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# Protective Effects of Erdosteine against Doxorubicin-induced Cardiomyopathy in Rats†

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Key words: doxorubicin; erdosteine; cardiotoxicity; oxidants; antioxidants; lipid peroxidation; catalase; superoxide dismutase; glutathione peroxidase.

**The usefulness of doxorubicin (DXR) is limited by its cardiotoxicity. In order to improve future DXR therapy by using a new antioxidant agent, an experimental study was designed. This study was undertaken to determine whether DXR-induced cardiotoxicity is prevented by erdosteine, a mucolytic agent showing antioxidant properties. Three groups of male Sprague-Dawley rats (60 days old) were used: one group was untreated as a control; the other groups were treated with DXR (single i.p. dosage of 20 mg kg$^{-1}$ body wt.) or DXR plus erdosteine (10 mg kg$^{-1}$ day$^{-1}$, orally), respectively. The DXR treatment without erdosteine increased antioxidant enzyme activities and also increased lipid peroxidation in myocardial tissue. The rats treated with DXR plus erdosteine produced a significant decrease in lipid peroxidation in comparison with control and DXR groups. Furthermore, erdosteine administration led to an increase in antioxidant enzyme activities in comparison with the control group. Erdosteine treatment also increased the activities of catalase (CAT) and glutathione peroxidase (GSH-Px) in comparison with the DXR group. There was no significant difference in lipid peroxidation of myocardial tissue between control and DXR plus erdosteine-treated rats. It was concluded that erdosteine caused an increase in the activities of antioxidant enzymes, especially GSH-Px and CAT, protecting the heart tissue sufficiently from oxidative damage to membrane lipids and other cellular components induced by DXR.** 

## INTRODUCTION

Most studies have shown that doxorubicin (DXR)—an anthracycline antibiotic with broad activity against haematological malignancies—has limited clinical usage owing to its cardiotoxicity (Shan *et al*., 1996; Cassidy *et al*., 1998; Nazeyrollas *et al*., 1999). There are several hypotheses to explain the mechanism of DXR-induced cardiotoxicity, including the formation of free oxygen radicals, which can damage cells by lipid peroxidation, and altered intracellular calcium homeostasis (Olson and Mushlin, 1990; Akimoto *et al*., 1993; Saeki *et al*., 1998).

Oxidative stress is a term denoting an imbalance between the production of oxidants and the respective defence systems of an organism. Oxidants encompass oxygen free radicals, reactive nitrogen species, sulphur-centred radicals and various others. The formation of oxygen free radicals by incomplete reduction of oxygen in the respiratory chain of mitochondria and the host defence systems, which include the 'oxidative burst' mediated by NADPH oxidase to produce superoxide radical, are the two main important sources of oxidative stress (Fig. 1) (Abuja and Albertini, 2001). These two mechanisms have a role in the generation of DXR-induced cardiotoxicity (Olson and Mushlin, 1990; Akimoto *et al*., 1993; Zhou *et al*., 2001). Because oxidative mechanisms have been implicated in the pathogenesis of DXR-induced cardiotoxicity, thiols and other types of free oxygen radical scavengers have been examined as potential cardioprotectors. One of these compounds, *N*-acetylcysteine, ameliorated acute high-dose DXR-induced cardiotoxicity (Venditti *et al*., 1998; Herman *et al*., 2000).

Erdosteine, which is a mucolytic agent, contains two blocked sulphydryl groups that are released following its catabolic process (Dechant and Noble, 1996; Braga *et al*., 2000). Its active metabolites exhibit free radical scavenging activity (Gazzani *et al*., 1989; Dechant and Noble, 1996). Erdosteine, administered orally once daily for 5 days, attenuated death due to paraquat toxicity in mice by preventing the detrimental effects of free oxygen radicals (Dechant 1996).

Thus, we designed the following study to establish the protective effect of erdosteine against DXR-induced cardiotoxicity in rats and the changes in oxidant–antioxidant status of the myocardium.

