Asian Journal of Chemistry Vol. 19, No. 5 (2007), 4035-4042

Dantrolene: In Doxorubicin Toxicity

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Doxorubicin (DOX) is a widely used anthracycline in the treatment of hematological and solid tumors. However, there are serious toxic effects on cardiovascular and hematopoietic system, which limits the use of the drug. This study examines the effect of dantrolene (5 and 10 mg kg^{-1}) on DOX (20 mg kg^{-1}, i.p.)-induced cardiotoxicity and hematotoxicity in normal rats. Change in nitric oxide ($NO^{\bullet}$) levels in cardiac tissue were measured and hemoglobin (Hb), white blood cells (WBC), red blood cells (RBC) and platelets were counted with an automatic counter. Pretreatment of rats with 5 and 10 mg kg^{-1} doses of dantrolene, markedly reduced the $NO^{\bullet}$ levels in the cardiac tissue. The hematotoxicity was characterized by leukopenia and thrombocytopenia that appeared after 48 h of single dose DOX (20 mg kg^{-1}) administration. Dantrolene (5 and 10 mg kg^{-1}) 0.5 h before doxorubicin injection ameliorated the hematotoxicity induced by doxorubicin. These results showed that dantrolene has provided a protective effect against DOX-induced nitrosative stress in the heart and hematotoxicity.

Key Words: Doxorubicin, Dantrolene, Cardiotoxicity, Hematotoxicity.

INTRODUCTION

Doxorubicin (DOX), an anthracycline antibiotic and broad-spectrum antineoplastic agent, is used primarily in variety of human cancers including acute leukemias, malignant lymphomas and a number of solid tumours[1]. Anthracycline antibiotics exert their therapeutic and toxic actions through

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number of mechanisms. Cytotoxicity of anthracyclines is attributed to DNA intercalation by an anthracycline semiquinone radical that is generated *via* redox-cycling[2]. The exact causal mechanism of DOX-induced cardiomyopathy remains unclear, but semiquinone radical, which causes the generetion of lipid peroxides, nitric oxide ($NO^{\bullet}$) and other destructive radicals, has now been established as being the cause of the drug's cardiotoxicity[3].

Irreversible cardiomyopthy is an important side-effect of anthracycline antibiotics. Abnormal electrocardiographic changes including ST-T wave alterations and arrhythmias are seen in acute form. Long-term administration of DOX causes a cumulative dose-dependent cardiac toxicity that is characterized dilated cardiomyopathy and congestive heart failure[1,4]. Cytopenia (anemia, neutropenia, thrombocytopenia), which is thought the result of myelosuppression, is also significant side effect of anthracyclines and may be dose limiting especially during combined chemotherapeutic regimens[3,5,6]. Neutropenia is more commonly observed than thrombocytopenia[3].

An inorganic free radical gas $NO^{\bullet}$ is synthesized by a group of isoenzymes called NO synthases (NOS) through sequential oxidation that converts L-arginine to L-citrulline. Three different forms of NOS have been identified; two of them the neuronal NOS (nNOS) and the endothelial NOS (eNOS) are constitutively expressed and 3rd the inducible NOS (iNOS) is expressed following immunological or inflammatory stimuli[7]. iNOS generates excessive amounts of $NO^{\bullet}$ for prolonged periods that have been linked to pathological manifestations observed in inflammatory or degenerative conditions. Peroxynitrite anion ($ONOO^{-}$) is a reactive oxidant synthesized from $NO^{\bullet}$ and superoxide anion ($O_2^{\bullet -}$), which reacts with a number of biomolecules including proteins, lipids and DNA[8,9]. $ONOO^{-}$ is more cytotoxic than $NO^{\bullet}$ or $O_2^{\bullet -}$ in a variety of experimental systems. It has been implicated in the pathogenesis of a wide variety of diseases and toxicologically relevant conditions.

