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Resveratrol treatment protects against doxorubicin-induced cardiotoxicity by alleviating oxidative damage

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Abstract

The possible protective effects of resveratrol (RVT) against cardiotoxicity were investigated in Wistar albino rats treated with saline, saline + doxorubicin (DOX; 20 mg/kg) or RVT (10 mg/kg) + DOX. Blood pressure and heart rate were recorded on the 1st week and on the 7th week, while cardiomyopathy was assessed using transthoracic echocardiography before the rats were decapitated. DOX-induced cardiotoxicity resulted in decreased blood pressure and heart rate, but lactate dehydrogenase, creatine phosphokinase, total cholesterol, triglyceride, aspartate aminotransferase and 8-OHdG levels were increased in plasma. Moreover, DOX caused a significant decrease in plasma total antioxidant capacity along with a reduction in cardiac superoxide dismutase, catalase and Na^+,K^+-ATPase activities and glutathione contents, while malondialdehyde, myelopreoxidase activity and the generation of reactive oxygen species were increased in the cardiac tissue. On the other hand, RVT markedly ameliorated the severity of cardiac dysfunction, while all oxidant responses were prevented; implicating that RVT may be of therapeutic use in preventing oxidative stress due to DOX toxicity.

Keywords: *Doxorubicin, toxicity, resveratrol, free radical*

Introduction

Adriamycin (doxorubicin, DOX) is one of the most effective chemotherapeutic antibiotics, widely used against leukaemia, lymphomas and various solid tumours of the lung, breast, thyroid and ovary [1]. Although DOX remains an important component in most chemotherapeutic regimens, owing to its efficacy in treating a broad spectrum of cancers, many tissues are susceptible to the unintended effects of chemotherapy [2]. Because cardiomyocytes are post-mitotic cells, major insults that befall them are generally irreversible and can irrevocably affect the cardiac functions. Consequently, chronic administration of DOX produces dose-dependent and irreversible cardiac toxicity and arrhythmia, restricting its full clinical potential as a chemotherapeutic agent [3].

Although the molecular basis for DOX-induced cardiotoxicity remains elusive, several mechanisms have been proposed for the cardiotoxic effects of DOX, including mitochondrial dysfunction, calcium overload, interaction with nuclear components and several membrane-bound molecules, alteration of fatty acid oxidation leading to depression of energy metabolism, induction of apoptosis and increased oxidative stress [4–10]. Available laboratory evidence suggests that increased oxidative stress with increased free radical production and decreased myocardial endogenous antioxidants plays an important role in the pathogenesis of DOX-induced heart failure [11,12]. DOX-induced production of reactive oxygen

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(ROS) and nitrogen species (RNS) involves the interplay of multiple processes in the heart, including redox cycling of the quinone moiety of DOX, disturbance of iron metabolism and DOX metabolites [13]. Since extensive free radical production is likely to overwhelm endogenous anti-radical defenses (e.g. glutathione, superoxide dismutase, catalase, vitamin E), the activation of free radical-induced unfavourable reactions could be protected by exogenous antioxidants that are expected to reduce the intracellular level of ROS.

Resveratrol is a polyphenol phytoalexin that is found in a number of edible materials, such as grape skins and seeds, peanuts, mulberries and red wine [14–16]. Resveratrol has been reported to have a wide range of pharmacological effects that include cardiovascular protection, neuroprotection, modulation of lipid metabolism, anti-carcinogenesis and anti-inflammatory effects [15–19]. Resveratrol, when added to cultured cardiomyocytes in low micromolar concentrations, was shown to induce a number of endogenous antioxidants and phase 2 enzymes, including superoxide dismutase (SOD), catalase, glutathione (GSH), glutathione reductase (GR), glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) [20]. Animal studies have shown that resveratrol reduces biliary cirrhosis-induced oxidative damage of the kidney and liver [21,22], as well as ischemia/reperfusion-induced tissue damage in the ovaries [23]. An immunohistochemical study has shown that resveratrol attenuated the expression of CD86 in the glomerular endothelium and peritubular vessels in rats with ischemia-reperfusion injury [24]. In accordance with these studies, we have previously demonstrated that resveratrol improves stomach, kidney, bladder and lung damage in rat models of ulcer, ischemia/reperfusion and sepsis [25–28]. Furthermore, we have also reported that resveratrol attenuates acetaminophen-induced hepatotoxicity and ifosfamide-induced nephrotoxicity [29,30].

Based on these above-mentioned findings, in the present study we aimed to investigate the possible beneficial activities of resveratrol against doxorubicin-induced oxidative damage in the cardiac tissues of rats.

Materials and methods

Animals

All experimental protocols were approved by the Marmara University Animal Care and Use Committee. Both sexes of Wistar albino rats (200–250 g) were kept at a constant temperature ($22 \pm 1^{\circ}C$) with 12 h light and dark cycles and fed a standard rat chow.

