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RESVERATROL PROTECTS AGAINST ACUTE CHEMOTHERAPY TOXICITY INDUCED BY DOXORUBICIN IN RAT ERYTHROCYTE AND PLASMA

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Doxorubicin (Dox), a widely used antitumor anthracycline antibiotic, plays an undisputed key role in the treatment of many neoplasic diseases. In this study, the protective role of resveratrol against Dox-induced erythrocytes and plasma toxicity has been evaluated in rats. Animals were treated with resveratrol (25 mg/kg b.w.) by intraperitoneal injection during 8 days. At the 4th day of treatment, rats were intraperitoneally injected with a single dose of Dox (20 mg/kg b.w.). At the end of the treatment, blood samples were collected following standard procedure and processed for oxidative stress parameters (malondialdehyde (MDA), carbonyl protein, free iron, calcium and H_2O_2 levels), transaminases and antioxidant enzymes as catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD). Data showed that Dox drastically increased erythrocytes and plasma MDA, free iron, H_2O_2 and carbonyl proteins but decreased calcium levels and also decreased erythrocytes CAT, POD and SOD activity. Besides, Dox decreased plasma CAT and SOD but unexpectedly increased POD activity. Dox also increased plasma ALT and AST levels and decreased them into erythrocytes. Co-treatment with resveratrol counteracted almost all Dox's effects. In conclusion, Dox induced a pro-oxidative stress into erythrocytes and resveratrol exerted real antioxidant properties which can be attributed, at least in part, to free iron and calcium modulation.

Key words: *antioxidant enzymes, doxorubicin, erythrocyte, oxidative stress, plasma, resveratrol, malondialdehyde*

INTRODUCTION

Doxorubicin (adriamycin) has been used in oncologic practice since the late 1960s. It held promise as a powerful drug in the fight against cancer (1). However, the clinical efficacy of this drug is limited due to damages toxicity in adults and pediatric cancer patients (2). The exact causal mechanism of Dox induced toxicity remains unclear, but most of the evidence indicates that free radicals are involved (3, 4). The chemical structure of Dox is prone to the generation of free radicals (5) and the oxidative stress that results correlates with cellular injury (6). In addition, Dox administration is associated with a decrease in endogenous antioxidants responsible for the scavenging of free radicals (7) leading to increased oxidative stress, which is followed by damages in organism (8).

Resveratrol (RVT) (3,4,5-trihydroxy-trans-stibene) is a polyphenol compound, classified as a phytoalexin, which is synthesized by several plants in response to adverse conditions such as stress, injury, UV irradiation and fungal infection (9). Resveratrol, abundantly found in grapes and red wine, exhibited a large spectrum of beneficial health effects including cardioprotective (10), antiproliferative (11) and neuroprotective properties (12). This polyphenol was suggested to play a role in the prevention of heart disease as it inhibits platelet aggregation (13) and modulate lipoprotein metabolism (14). Resveratrol was also shown to inhibit cellular events associated with tumor initiation, promotion and progression (15), and due to its weak toxicity should be envisaged as a chemopreventive agent (16). Resveratrol has also estrogenic, vaso-relaxing activity (17) and cardiovascular benefits (18). It has been proposed as a major constituent of the polyphenol fraction to which the health benefits of red wine consumption are attributed. *In vivo* and *in vitro* studies have also shown that resveratrol plays a role in the prevention of inflammation, atherosclerosis and carcinogenesis (19). In cancer chemoprevention, resveratrol seems to exert anti-initiation activity (16) and has strong antioxidant activity (20). It was also demonstrated that resveratrol protects acetaminophen-induced hepatotoxicity (21) and reduces mortality and liver damage after chronic alcohol intoxication (22, 23).

The present study was designed to evaluate the toxic effect of Dox on erythrocytes and plasma antioxidant status, as well as the putative protection offered by resveratrol co-treatment. Data reinforced the pro-oxidant effect of Dox as well as the potential protective one of resveratrol. The mode of action seems to involve free iron and H_2O_2 disturbances leading to calcium depletion. Dox induces a pro-oxidative state and resveratrol exerts potent antioxidative properties.

* Contributed equally to this work.