Periferally acting skeletal muscle relaxant dantrolene is hydantoin derivative related to phenytoin; it depresses excitation-contraction coupling in the muscle fibre by inhibiting calcium release from sarcoplasmic reticulum. It is used in the treatment of muscle spasticity, malignant hyperthermia and neuroleptic malignant syndrome[3]. It is shown that the dantrolene sodium has cytoprotective effect on the neuron or heart cells and *in vivo* and *in vitro* antioxidant properties[10-13]. Antioxidant activity of exogenous antioxidant agents have been attributed to various mechanisms, among which are prevention of the onset of damage, delaying of pathologic process, decomposition of peroxides, prevention of continued hydrogen abstraction, radical scavenging and stimulation of both enzymatic and/or

non-enzymatic antioxidant defence and repair mechanisms[14,15]. Antioxidant defences are believed to have an important role in protecting cells against the toxicity of DOX and these defences can be augmented by exogenous antioxidants such as vitamin E, melatonin or L-carnitine[16,17]. Therefore, some antioxidant agents have been tested to ameliorate the patients from DOX-induced cardiac damage until now. Due to cytoprotective and *in vivo* or *in vitro* antioxidant properties, we investigated the possible protective activity of dantrolene in acute DOX-induced cardiotoxicity and hematotoxicity in rats.

EXPERIMENTAL

Dantrolene were purchased from Sigma Chemical co. (St Luois, MO, USA), Doxorubicin (adriamycin) was purchased from Carlo Erba A.S., Turkey. Nicotinamide adenine dinucleotide, sulphanilamide and N-(1-napthyl) ethylenediamine were purchased from Sigma Chemical Co (St Louis, MO, USA). All other chemicals were of analytical grade.

Animals: 32 Albino Sprague-Dawley male rats weighing 150-180 g were used and housed in an air-conditioned room (22 ± 1ºC) with controlled 14 h light/10 h dark cycles. The rats were fed with standard laboratory chow and water before the experiment, *ad libitum* and randomly divided into 4 equal groups (n = 8) and housed in a separate cages. Groups 1 and 2 were injected with 5 and 10 mg kg^{-1} dantrolene, group 3 was injected with saline solution. Group 4 was received no dantrolene or DOX (it was used as control). Following a 0.5 h period, all the animals in groups 1-3 were given 20 mg kg^{-1} of DOX dissolved in distilled water by a single injection. All of drugs were administered by intraperitoneally and at the same volume (0.5 mL). 48 h later, animals in all groups were anesthetized by tiopenthal sodium (50 mg kg^{-1}) and 2 mL blood samples were taken from the aortas and hearts were rapidly excised, sectioned and washed in physiological saline to remove blood and immediately stored at -80ºC until being processed for analysis of $NO^{\bullet}$. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Biochemical analysis: Cardiac $NO^{\bullet}$ (nitrite + nitrate) levels were measured using the Griess reagent as previously described[18,19]. Griess reagent, the mixture (1:1) of 0.2 % N-(1-naphthyl) ethylenediamine and 2 % sulphanilamide in 5 % phosphoric acid, gives a red-violet diazo dye with nitrite and the resultant colour was measured at 540 nm. First nitrate was converted to nitrite using nitrate reductase. The second step was the addition of Griess reagent, which converts nitrite to a deep purple azo compound; photometric measurement at the absorbance of 540 nm determines the nitrite concentration. $NO^{\bullet}$ levels are expressed as μmol/mg protein.

The protein was determined using the Bradford method[20]. Biochemical measurements were carried out at room temperature using a spectrophotometer (CECIL CE 3041, Cambridge, UK).

Blood analysis: Hemoglobin (Hb), white blood cells (WBC), red blood cells (RBC) and platelets were counted with an automatic counter (Coulter Gen-S, Coulter Corporation, Miami.).

Statistical analyses: Results are presented as mean ± SEM. All parameters were analyzed using a one-way variance analysis (Anova). Least significant difference multiple range test was used to compare the mean values. Acceptable significance was recorded when $p < 0.05$. Statistical analysis was performed with Statistical Package for the Social Sciences for Windows (SPSS, version 10.0, Chicago, IL, USA).

RESULTS AND DISCUSSION

DOX administarion caused an increase in the cardiac levels of $NO^{\bullet}$ (measured as nitrate/nitrite), these values were significant (Fig. 1) and reduced when dantrolene was treated. There were no differences between 5 and 10 mg/kg doses of dantrolene and between the dantrolene treated and control groups.