Experimental design

The rats ($n = 32$) were randomly divided into four groups as: control, resveratrol (RVT), doxorubicin (DOX) and DOX + RVT. In the RVT group, resveratrol (10 mg/kg/day) was administered intraperitoneally for a total of 7 weeks. The rationale for the selected dose of RVT depends on our previous reports demonstrating its protective action in several oxidative injury models [25,26]. In the DOX group, following a 2-week saline injection, animals were additionally administered intraperitoneally with doxorubicin every other day six times to reach a cumulative dose of 20 mg/kg over a 2-week period, while saline injections were continued for the following 3 weeks. In the DOX + RVT group, after a 2-week RVT pre-treatment (10 mg/kg/day), DOX was administered at the same cumulative dose in the following 2 weeks along with RVT treatment, then only RVT was continued for the next 3 weeks. Animals in the control group were treated with saline only. Body weight, systolic blood pressure (BP) and heart rate (HR) measurements were recorded at the beginning of the study to obtain the basal levels. At the end of the 7th week of the initial treatments, BP and HR measurements were repeated, while transthoracic echocardiography was performed to assess the cardiac function of the rats. Then, the animals were decapitated, trunk blood was collected and immediately centrifuged at $3000 \times g$ for 10 min to assay lactate dehydrogenase, creatine kinase, total cholesterol, triglyceride, aspartate aminotransferase, total antioxidant capacity and 8-hydroxy-2′-deoxyguanosine levels in the plasma. In order to evaluate the presence of oxidative tissue injury, cardiac samples were taken and stored at $-80^{\circ}C$ for the determination of malondialdehyde and glutathione levels, myelopreoxidase, superoxide dismutase (SOD), catalase (CAT) and Na^+,K^+-ATPase activities and collagen content. To assess the role of reactive oxygen species in doxorubicin-associated cardiotoxicity, luminol- and lucigenin-enhanced chemiluminescences were measured as indicators of radical formation. Additional tissue samples were taken for histological analyses.

Measurement of blood pressure

Indirect blood pressure measurement was made by the tail cuff method (Rhma-Labor Technique, 2 channel blood pressure monitor 8002, model MK-2000). Rats were allowed to get accustomed to this procedure for 7 days before the basal measurements were obtained. Initially, the rats were placed for 10 min in a chamber heated to $35^{\circ}C$. Then the rats were placed in individual plastic restrainers and a cuff with a pneumatic pulse sensor was wrapped around their tails. Blood pressure and HR values recorded

during each measurement period were averaged from at least three consecutive readings on that occasion obtained from each rat.

Echocardiography

Echocardiographic imaging and calculations were done according to the guidelines published by the American Society of Echocardiography [27] using a 12 MHz linear transducer and 5–8 MHz sector transducer (Vivid 3,General Electric Medical Systems Ultrasound, Tirat Carmel, Israel). Under ketamine (100 mg/kg, ip) anaesthesia, measurements were made from M-mode and two-dimensional images obtained in the parasternal long and short axes at the level of the papillary muscles after observation of at least six cardiac cycles. Interventricular septal thickness (IVS), left ventricular diameter (LVD) and left ventricular posterior wall thickness (LVPW) were measured during systole (s) and diastole (d). Ejection fraction, fractional shortening and left ventricular mass were calculated from the M-mode images using the formulae [% EF $= (\mathrm{LVDd})^3 - (\mathrm{LVDs})^3/(\mathrm{LVDd})^3 \times 100$] for ejection fraction and [% FS $= \mathrm{LVDd} - \mathrm{LVDs}/\mathrm{LVDd} \times 100$] for fractional shortening, Left Ventricular Mass $= 1.04 \times ((\mathrm{LVDd} + \mathrm{LVPWd} + \mathrm{IVSd})^3 - (\mathrm{LVDd})^3) \times 0.8 + 0.14$, Relative wall thickness $= 2 \times (\mathrm{LVPWd}/\mathrm{LVDd})$.

Blood assays

Plasma levels of lactate dehydrogenase (LDH), creatine kinase (CK), total cholesterol (TCH), triglyceride (TG) and aspartate aminotransferase (AST) were determined spectrophotometrically using an automated analyser (Bayer Opera biochemical analyser, Germany), while total antioxidant capacity (AOC) was measured by using a colorimetric test system (ImAnOx, cataloge no.KC5200, Immunodiagnostic AG, D-64625 Bensheim), according to the instructions provided by the manufacturer. The 8-hydroxy-2′-deoxyguanosine (8-OhdG) content in the extracted DNA solution was measured as an indicator of oxidative DNA damage using an enzyme-linked immunosorbent assay (ELISA) method (Highly Sensitive 8-OHdG ELISA kit, Japan Institute for the Control of Ageing, Shizuoka, Japan). These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intra-assay precision and small amount of plasma sample required for conducting the assay.

Measurement of luminol and lucigenin chemiluninescence in the cardiac tissue

Lucigenin (bis-N-methylacridiniumnitrate) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were obtained from Sigma (St Louis, MO). Measurements were made at room temperature using Junior LB 9509 luminometer (EG&G Berthold, Germany). Specimens were put into vials containing PBS-HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). ROS were quantitated after the addition of enhancers such as lucigenin or luminol for a final concentration of 0.2 mM. Luminol detects a group of reactive species, i.e. OH^-, H_2O_2, HOCl radicals, and lucigenin is selective for O_2^- [23,24]. Counts were obtained at 1 min intervals and the results were given as the area under curve (AUC) for a counting period of 5 min. Counts was corrected for wet tissue weight (rlu/mg tissue) [28].

Measurement of malondialdehyde and glutathione in the cardiac tissue

Cardiac tissue samples were homogenized with ice-cold 150 mM KCl for the determination of malondialdehyde (MDA) and glutathione (GSH) levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously [29]. Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of $1.56 \times 10^5\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$ and results are expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure [30]. Briefly, after centrifugation at 3000 rev/min for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 mol/l $Na_2HPO_4.2H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. GSH levels were calculated using an extinction coefficient of $1.36 \times 10^4\ \mathrm{M}^{-1}\ \mathrm{cm}^{-1}$. Results are expressed in μmol GSH/g tissue.

