroxidizing action. However, the result was not significant in post-treatment group, which suggests that post-treatment with telmisartan, does not restore the DXR-induced GSH depletion.

The rapid cell swelling of sub-sarcolemmal bulbs and injured myocardium could facilitate the loss of intracellular enzymes in DXR-treated rats; this might be the possible mechanism for the increased levels of LDH in serum [3]. Our results confirm the excess activity of LDH in cardiac tissues. This might also be due to induction or activation of LDH in cardiac tissue following DXR treatment. Pacher et al. [33] and Buyukokuroglu et al. [7] also observed similar change in levels of LDH in DXR treated rats. The reduction in the activity of serum LDH in animals pre- and

post-treated with telmisartan showed the suppression of cardiac injury by AT1 receptor antagonist.

H_2O_2 is decomposed into water and oxygen molecules by the enzyme CAT present in the cytoplasm. The increase in CAT activity in heart of rats treated with DXR indicates induction of this antioxidant enzyme as reported earlier [11, 16]. It seems that myocardial tissue tried to detoxify the oxygen free radicals but was not able to do that. The tissue injury was not prevented by insufficient antioxidant activities and, hence, the increased CAT activity might be an adaptive response to protect the heart against the deleterious effects of hydrogen peroxide. Pre- and post-treatment with telmisartan modulated the DXR-induced rise in CAT level which reflects the possible protective effect of telmisartan.

DXR-induced morphological changes in the cardiac tissues were observed by light microscopy. Some studies have reported that DXR treatment induces significant morphological changes [36], while others have reported that there is no significant morphological changes until 5 days after acute treatment [6, 20] or until 8-weeks after the beginning of chronic-treatment [30]. In the present acute study, the biochemical changes support the histopathological changes where single (20 mg/kg) *ip* dose of DXR produced minor morphological changes after 48 h but major changes were observed on day seven, which were reduced by groups pre- and post-treated with telmisartan, respectively, as seen in Figure 3.

Our results support the earlier finding that AT1-mediated angiotensin pathway plays an important role in DXR-induced cardiac impairment as pretreatment with telmisartan attenuates DXR-induced biochemical and morphological changes. Although some protection occurred as evidenced by a decrease in LDH activity and CAT level, post-treatment with telmisartan could not prevent completely the changes associated with DXR cardiotoxicity, such as lipid peroxidation and GSH level. This finding suggests that inhibition of AT1-mediated angiotensin pathway by pretreatment with telmisartan is more beneficial against DXR cardiotoxicity.

In conclusion, our results suggest that telmisartan (AT1 receptor antagonist) has a protective effect on DXR-induced cardiotoxicity and may be an available agent to protect the myocardium in DXR chemotherapy. However, before a conclusive statement can be made on the potential usefulness of AT1 receptor antagonist (telmisartan) as an adjunct to DXR therapy, there is a need for further long-term chronic studies with angiotensin receptor blockers. Although notable effects of Ang II receptor blocker, valsartan on acute cardiotoxic changes after standard chemotherapy with cyclophosphamide, DXR, vincristine, and prednisolone (CHOP) has been demonstrated clinically [29], but long-term effect of Ang II receptor blocker for patients undergoing repeated DXR therapy is required.

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