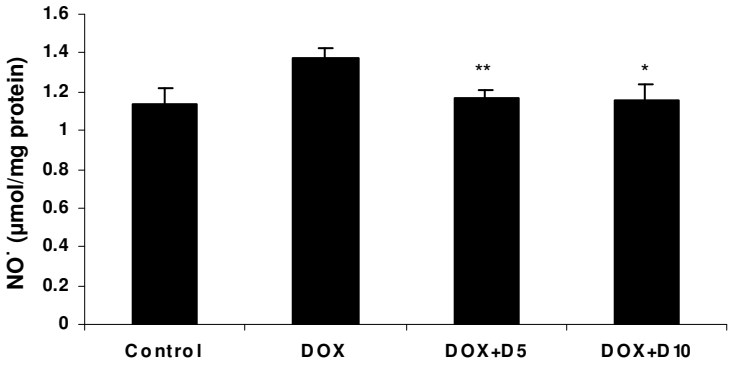

Fig. 1. Effects of 5 and 10 mg kg^{-1} doses of dantrolene (D5 and D10) on cardiac NO level in doxorubicine (DOX; 20 mg/kg)-administered rats (N = 8). *$p < 0.05$, **$p < 0.01$, *vs.* DOX group

The mean levels of WBC, RBC, Hb and platelet counts in the groups are shown in Table-1. DOX caused a decrease in the mean number of WBC ($p < 0.001$) and platelets ($p < 0.05$) and an increase in the levels of RBC ($p < 0.05$) and haemoglobin ($p < 0.005$). The decreases in WBC were also significant in dantrolene treated groups. However, mean WBC counts were higher in groups 1 and 2 than group 3 ($p < 0.005$). 5 mg kg^{-1} dantrolene treatment caused less decrease in the mean number of WBC as compared

with the mean value of 10 mg kg^{-1} dantrolene treated group. The mean platelet counts in dantrolene treated groups were similar to control ($p > 0.05$). Group 1 had higher value than group 3 ($p < 0.05$). The mean values of RBC and hemoglobin were higher in DOX treated group than controls. There were no differences between the DOX alone and dantrolene treated groups and between the dantrolene treated and control groups.

TABLE-1
EFFECTS OF THE DANTROLENE ON WHITE BLOOD CELL (WBC), RED BLOOD CELL (RBC), HEMOGLOBIN (Hb) AND PLATELET COUNTS IN DOX-TREATED RATS

Groups	WBC ($10^3/mm^3$)	RBC ($10^6/mm^3$)	Hb (g/dL)	Platelets ($10^3/mm^3$)
Control	8.97±0.45	8.2±0.08	14.8±0.14	729.6±32.39
DOX	4.26±0.26^{c}	8.6±0.13^{a}	15.9±0.26^{b}	614.1±28.75^{a}
DOX+D5	6.70±0.46***^{,b}	8.1±0.21	14.8±0.27	726.5±42.15*
DOX+D10	5.40±0.21**^{,b}	8.4±0.23	15.3±0.38	647.6±30.19

DOX, 20 mg/kg doxorubicine; D5, 5 mg kg^{-1} dantrolene; D10, 10 mg kg^{-1} dantrolene; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* DOX; $^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$ *vs.* Control.

The cardiotoxicity of the DOX is one of the main factors, which limits its prolonged use. DOX-related cardiotoxicity results in a dilated cardiomyopathy with irreversible and incurable congestive heart failure, with high mortality. Several hypotheses are suggested the molecular mechanisms, which can explain this cardiac toxicity, but induction of an oxidative stress within myocardial tissue is implicated in the pathogenic process[21]. Protection of myocardium with cardioprotective agents targeting oxidative stress during chemotherapy is important for an optimal use of the anthracyclines.

$NO^{\bullet}$ is necessary for normal cardiac physiology and has a protective role in the ischemic heart and nonischemic heart failure and myocarditis. However, reactive nitrogen species (RNS) can also exert cytotoxic effects. Many of the toxic actions of $NO^{\bullet}$ are not due directly to $NO^{\bullet}$ itself, but are mediated by the highly reactive oxidant $ONOO^{-}$, which is produced following interaction[22] of $NO^{\bullet}$ with $O_2^{\bullet -}$. The processes triggered by $ONOO^{-}$ include initiation of lipid peroxidation, inhibition of mitochondrial respiration, inhibition of membrane pumps, depletion of glutathione and damage to DNA[23]. The generation of $ONOO^{-}$ can also inhibit superoxide dismutase and other antioxidant molecules and systems, which leads to positive feedback cycles of intracellular oxidant generation and oxidative injury and the reaction of $ONOO^{-}$ with lipids can lead to peroxidation (malondialdehyde formation). It is suggested that nitrosative stress and

generation of $ONOO^-$ play an important role in the pathogenesis of DOX-related cardiomyopathy and $ONOO^-$-mediated nitration of myofibrillar creatine kinase impairs myocardial contractility[22].