Measurement of superoxide dismutase (SOD) and catalase (CAT) activities in the cardiac tissue

SOD activity was measured according to the previously described method [31]. Briefly, measurements were performed in cuvettes containing 2.8 mL 50 mM potasium phosphate (pH = 7.8) with 0.1 mM EDTA, 0.1 mM 0.39 mM riboflavin in 10 mM potasium phosphate (pH 7.5), 0.1 mL of 6 M M O-dianisidin.2 HCl in deionized water and tissue extract (50, 100 μL). Cuvettes with all their components were illuminated with 20-W Slylvania Grow Lux fluorecent tubes that were placed 5 cm above and to one side of cuvettes. Absorbances were mesaured at 460 nm with a Schimadzu UV-02 model spectrophotometer. A standard curve was prepared routinely with bovine SOD (Sigma Chemical Co, S-2515-3000 U) as reference. Absorbance readings were taken at 0 and 8 min of illumunation and the net absorbances were calculated.

The method for the measurement of catalase activity is based on the catalytic activiy of the enzyme which catalyses the decomposition reaction of H_2O_2 to give H_2O and O_2 [32]. Briefly, the absorbances of the sample containing 0.4 ml homogenate and 0.2 ml H_2O_2 were read at 240 nm against a blank containing 0.2 mL phospahate buffer and 0.4 mL homogenate for $\sim$1 min.

Measurement of myeloperoxidase and Na^+,K^+-ATPase activities in the cardiac tissue

Myeloperoxidase (MPO) is an enzyme that is found predominantly in the azurophilic granules of polymorphonuclear leukocytes (PMN). Tissue MPO activity is frequently utilized to estimate tissue PMN accumulation in inflamed tissues and correlates significantly with the number of PMN determined histochemically in tissues. MPO activity was measured in tissues in a procedure similar to that documented by Hillegass et al. [33]. Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41 400 g (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41 400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Measurement of Na^+,K^+-ATPase activity is based on the measurement of inorganic phosphate released by ATP hydolysis during incubation of homogenates with an appropriate medium. Since the activity of Na^+,K^+-ATPase, a membrane-bound enzyme required for cellular transport, is very sensitive to free radical reactions and lipid peroxidation, reductions in this activity can indicate membrane damage indirectly. The total ATPase activity was determined in the presence of 100 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$, 0.1 mM EDTA, 30 mM Tris HCl (pH 7.4), while the Mg^{2+}-ATPase activity was determined in the presence of 1 mM ouabain. The difference between the total and the Mg^{2+}-ATPase activities was taken as a measure of the Na^+,K^+-ATPase activity [34,35]. The reaction was initiated with the addition of the homogenate (0.1 ml) and a 5-min pre-incubation period at 37°C was allowed. Following the addition of 3 mM Na_2ATP and a 10-min re-incubation period, the reaction was terminated by the addition of ice-cold 6% perchloric acid. The mixture was then centrifuged at 3500 g and Pi in the supernatant fraction was determined by the method of Fiske and Subarrow [36]. The specific activity of the enzyme was expressed as nmol Pi mg^{-1} protein h^{-1}. The protein concentration of the supernatant was measured by the Lowry et al. [37] method.

Measurement of cardiac collagen content

Tissue collagen was measured as a free radical-induced fibrosis marker. Cardiac tissue samples were cut with a razor blade, immediately fixed in 10% formalin in 0.1 M phosphate buffer (pH; 7.2) in paraffin and $\sim$ 15 μm thick sections were obtained. Evaluation of collagen content was performed according to the method published by Lopez de Leon and Rojkind [38] based on the selective binding of the dyes Sirius Red and Fast Green FCF to collagen and non-collagenous components, respectively. Both dyes were eluted readily and simultaneously using 0.1 N NaOH-methanol (1:1, v/v). Finally, the absorbances at 540 and 605 nm were used to determine the amount of collagen and protein, respectively.

Histopathological analysis

For light microscopic analysis, samples from myocard tissues were fixed in 10% buffered formalin for 48 h, dehydrated in ascending alcohol series and embedded in paraffin wax. Approximately 7-μm-thick sections were stained with hematoxylin and eosin for general morphology. Stained sections were observed under an Olympus BX50 photomicroscope (Tokyo, Japan). All tissue sections were examined by an experienced histologist who was unaware of the treatments.

Statistics

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA). Each group consisted of eight animals. All data were expressed as means $\pm$ SEM. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were regarded as significant.

Results

The basal blood pressure and heart rate measurements (t_1) recorded before the initiation of the treatments were not different among the four experimental groups ($p > 0.05$; Table I). The HR and BP measurements made at the end of the 7th week of the treatment schedules (t_2) were not changed in either the saline-treated or RVT-treated control groups. However, in the saline-treated DOX group with cardiotoxocity, both systolic BP and HR were found to be significantly lower as compared to saline-treated control group ($p < 0.001$). On the other hand, in the RVT-treated DOX group, resveratrol treatment prevented BP and

Table I. Systolic blood pressure (BP), heart rate (HR), body weight, heart weight and heart/body weight ratio values in the saline- or resveratrol (RVT)-treated control and doxorubicin groups. Each group consists of eight animals.

	Control		Doxorubicin	
	Saline-treated	RVT-treated	Saline-treated	RVT-treated
BP (mmHg)				
t_1	120±1.4	117±2.4	117±1.2	119±0.5
t_2	121±0.7	118±1.7	99±1.1***	116±2.0^{+++}
HR (beats/min)				
t_1	394±12.8	403±6.6	396±4.8	384±11.8
t_2	395±5.6	401±5.5	297±7.8 ***	396±19.4^{+++}
Body weight (g)				
t_1	212±5.6	208±4.4	206±3.4	206±7.2
t_2	260±5.7***	262±9.4***	218±9.9$^{\&\&\&}$	244±6.3**
Heart weight (g)	0.567±7.2	0.557±8.5	0.440±24.3$^{\&\&\&}$	0.508±10.3$^{\&\&,+}$
Heart/body weight ratio ($\times 10^3$)	2.18±0.03	2.10±0.07	1.96±0.09	2.02±0.05

**$p<0.01$, ***$p<0.001$: vs t_1 value; $^{+}p<0.05$, $^{+++}p<0.001$: vs saline-treated doxorubicin group; $^{\&\&}p<0.01$, $^{\&\&\&}p<0.001$: vs saline-treated control group; t_1: basal values; t_2: values at the end of the 7th week.