MATERIAL AND METHODS

Reagents

2-thiobarbituric acid (TBA); 2,6,-di-tert-butyl-4-hydroxy-toluene (BHT); trichloroacetic acid (TCA); hydrogen peroxide (H_2O_2); 2-methoxyphenol (gaiacol); bovine catalase 4-(1-hydroxy-2-methylamino-ethyl)-benzene-1,2-diol (epinephrine) and, 2,4,dinitrophenyl hydrazine (DNPH) were obtained from Sigma-Aldrich Co (Germany). Dox hydrochloride was purchased from Pharmacia Italia (Italy). Resveratrol (trans 3,4',5 trihydroxystilbene) was purchased from Selmedica Healthcare (Korea).

Animals and treatment

Twenty-four female Wistar rats (220–240 g) were used in these experiments in accordance with the ethic committee of Tunis University for care and use of animals in conformity with NIH guideline (24). They were provided with food and water *ad libitum* and maintained in animal facility at fixed temperature of 22±2°C with a 12 h light-dark cycle. Rats were randomly divided into four groups of six animals each and daily intraperitoneally (i.p.) injected with either vehicle (C: ethanol 10%) or resveratrol (RVT: 25 mg/kg bw) for 8 days. Dox was dissolved in saline and administered at 20 mg/kg bw by a single i.p. injection on the fourth day. At the end of the treatment, rats were anesthetized with 0.5 ml urethane (40 mg/ml) and their blood collected and processed for biochemical determination of erythrocytes and plasma antioxidant parameters.

Blood processing

Whole blood was obtained by cardiac puncture and collected into heparinized tubes. Erythrocytes were isolated from plasma by centrifugation at 1000 g for 10 min at 4°C and homogenized using a hypotonic buffer Tris-HCl 10 mM pH 7.5, $MgCl_2$ 5 mM, NaCl 10 mM.

Plasma and erythrocytes transaminases

Plasma and erythrocytes aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured enzymatically using a commercial kit from Biomaghreb (Tunisia) following the manufacturer's procedure.

Lipoperoxidation

Lipoperoxidation was determined by MDA measurement according to the double heating method (25). Briefly, aliquots from erythrocytes homogenates or plasma were mixed with BHT-TCA solution containing 1% BHT (w/v) dissolved in 20% TCA (w/v) and centrifuged at 1000 g for 5 min at 4°C. Supernatant was blended with 0.5N HCl, 120 mM TBA in 26 mM Tris and then heated at 80°C for 10 min. After cooling, absorbance of the resulting chromophore was determined at 532 nm using a BIORAD UV-visible spectrophotometer. MDA levels were determined by using an extinction coefficient for MDA-TBA complex of $1.56\ 10^5\ M^{-1}cm^{-1}$.

Protein carbonylation

Oxidative damage to proteins was evaluated by quantifying protein carbonylation in erythrocytes homogenates and plasma according to Levine *et al.* (26). After proteins precipitation with 20% TCA and centrifugation at 11000 g for 3 min at 4°C (Beckman J20), pellet was dissolved in 10 mM DNPH-containing buffer. After 3 washings with ethanol-ethylacetate (1:1), pellet was dissolved in 20 mM potassium phosphate (pH 2.3) containing 6 M guanidine HCl and absorbance measured at 366 nm using the molar extinction coefficient of 22000 $M^{-1}cm^{-1}$. Results were expressed as nmol carbonyl residues/mg protein.

Protein determination

Total soluble proteins were determined according to the biuret method (27). Briefly, at acidic pH soluble proteins constituted with copper a colourful complex measurable at 546 nm using a SmartSpec 3000 BIORAD UV-visible spectrophotometer.

Antioxidant enzyme activities

All spectrophotometric analyses of erythrocytes or plasma antioxidant enzyme activities were performed with a SmartSpec 3000 BIORAD UV-visible spectrophotometer. Catalase (CAT) activity was assayed by measuring the initial rate of H_2O_2 disappearance at 240 nm (28). The reaction mixture contained 33 mM (1000 µl) H_2O_2 in 50 mM (1995 µl) phosphate buffer pH 7.0 and 5 µl of erythrocytes extract or plasma. CAT activity was calculated using the extinction coefficient of 40 $mM^{-1}cm^{-1}$ for H_2O_2 (28).