DOX can catalytically increase $O_2^{\bullet -}$ levels at the expense of cellular reductants[24]. The mechanism involves one-electron reduction of DOX by reduced flavoenzymes to yield the DOX semiquinone radical, which, in the presence of oxygen, is oxidized back to DOX and produces $O_2^{\bullet -}$:

$$DOX + e^- \rightarrow DOX^{\bullet -}$$
$$DOX^{\bullet -} + O_2 \rightarrow DOX + O_2^{\bullet -}$$

In the present investigation, when rats given DOX (20 mg/kg), the $NO^{\bullet}$ levels significantly increased in the cardiac tissue. The present $NO^{\bullet}$ result is in agreement with the previous study in rat cardiac tissue[25]. In previous study, Buyukokuroglu *et al.*[26] showed that dantrolene administration improved the antioxidant enzyme activity and cardiac enzymes' profiles and decreased the lipid peroxidation in adriamycin-treated rat[26]. Again, in the present study, both doses of dantrolene significantly decreased the $NO^{\bullet}$ levels in the cardiac tissue. On the basis of these results, it is suggested that, besides augmenting endogenous antioxidant defense capacity, decreasing the lipid peroxidation activity and improving cardiac enzymes' profiles by dantrolene, its inhibitor effect on $NO^{\bullet}$, at least in part, may play a protective role in DOX cardiotoxicity. Thus, it may be concluded that dantrolene administration decreases lipid peroxidation and $NO^{\bullet}$ generation in DOX-treated cancer patients and alleviates the DOX toxicity to heart.

Hematologic toxicities of chemotherapeutic agents have served to limit their full therapeutic potential. There has been some approaches to lower the risk of hematotoxicity such as growth factor usage. In this study, the effect of dantrolene on the hematotoxicity induced by doxorubicin was investigated.

A single dose injection of 20 mg kg^{-1} doxorubicin in the rats produced a marked leukopenia and thrombocytopenia. These results were in agreement with Lahouel *et al.*[27], who reported that leukopenia and thrombocyopenia after 10 mg kg^{-1} DOX administration. Al-Shabanah *et al.*[28] reprted that severe leukopenia and anemia after 72 h of 15 mg kg^{-1} of DOX administration. These findings may be attributed to the destructive effects of DOX on peripheral blood cells and bone marrow. Dantrolene treated groups had higher WBC counts than DOX treated group. Also platelet counts were higher in 5 mg kg^{-1} but not 10 mg kg^{-1} dantrolene treated group. These results indicated that dantrolene had a protective effect against DOX induced hematotoxicity. This effect may be attributed to its cytoprotective effect which is shown in neuron and cardiac cells[10,11] and antioxidant properties[12,13]. Calcium influx induced by DOX may be associated with acute

leukopenia and thrombocytopenia. Sacco *et al.*[29] reported that zofenopril, a angiotensin converting enzyme (ACE) inhibitor, could play a significant role in the preservation and regulation of Ca homeostasis in cardiomyocytes affected by doxorubicin treatment. Al-Shabanah *et al.*[28] reported that an ACE inhibitor, captopril, may protect against hematotoxicity induced by DOX. Meanwhile, dantrolene is a calcium antagonist and may ameliorate the toxic effect of DOX by this way.

Hemoglobin and RBC counts were higher in DOX alone treated group. Here it is speculated that these are the result of hemoconsantration and are related to long life-time of the erythrocytes. We observed ascites fluid in all DOX treated rats. The amount of ascites was more in DOX alone treated rats than the others.

In conclusion, dantrolene has provided a protective effect against DOX-induced nitrosative stress in the heart and hematotoxicity. Neutralization of reactive nitrogen species and elimination of $ONOO^-$ may reveal as novel approaches for the protection against DOX-related cardiomyopathy. Again, dantrolene may be useful against DOX-related cytopenia. However, further studies are needed to reveal the long term or exact mechanism(s) of the protective effect of dantrolene on heart and blood cell counts.

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(*Received*: 1 November 2006; *Accepted*: 9 March 2007) AJC-5513