HR alterations significantly, as compared to the saline-treated DOX group ($p<0.001$).

As expected, by the end of the 7 weeks, body weight measurements exhibited marked elevations in all groups, except the saline treated-DOX group ($p<0.001$). Similarly, heart weight of the saline treated-DOX group showed a significant reduction when compared with the control group ($p<0.001$). When added to DOX treatment, resveratrol prevented the reductions in the heart weights ($p<0.05$).

Table II summarizes the transthoracic echocardiograpy measurements of both saline- and RVT-treated rats with DOX toxicity as compared to control group. LV posterior wall thickness, LV end-diastolic and end-systolic dimensions, as well as relative wall thickness and percentage fractional shortening and ejection fraction were increased significantly ($p<0.05$–0.001) in the saline-treated rats with DOX cardiotoxicity. However, in the RVT-treated rats with DOX toxicity, these mesurements were significantly reduced ($p<0.05$–0.001).

Plasma LDH activity showed a 2.5-fold increase in the saline-treated doxorubicin group, indicating generalized tissue damage ($p<0.001$), while treatment with the antioxidant resveratrol suppressed the LDH activity ($p<0.001$, Table III). In accordance with the plasma LDH activity, doxorubicin caused significant increases in the plasma levels of CK and AST ($p<0.001$). When resveratrol was administered in addition to doxorubicin treatment, all of these parameters were significantly reversed ($p<0.001$). On the other hand, increased total cholesterol and triglyceride levels in the saline-treated DOX group were also reversed in the plasma of resveratrol-treated rats with cardiotoxicity (Table III).

Doxorubicin treatment caused a significant decrease in total antioxidant capacity ($p<0.001$), which increased the plasma level of 8-OH dG ($p<0.001$), indicating oxidative DNA damage (Table III). On the other hand, when resveratrol was coupled with DOX treatment, the reduction in AOC was significantly less than that of the saline-treated DOX group ($p<0.05$), while the elevation in 8-OH dG level was significantly reduced ($p<0.001$).

Chemiluminescence levels in the cardiac samples detected by both luminol and lucigenin probes showed significant increases in the saline-treated DOX group as compared to the CL levels of the control group ($p<0.05$–0.01; Figure 1A and B). On the other hand, in the RVT plus DOX treated group,

Table II. Transthoracic echocardiograpy measurements in in the saline- or resveratrol (RVT)-treated control and doxorubicin groups. Each group consists of eight animals.

	Control		Doxorubicin	
	Saline-treated	RVT-treated	Saline-treated	RVT-treated
IVS (mm)	2.1±0.05	2.3±0.10	2.5±0.19	2.4±0.12
PW (mm)	2.2±0.12	2.1±0.07	1.6±0.12**	2.1±0.08^{+}
RWT	0.7±0.02	0.8±0.04	0.9±0.06***	0.8±0.03^{++}
LVDs (mm)	2.5±0.12	2.6±0.15	3.5±0.14***	2.8±0.12^{++}
LVDd (mm)	4.1±0.10	4.2±0.16	5.6±0.19***	4.5±0.17^{+++}
EF (%)	77.3±1.3	75.5±1.4	63.8±2.6***	75.4±1.5^{+++}
FS (%)	39.8±3.9	37.9±4.5	23.6±1.9*	37.2±3.4^{+}

IVS: interventricular septal thickness (IVS); PW: left ventricular posterior wall thickness; RWT: relative wall thickness; LVDs: left ventricular diameter in sistole; LVDd: left ventricular diameter in diastole; EF: ejection fraction; FS: fractional shortening.

Table III. Plasma lactate dehydrogenase (LDH), creatinine phosphokinase (CK), total cholesterol (TCH), triglyceride (TG), aspartate amino transferase (AST), total antioxidant capacity (AOC) and 8-OH dG levels in the saline- or resveratrol (RVT)-treated control and doxorubicin groups. Each group consists of eight animals.

	Control groups		Doxorubicin-treated groups	
	Saline-treated	RVT-treated	Saline-treated	RVT-treated
LDH (U/L)	1585±107	1337±116	4101±173***	2320±255***, +++
CK (U/L)	1673±137	1735±121	8858±816***	3967±821***, +++
AST (U/L)	67.3±3.801	71.8±3.2	149.8±4.7***	74.8±6.5+++
TCH (mg/dL)	93.5±4.5	93.3±4.5	124.5±9.4**	89.3±4.1**, ++
TG (mg/dL)	70.17±2.5	68.7±3.6	100.7±4.6***	71.0±2.3***, +++
AOC (pg/ml)	359.3±20.6	341.3±14.4	210.7±20.0***	284.3±13.8***, +
8-OH dG (ng/ml)	0.33±0.1	0.34±0.1	5.8±0.4***	2.7±0.3***,+++

**$p<0.01$, ***$p<0.001$; compared to saline-treated control group; $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$; compared to saline-treated doxorubicin group.

the elevations in CL values of lucigenin and luminol were abolished ($p<0.05$–0.01). Similarly, MDA content of the cardiac tissue in the control group (8.8±0.4 nmol/g) was elevated by DOX treatment (18.5±2.5 nmol/g, $p<0.01$), while resveratrol treatment significantly decreased DOX-induced MDA elevation (11.8±1.4 nmol/g, $p<0.05$; Figure 2A). In accordance with that, DOX caused a significant decrease in cardiac GSH level (1.22±0.1 μmol/g; $p<0.001$) when compared to the control group (2.2±0.1 μmol/g); while in the resveratrol-treated DOX group, GSH content of the cardiac tissue was found to be preserved (2.14±0.1 μmol/g; $p<0.01$), being not significantly different from that of the control group (Figure 2B).