Peroxidase (POD) activity was measured at 25°C using guaiacol as hydrogen donor. The reaction mixture contained 9 mM (25 µl) guaiacol, 19 mM (100 µl) H_2O_2 in 50 mM (870 µl) phosphate buffer pH 7 and 5 µl of erythrocytes extract or plasma in 1 ml final volume. The reaction was initiated by the addition of H_2O_2 and monitored by measuring the increase in absorbance at 470 nm each 30 s for 3 min. Peroxidase activity was expressed as nmol of guaiacol oxidized per min with a molecular extinction coefficient of 26.2 mM^{-1} for calculation (29).

Superoxide dismutase (SOD) activity was determined by using modified epinephrine assay (30). At alkaline pH, superoxide anion (O_2^-) causes the auto-oxidation of epinephrine to adenochrome. One unit of SOD is defined as the amount of extract that inhibits the rate of adenochrome formation by 50%. Erythrocytes extract or plasma were added in 2 ml reaction mixture containing 10 µl bovine catalase (0.4 U/µl), 20 µl epinephrine (5 mg/ml) and 62.5 mM sodium carbonate/sodium bicarbonate buffer pH 10.2. Changes in absorbance were recorded at 480 nm. Characterization of SOD isoforms was performed using KCN (3 mM) which inhibited Cu/Zn-SOD or H_2O_2 (5 mM) affecting both Cu/Zn-SOD and Fe-SOD. Mn-SOD was insensitive to both inhibitors.

Free iron determination

Erythrocytes and plasma free iron levels were determined according to Leardi *et al.* (31) using a commercially available kit from Biomaghreb, Tunisia. At acidic pH 4.8 all Fe^{3+} released from transferrine was reduced by ascorbic acid into Fe^{2+}, which constituted with ferrozine a purple colourful complex measurable at 560 nm. Briefly, 50 µl of erythrocytes homogenates or plasma were added to 250 µl of reaction mixture containing ascorbic acid (5 g/L) and ferrozin (40 mM) and incubation performed at 37°C for 10 min.

H_2O_2 determination

Erythrocytes and plasma H_2O_2 were determined enzymatically according to Kakinuma *et al.* (32) using a commercially available kit from Biomaghreb (Tunisia). Briefly, in the presence of peroxidase H_2O_2 reacts with 4-amino-antipyrine and phenol to give a red colored quinoeimine

which absorbed at 505 nm. Results are expressed as mmol H_2O_2/mg protein.

Calcium measurement

Erythrocytes and plasma ionizable calcium was determined according to Stern and Lewis (33) using a commercially available kit from Biomaghreb, Tunisia. At basic pH calcium constituted with cresolphtalein a purple colourful complex measurable at 570 nm. Briefly, 50 µl of erythrocytes homogenates or plasma were added to 650 µl of reaction mixture containing 2-amino-2-methyl 1-propanol buffer (500 mmol/L), cresolphtalein (0.62 mmol/L) and hydroxy-8 quinoleine (69 mmol/L). Incubation was carried out at room temperature for 5 min assuming the complex was stable during 1 hour.

Statistical analysis

Data were analyzed by unpaired Student's t-test or one-way analysis of variance (ANOVA) and expressed as means ± standard error of the mean (S.E.M.). All statistical tests were 2-tailed, and $p<0.05$ was considered significant.

RESULTS

Erythrocytes and plasma lipoperoxidation and carbonylation

We reported in *Fig. 1* the effect of Dox and resveratrol on erythrocytes (*Fig. 1A*) and plasma (*Fig. 1B*) lipoperoxidation and on erythrocytes (*Fig. 1C*) and plasma protein carbonylation (*Fig. 1D*). Dox increased erythrocytes and plasma MDA and carbonyl protein. Resveratrol, which had no significant effect per se on plasma protein carbonylation, decreased it into erythrocytes. It also decreased MDA levels into erythrocytes and plasma. Moreover, resveratrol counteracted all Dox-induced effect on erythrocytes and plasma lipoperoxidation and carbonylation.