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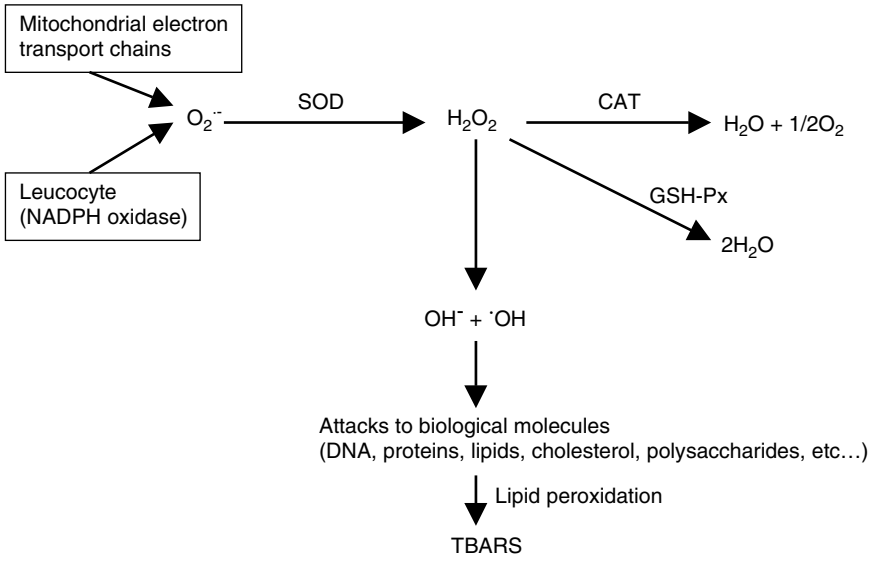


**Figure 1**. Schematic representation of the relationships between free oxygen radical formation, the antioxidant system and lipid peroxidation: $O_2^{\cdot-}$, superoxide anion radical; SOD, superoxide dismutase; CAT, catalase; $H_2O_2$, hydrogen peroxide; GSH-Px, glutathione peroxidase; $OH^-$, hydroxyl ion; $^{\cdot}OH$, hydroxyl radical (the most potent oxygen free radical); TBARS, thiobarbituric acid reactive substances (the index of lipid peroxidation).

## MATERIALS AND METHODS

Male Sprague-Dawley rats (60 days old) were used in the experiments. The animals were housed in quiet rooms with a 12-h light/dark cycle (7 a.m. to 7 p.m.) and the experiments were performed in accordance with the 'Guide for the Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 85–23, 1985' and approved by the local ethical committee at the Medical School of Inonu University.

Rats were assigned randomly to one of three groups: control untreated rats ($n = 8$); animals ($n = 7$) treated with a single i.p. injection of DXR (20 mg $kg^{-1}$ body wt.; Carlo Erba, Turkey) (Venditti *et al*., 1998); and animals ($n = 10$) treated for 12 days with oral administration of erdosteine (10 mg $kg^{-1}$ body wt. $day^{-1}$; Ilsan, Turkey) (Dechant and Noble, 1996) beginning 2 days before a single i.p. injection of DXR. Three rats from the DXR group (formerly $n = 10$) were excluded from the study because they were dead after i.p. DXR injection in the following days.

On the 10th day of DXR treatment, the animals were anaesthetized with urethane (i.p., 1.2 g $kg^{-1}$) and then the hearts were excised rapidly and stored at $-70\,^{\circ}C$ until analysed. After weighing the heart, the homogenate, supernatant and extracted samples were prepared as described elsewhere (Irmak *et al*., 2001), and the following determinations were made on the samples using commercial chemicals supplied by Sigma (St. Louis, MO, USA).

### Determination of catalase activity

Catalase (CAT, EC 1.11.1.6) activity was determined according to Aebi's method (Aebi, 1974). The principle of the assay is based on determination of the rate constant $k$ ($s^{-1}$) or the $H_2O_2$ decomposition rate at 240 nm. Results were expressed as $k$ (rate constant) per gram of protein ($k$ $g^{-1}$ protein).

### Determination of glutathione peroxidase activity

Glutathione peroxidase (GSH-Px, EC 1.6.4.2) activity was measured by the method of Paglia and Valentine (1967). The enzymatic reaction in the tube containing NADPH, reduced glutathione (GSH), sodium azide, and glutathione reductase was initiated by the addition of $H_2O_2$, and the change in absorbance at 340 nm was monitored by a spectrophotometer. Activity was given in units per gram of protein (U $g^{-1}$ protein).