In parallel with the GSH results, SOD and CAT activities were reduced in the saline-treated DOX groups (0.9±0.04 U/mg prot and 60.8±2.5 U/mg prot; $p<0.001$), as compared to those of the control group (1.25±0.06 U/mg prot and 77.4±2.7 U/mg

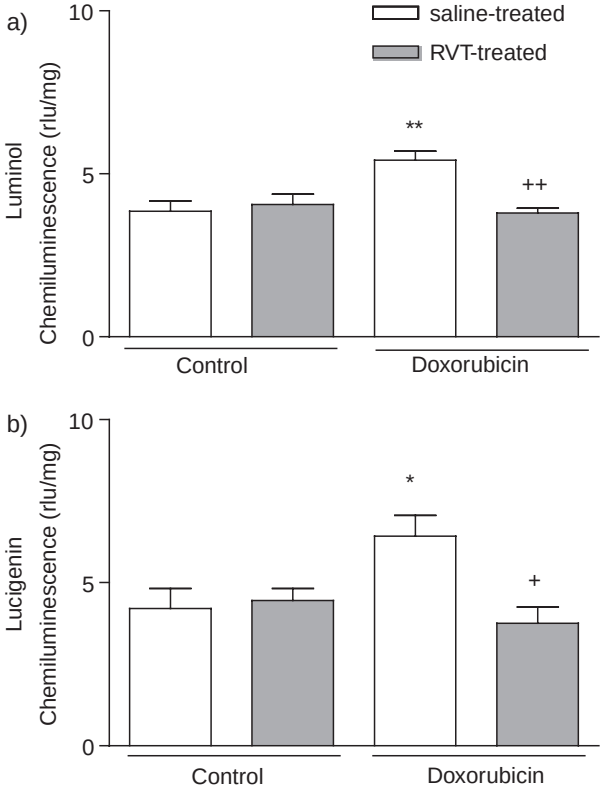

Figure 1. (a) Luminol and (b) lucigenin chemiluminescence levels in the heart tissue of saline- or resveratrol-treated doxorubicin and control groups. Data are presented as means±SEM. *$p<0.05$; **$p<0.01$ vs saline-treated control group; $^{+}p<0.05$; $^{++}p<0.01$ vs saline treated-doxorubicin group. For each group $n=8$.

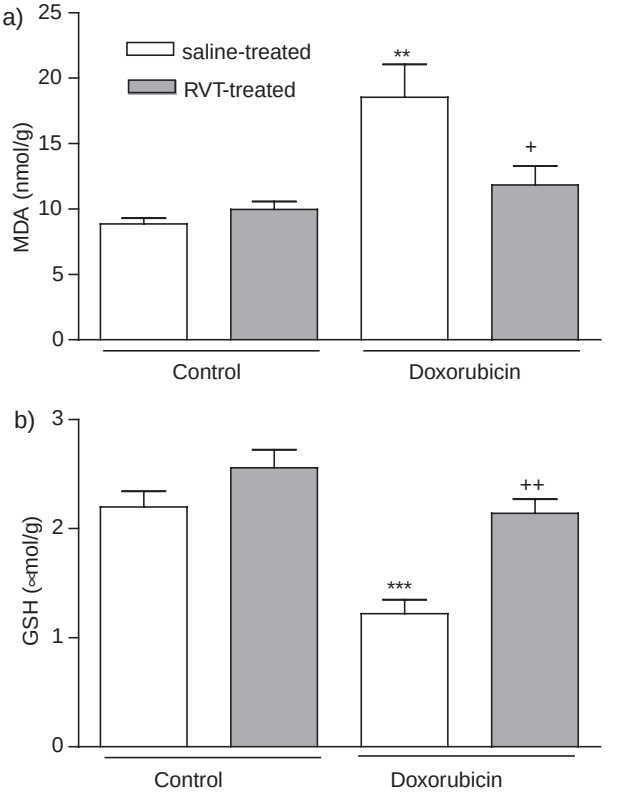

Figure 2. (a) Malondialdehyde and (b) glutathione levels in the heart tissue of saline- or resveratrol-treated doxorubicin and control groups. Data are presented as means±SEM. ** $p<0.01$; ***$p<0.001$ vs saline-treated control group; $^{+}p<0.05$; $^{++}p<0.01$ vs saline treated-doxorubicin group. For each group $n=8$.