Erythrocytes and plasma transaminases

We further evaluated erythrocytes and plasma transaminases (*Fig. 2*). Dox significantly decreased erythrocytes ALT (*Fig. 2A*) and AST (*Fig. 2B*) and increased plasma ALT (*Fig. 2C*) and AST (*Fig. 2D*). Co-treatment with resveratrol counteracted all Dox-induced disturbances in transaminases to near control level.

Erythrocytes antioxidant enzyme activities

We further asked whether Dox affected antioxidant enzymes. Dox down-regulated CAT (*Fig. 3A*), POD (*Fig. 3B*) and SOD (*Fig. 3C*) activities, mainly the Fe isoform in this latter case. Resveratrol *per se* increased erythrocytes CAT, POD and SOD mainly the Cu/Zn isoform in this latter case. Resveratrol abrogated all Dox-induced in antioxidant enzyme activities.

Plasma antioxidant enzyme activities

Dox and resveratrol effect on plasma antioxidant enzyme activities was also evaluated. Dox up-regulated POD (*Fig. 4B*) but decreased CAT (*Fig. 4A*) and SOD (*Fig. 4C*) activities, mainly the Cu/Zn and Fe isoforms in this latter case. Resveratrol per se increased CAT, decreased POD but had no effect on SOD. Resveratrol treatment abrogated the Dox-induced disturbances in antioxidant enzyme activities.