### Determination of thiobarbituric acid reactive substance level

The tissue thiobarbituric acid reactive substance (TBARS) level was determined by a method (Esterbauer and Cheeseman, 1990) based on the reaction with thiobarbituric acid (TBA) at 90–100 °C. In the TBA test reaction, malondialdehyde (MDA) or MDA-like substances and TBA react to form a pink pigment with an absorption maximum at 532 nm. The reaction was performed at pH 2–3 at 90 °C for 15 min. The sample was mixed with 2 volumes of cold 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water-bath for 10 min. After cooling, the absorbance was read at 532 nm. The results were expressed as nanomoles per gram of wet tissue (nmol $g^{-1}$ wet tissue) according to a standard graph that was prepared from the measurements made with a standard solution (1,1,3,3-tetramethoxypropane).

### Determination of superoxide dismutase activity

Total (Cu–Zn and Mn) superoxide dismutase (SOD, EC 1.15.1.1) activity was determined according to the method of Sun *et al*. (1988) and a slightly modified method by Durak *et al*. (1993). The principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of

the lysate after 1.0 ml of an ethanol–chloroform mixture (5 : 3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. The SOD activity was also expressed as units per milligram of protein (U $mg^{-1}$ protein).

### Determination of protein content

Protein measurements were made at all stages according to Lowry's method (Lowry *et al*., 1951).

### Statistical analysis

Data were analysed by using a commercially available statistics software package (SPSS® for Windows, version 9.0, Chicago, USA). Distribution of the groups was analysed with a one-sample Kolmogrov–Smirnov test. All groups showed a normal distribution, so parametric statistical methods were used to analyse the data. A one-way ANOVA test was performed and *post hoc* multiple comparisons were done using the least significant difference. Results are presented as means ± SEM; $P < 0.05$ was regarded as statistically significant.

## RESULTS AND DISCUSSION

The results are summarized in Table 1. The TBARS level was increased in the DXR group in comparison with control and DXR + erdosteine groups ($P < 0.001$). There is a higher GSH-Px activity in the DXR + erdosteine group than in the control ($P < 0.001$) and DXR groups ($P < 0.05$). There is also a higher SOD activity in both study groups than in the control group ($P < 0.05$). The activity of CAT was increased in the DXR and DXR + erdosteine groups in comparison with the control group ($P < 0.001$) and in the DXR + erdosteine group in comparison with the DXR group ($P < 0.05$). There was no significant difference in TBARS levels between control and DXR + erdosteine groups.

The results of this study have confirmed that a cumulative dose of DXR (20 mg $kg^{-1}$) induces cardiac toxicity in rats (Falcone *et al*., 1998). In agreement with previous studies using an acute treatment with DXR (Doroshow, 1983; Venditti *et al*., 1998), our results show that this anthracycline gives rise to a high TBARS level. It is well known that DXR significantly increases oxygen free radicals in the myocardium (Doroshow, 1983). The reactive species mediating oxidative stress in organisms are thought to be radicals. Lipid peroxidation reactions are chain reactions driven by oxygen free radicals in which one radical can induce the oxidation of a comparatively large number of substrate molecules. On the other hand, antioxidants are molecules that can prevent or reduce the extent of oxidative destruction of biomolecules (Fig. 1) (Abuja and Albertini, 2001; Herken *et al*., 2001). The formation of free oxygen radicals damages cells and its organelles by lipid peroxidation. This process explains the pathological picture of anthracycline cardiac toxicity, characterized by disruption of heart mitochondrial and sarcoplasmic reticular formation in myocardial compartments (Doroshow, 1983; Venditti *et al*., 1998). The TBARS level is one of the indices of lipid peroxidation and a high TBARS level indicates membrane lipid peroxidation and cellular injury (Herken *et al*., 2001). Two sources of oxidants that may contribute to DXR-induced myocardial injury include: the mitochondrial electron transport chain, which leaks superoxide radicals ($O_2^{\cdot -}$) (Zhou *et al*., 2001); and neutrophils (Arnhold *et al*., 2001), which may secrete $O_2^{\cdot -}$ and $H_2O_2$ through the action of NADPH oxidase.