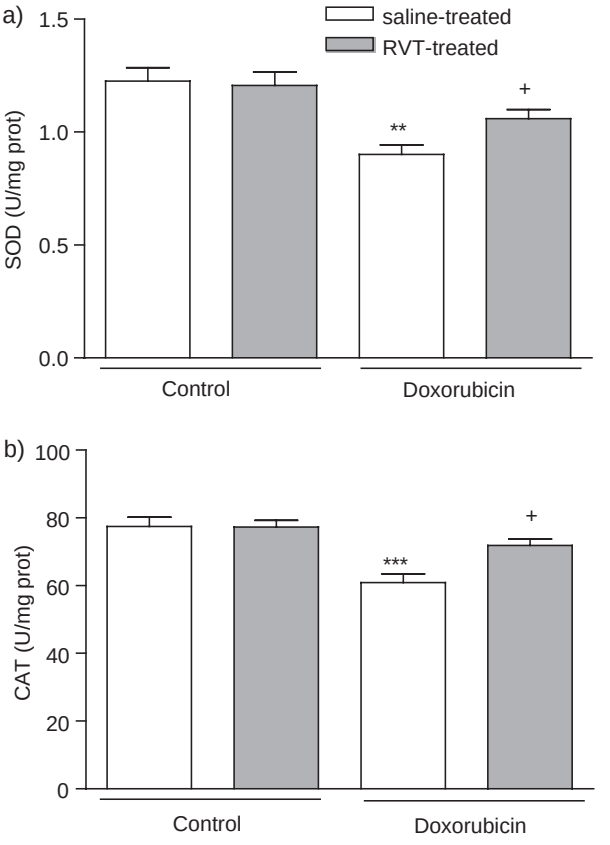

Figure 3. (a) Superoxide dismutase (SOD) and (b) Catalase (CAT) activities in the heart tissue of saline- or resveratrol-treated doxorubicin and control groups. Data are presented as means ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$ vs saline-treated control group; $^{+}p < 0.05$ vs saline treated-doxorubicin group. For each group $n = 8$.

prot) (Figure 3A and B). However, in the RVT-treated DOX group both SOD and CAT activities were found to be elevated back to control levels (1.06 ± 0.04 U/mg prot and 71.8 ± 1.8 U/mg prot) ($p < 0.05$–0.01).

Myeloperoxidase activity, which is accepted as an indicator of neutrophil infiltration, was significantly higher in the cardiac tissues of saline-treated DOX group (10.1 ± 1.2 U/g, $p < 0.01$) as compared to that of the control group (5.6 ± 0.4 U/g; Figure 4A). However, in the RVT treated DOX group MPO activity of the cardiac tissue was significantly depressed (6.2 ± 0.5, $p < 0.01$), reaching a level that was not different than that of the control group.

When compared to the control group (0.97 ± 0.01 mmol/mg protein/h), Na^+,K^+-ATPase activity was reduced in the saline treated-DOX group (0.42 ± 0.01 mmol/mg protein/h, $p < 0.05$), indicating impaired transport function and membrane damage in the cardiac tissue (Figure 4B). On the other hand, in the group treated with both DOX and RVT, the measured Na^+,K^+-ATPase activity (0.72 ± 0.01 mmol/mg protein/h) was not different to either the saline-treated DOX or the control groups.

The collagen content in the cardiac tissue of saline-treated DOX group (12.1 ± 0.9 μg/mg prot) was markedly increased, indicating enhanced fibrotic activity as compared to the control group (8.7 ± 0.4 μg/mg prot; $p < 0.01$). Resveratrol treatment significantly reduced DOX-enhanced increase in the cardiac collagen content (9.1 ± 0.6 μg/mg prot; $p < 0.05$) back to control levels (Figure 4C).

Light microscopic evaluation of the cardiac muscle cells in the control groups, treated with either resveratrol or saline, revealed regular nuclei with central alignment (Figure 5A). In the saline-treated doxorubicin group, the capillaries present in the connective tissue of muscle fibres showed congestion besides vacuolization in the cytoplasm of most of

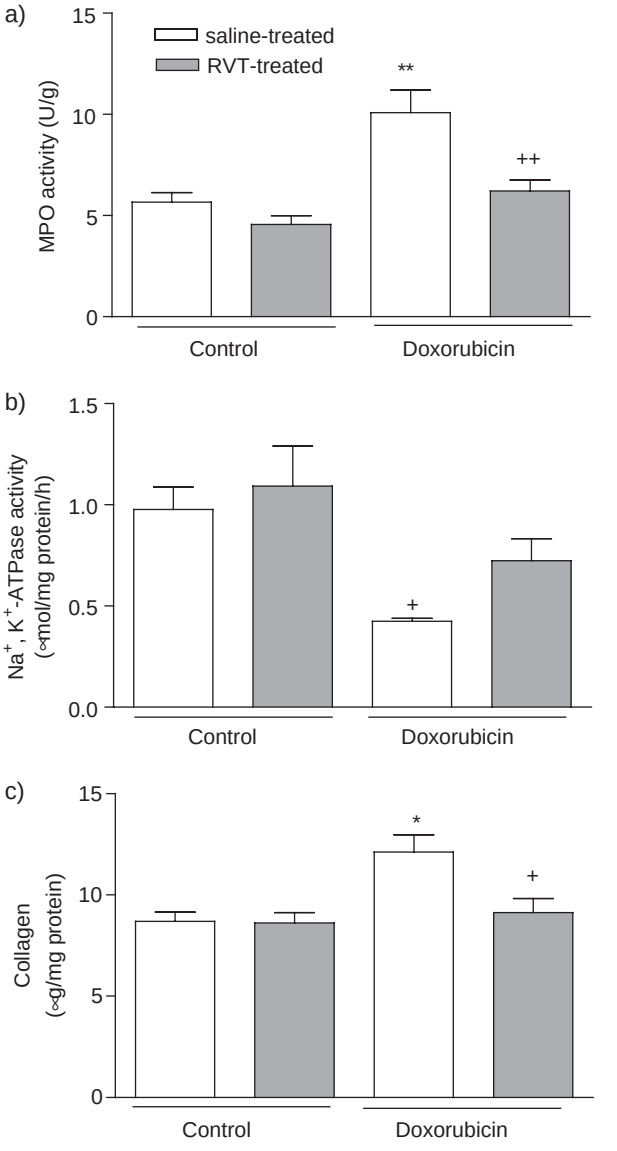

Figure 4. (a) Myeloperoxidase (MPO), (b) Na^+,K^+-ATPase activities and (c) Collagen content in the heart tissue of saline- or resveratrol-treated doxorubicin and control groups. Data are presented as means ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$ vs saline-treated control group; $^{+}p < 0.05$; $^{++}p < 0.01$ vs saline treated-doxorubicin group. For each group $n = 8$.

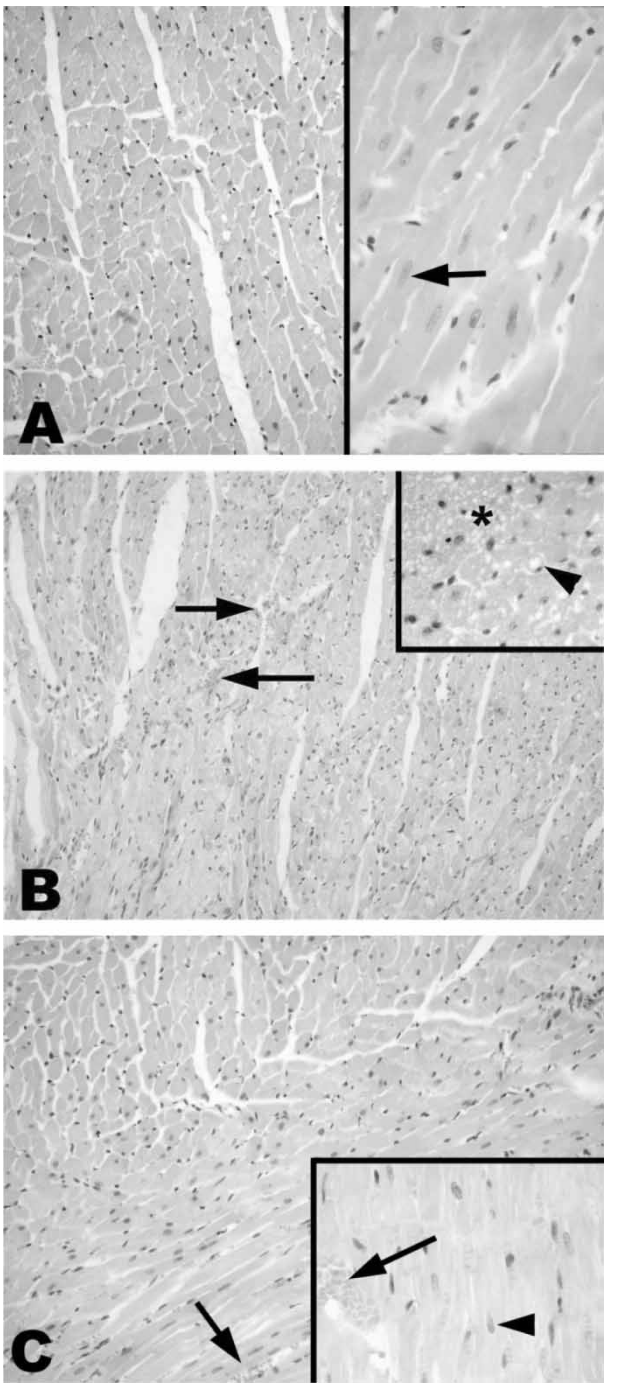

Figure 5. (A) Control group: regular alignment of muscle cells with centally located nuclei (arrow). (B) Saline-treated doxorubicin group: capillary vasocongestion among the connective tissue of the muscle cells (arrows), vacuolization in the cytoplasm of the cardiac muscle cells (*) capillary (arrowhead). (C) Resveratrol-treated doxorubicin group: vasocongestion present in some areas (arrows), centrally located nucleus in the cells (arrowhead). HE staining, × 200 insets × 400.

the cardiomyocytes (Figure 5B). On the other hand, in the resveratrol-treated doxorubicin group, the capillary vasocongestion was reduced, the vacuolization in the cytoplasm of the cells was decreased and a regular cellular morphology was settled (Figure 5C).

Discussion

In the present study, increases in lipid peroxidation, collagen content and myeloperoxidase activity due to toxic effects of DOX were accompanied by significant reductions in glutathione level, antioxidant capacity and Na^+,K^+-ATPase activity in the cardiac tissue, implicating the presence of oxidative tissue damage. In addition, elevated serum levels of LDH, CK and AST levels, as well as the histological findings demonstrate the severity of DOX-induced toxicity. Moreover, systolic blood pressure and heart rate were significantly reduced and myocardial functions were improved. The results also demonstrate that resveratrol treatment through its antioxidant effects, as observed by reversal of the changes in all the measured parameters, prevented the severity of DOX-induced cardiotoxicity by alleviating the extent of oxidative stress.

The clinical use of anthracyclines, namely doxorubicin, is associated with the development of life-threatening cardiomyopathy [7], which is closely related with the generation of highly reactive oxygen species that cause direct damage to cardiac myocyte membranes by means of membrane lipid peroxidation. Cellular damage induced by DOX 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 [39]. Several studies suggest that DOX produces DNA strand breaks by a free radical process [40,41], because the quinone structure of DOX permits it to act as an electron acceptor in reactions mediated by oxoreductive enzymes, including cytochrome P450 reductase, NADH dehydrogenase and xanthine oxidase. The addition of free electrons converts the quinones to semiquinone free radicals, which may induce free radical injury to DNA themselves as well as after interaction with molecular oxygen to form superoxides, hydroxyl radicals and peroxides. In the present study, marked elevation of luminol and lucigenin chemiluminescence levels in the cardiac tissue of DOX-treated rats indicate the generation of H_2O_2, OH^-, hypochlorite, peroxynitrite, lipid peroxyl radicals and superoxide radicals [28]. In accordance with CL increases, MDA level in the hearts was also increased significantly, implicating the presence of enhanced lipid peroxidation, which is an autocatalytic mechanism leading to oxidative destruction of cellular membranes [42–44].