In the living organism, the oxidant–antioxidant system is in equilibrium. When there is an imbalance in this system, tissue injury can be seen. Doxorubicin has been accepted as one of the causative factors of the disruption and may change the oxidant–antioxidant status. In the present study, the activities of SOD, CAT and GSH-Px were increased in the DXR-treated groups over the control. We believe that myocardial tissue was attempting to detoxify the oxygen free radicals; however, it was insufficient and the defence system was overwhelmed. The insufficient antioxidant activities could not cope with the oxidative stress produced and did not prevent the lipid peroxidation.

Erdosteine is a molecule containing two sulphur atoms, one of which is blocked in the aliphatic side-chain and the other is enclosed in the heterocyclic ring (Braga *et al*., 1999). Erdosteine does not posses a free thiol group itself, but does produce three active metabolites containing an—SH group (Braga *et al*., 1999). Erdosteine, owing to the presence of two sulphydryl group in its metabolites, can act as a free oxygen radical scavenger and this component of the mechanism of action is likely to be involved in the cardioprotection afforded towards cardiac injuries induced by oxidative stress. An increase in free radical formation has been well documented in DXR-induced cardiotoxicity (Venditti *et al*., 1998; Sacco

Table 1—The activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and the thiobarbituric acid reactive substance (TBARS) level in control, doxorubicin (DXR) and DXR + erdosteine groups

| | CAT (k $g^{-1}$ protein) | SOD (U $mg^{-1}$ protein) | GSH-Px (U $g^{-1}$ protein) | TBARS (nmol $g^{-1}$ wet tissue) |
|---|---|---|---|---|
| 1. Control ($n = 8$) | 0.195 ± 0.015 | 0.167 ± 0.019 | 2.650 ± 0.147 | 22.05 ± 3.01 |
| 2. DXR ($n = 7$) | 0.332 ± 0.022 | 0.270 ± 0.027 | 3.030 ± 0.184 | 60.31 ± 3.76 |
| 3. DXR + erdosteine ($n = 10$) | 0.408 ± 0.023 | 0.273 ± 0.009 | 3.747 ± 0.238 | 21.46 ± 3.73 |
| *P* | | | | |
| 1–2 | 0.0001 | 0.001 | N.S. | 0.0001 |
| 1–3 | 0.0001 | 0.0001 | 0.001 | N.S. |
| 2–3 | 0.019 | N.S. | 0.022 | 0.0001 |

*et al*., 2001), therefore a contribution of indirect scavenger action of erdosteine in preventing DXR-induced cardiotoxicity appears likely. It was demonstrated that erdosteine was effective *in vitro* against the inactivation of human $\alpha_1$-antitripsin induced by cigarette smoke. It was known that $\alpha_1$-antitripsin protects the lungs against oxidation reaction due to smoking by re-establishing the oxidant–antioxidant balance in favour of the antioxidants (Gazzani *et al*., 1989). Gazzani *et al*. suggested that erdosteine might either exert antioxidant activity by behaving as a reducing compound and as an electron donor or react directly with peroxynitrates to destroy radicals. It was demonstrated that the DXR treatment significantly reduces antioxidant capacity in heart and blood, which accounts for the increased susceptibility to oxidative stress of the cellular structures (Venditti *et al*., 1998). Antioxidant capacity contains not only CAT, SOD and GSH-Px but also Vitamin E, Vitamin C selenium, etc. Thus, antioxidant enzyme activities alone do not reflect total antioxidant capacity. Increased antioxidant enzymes activities induced by DXR treatment as a response to oxidative stress might consume the other cellular antioxidants. To test this hypothesis, another study should be undertaken in which all the antioxidant systems are measured separately. Venditti *et al*. explained that vitamin E and *N*-acetylcysteine prevented the lipid peroxidation of the heart due to DXR toxicity in rats (Venditti *et al*., 1998). Erdosteine administration resulted in high antioxidant capacity as well as reacting directly with radicals by its active metabolites. Erdosteine administration resulted in an increase in the activities of antioxidant enzymes GSH-Px and CAT, protecting the myocardium from oxidative damage to cellular membrane components due to DXR treatment.

In conclusion, erdosteine may be used to prevent cardiotoxicity during anthracycline antibiotic administration as a chemotherapeutic agent for malignancies. However, it should be determined whether erdosteine has a protective role in the electrophysiological changes due to DXR-induced cardiotoxicity. Thus the subject needs further investigation, with both biochemical and physiological parameters being measured together.

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