Since the research on the mechanisms of DOX-induced cardioxicity suggests that ROS are intimately involved in DOX-induced cardiotoxicity [45], several studies have focused on the search of antioxidative agents that could be administered to protect against the cardiotoxic effects of the anti-tumour antibiotic [46,47]. Thus, in attempts to eliminate the cardiotoxic effects of the drug, which limit its clinical use, several

antioxidants containing sulphydryl groups have been studied and proved to be effective against DOX toxicity [48–51]. Recently, resveratrol was shown to relieve the toxic effects of DOX on myocardium by inducing nitric oxide production [52]. In an *in vitro* study, resveratrol and its analogues exhibited potencies in inhibiting lipid peroxidation in rat brain, kidney and liver homogenates [53]. Similarly, in another *in vitro* study, blood platelets after their incubation with resveratrol were shown to exhibit a protective effect against lipid peroxidation in cell membranes and DNA damage caused by ROS [54]. In accordance with the aforementioned observations, the reduction in the cardiac MDA levels of rats treated with resveratrol indicates that resveratrol protects the myocardium against DOX-induced lipid peroxidation. Thus, the current findings suggest that resveratrol reduces lipid peroxidation and oxidative injury of the heart and thereby improves cardiac function, as evident by echocardiography.

It is well known that increased ROS generation may cause a significant amount of single-strand DNA breaks which activate the nuclear poly(ADP-ribose) polymerase (PARP), a chromatin-bound enzyme involved in the repair of DNA breaks [55]. However, activation of the nuclear PARP accelerates NAD+ catabolism and depletion of NAD+ compromises the mitochondrial energy metabolism, which contributes to the cardiotoxicity of DOX [56]. 8-OHdG is one of the most common adducts formed by oxidative DNA damage [57] and a reliable marker of oxidative DNA damage [58]. The present results demonstrate that DOX treatment leads to a highly elevated plasma level of 8-OhdG, which is reduced when the rats are treated with resveratrol. Thus, DOX-induced cardiotoxicity appears to be alleviated by resveratrol through a mechanism that involves the protection against oxidative DNA damage and associated changes in mitochondrial energy metabolism.

The sources of oxidants in DOX-induced myocardial injury may include mitochondrial electron transport chain and neutrophils [59,60]. Since myeloperoxidase (MPO) plays a fundamental role in oxidant production by neutrophils and is used as an index for the neutrophil infiltration, the increased MPO activity observed in the current study clearly demonstrates that DOX-induced cardiac damage is neutrophil-dependent. The neutrophils have a role in oxidant injury via the mechanisms that include the activation of NADPH oxidase or MPO enzyme systems. Activated neutrophils release MPO, causing production of large amounts of HOCl, which oxidizes and damages macromolecules, including proteins, lipids, carbohydrates and nucleic acids [61]. Increasing evidence also suggests that neutrophils release chemotactic substances, which further promote neutrophil migration to the tissue, activate neutrophils and increase the damage [62]. Our results are in agreement with similar studies, which found that increased MPO activity plays a role in DOX-induced cardiotoxicity and the anti-inflammatory drugs prevent cardiotoxicity by inhibiting neutrophil infiltration [63,64]. Similarly, the red wine constituent resveratrol in the current study suppressed the enzyme activity through its anti-inflammatory properties, which may be explained by its inhibitory effect on the activation of COX-1 [65] and may be the primary basis for its protective effects against DOX cardiotoxicity.

Blood levels of CK, LDH and AST are extensively used in the everyday clinical practice as markers for the diagnosis of cardiac toxicity. DOX-induced experimental cardiotoxicity was manifested biochemically by a significant elevation of serum CK activity and LDH [66]. In the present study, the plasma levels of these enzymes were significantly elevated in the saline-treated DOX group, while resveratrol treatment significantly prevented these increases and reversed DOX-induced hypotension and bradycardia. Recently, resveratrol has been found to prevent and cure cardiovascular diseases and improve microcirculatory disorders by protecting the vascular endothelium, inhibiting platelet aggregation, increasing cellular nitric oxide levels, as well as by modulating lipid metabolism [15–20]. Accordingly, in the current study, the rats treated with DOX showed an increase in plasma lipids, while resveratrol depressed these back to control levels. Similarly, a previous report has suggested that a better protection against DOX-induced cardiotoxicity can be achieved if ROS scavenging is accompanied by lipid lowering activity [67].

As it is observed in the present study, DOX treatment in experimental animals was shown to reduce cardiac GSH levels and superoxide dismutase and catalase activities [59,68], which is one of the essential compounds for maintaining cell integrity. Furthermore, in relation to DOX-induced cardiotoxicity, it was previously known that fewer antioxidant defenses are present in the heart as compared to other organs [69,70]. As a consequence, deficiency or depletion of GSH and other antioxidant enzymes causes damage to macromolecules or to membrane lipids when there is consistent formation of oxygen free radicals by the toxic effects of DOX. On the contrary, resveratrol appears to support the preservation of heart antioxidants and thereby alleviates DOX-induced oxidative damage of the heart. The present findings have demonstrated that the cardioprotective effects of resveratrol in DOX-induced oxidative damage may be due to an augmentation of the endogenous antioxidants and the inhibition of lipid peroxidation by maintaining a balance in oxidant-antioxidant status, inhibiting neutrophil infiltration and modulating lipid metabolism, nominating resveratrol as a highly promising agent in avoiding the development of DOX-induced cardiotoxicity.

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