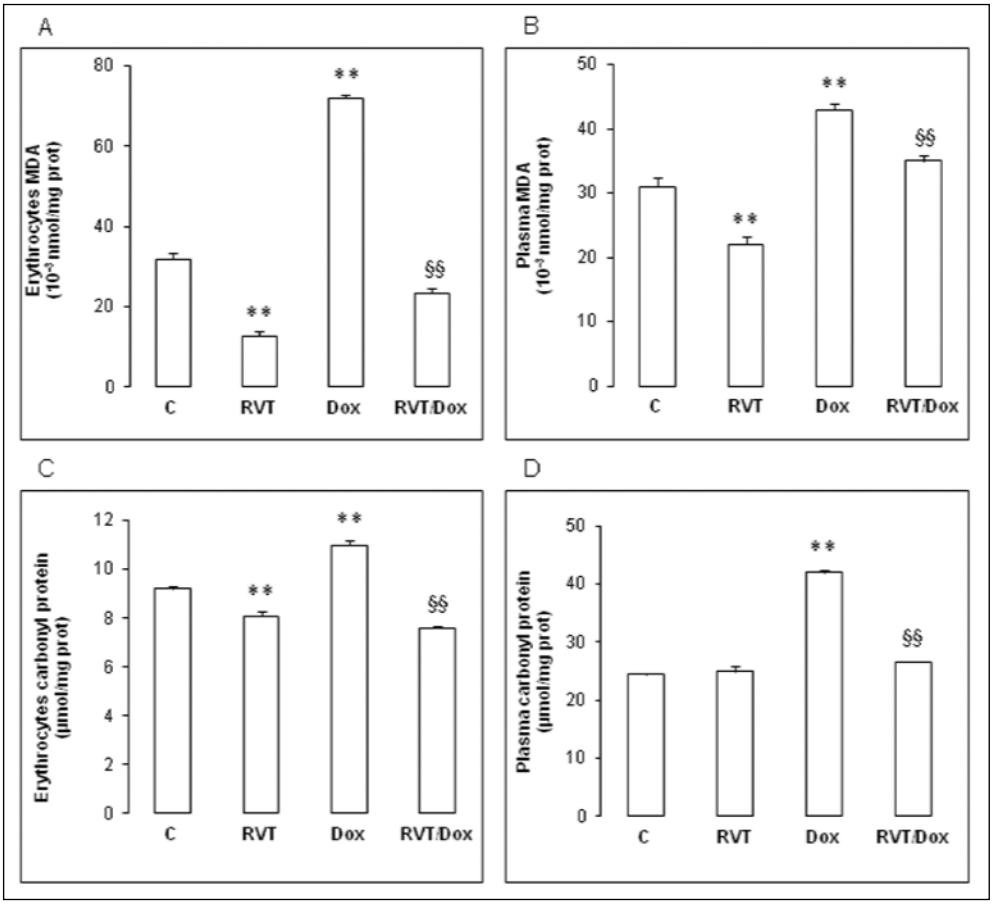

Fig. 1. Effect of doxorubicin (Dox) and resveratrol on erythrocytes and plasma oxidation. Rats were pre-treated or not with resveratrol during 8 days and challenged with a single dose of Dox at the fourth day. Erythrocytes (*Fig. 1A*) and plasma (*Fig. 1B*) MDA and erythrocytes (*Fig. 1C*) and plasma (*Fig. 1D*) protein carbonylation were determined. Results are expressed as mean ±S.E.M. (n=6); ** indicated $p<0.01$ *vs*. C; §§ indicated $p < 0.01$ *vs*. Dox.

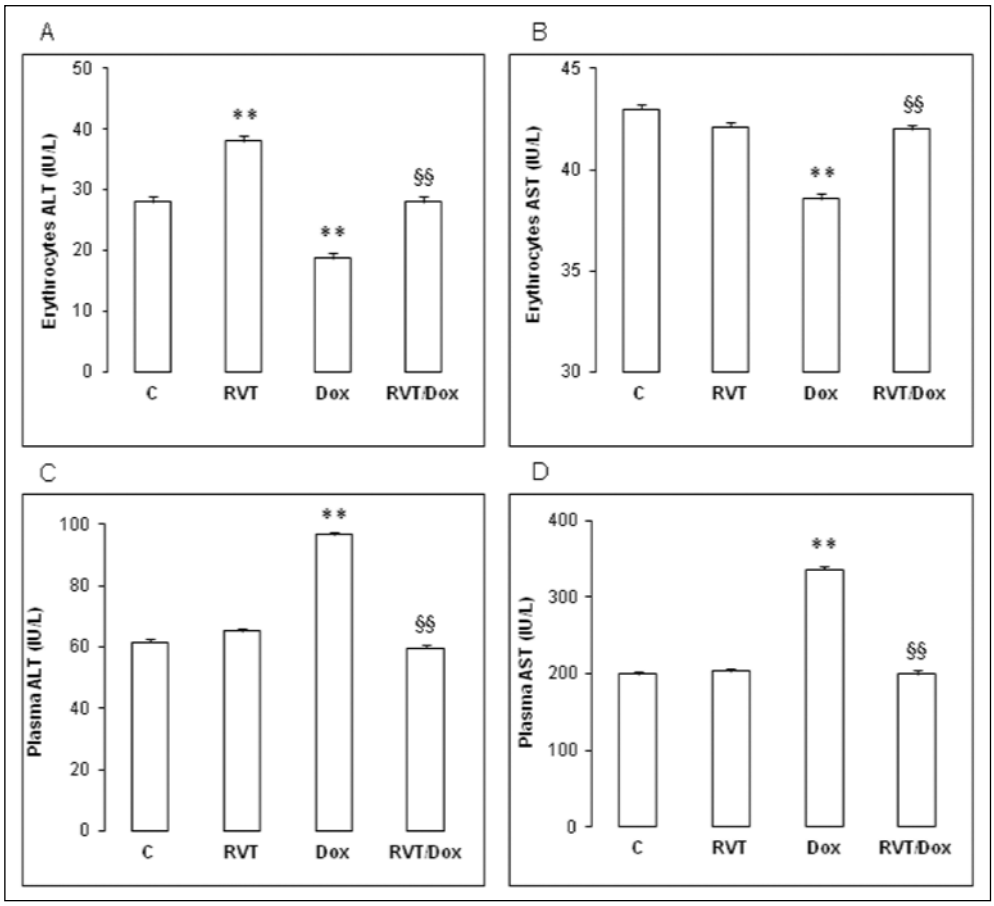

Fig. 2. Effect of Dox and resveratrol on erythrocytes and plasma transaminases. Rats were pre-treated or not with resveratrol during 8 days and challenged with a single dose of Dox at the fourth day. Erythrocytes (*Fig. 2A*) and plasma (*Fig. 2C*) ALT and erythrocytes (*Fig. 2B*) and plasma (*Fig. 2D*) AST were determined. Results are expressed as mean ±S.E.M. (n=6); ** indicated $p<0.01$ *vs*. C; §§ indicated $p<0.01$ *vs*. Dox.

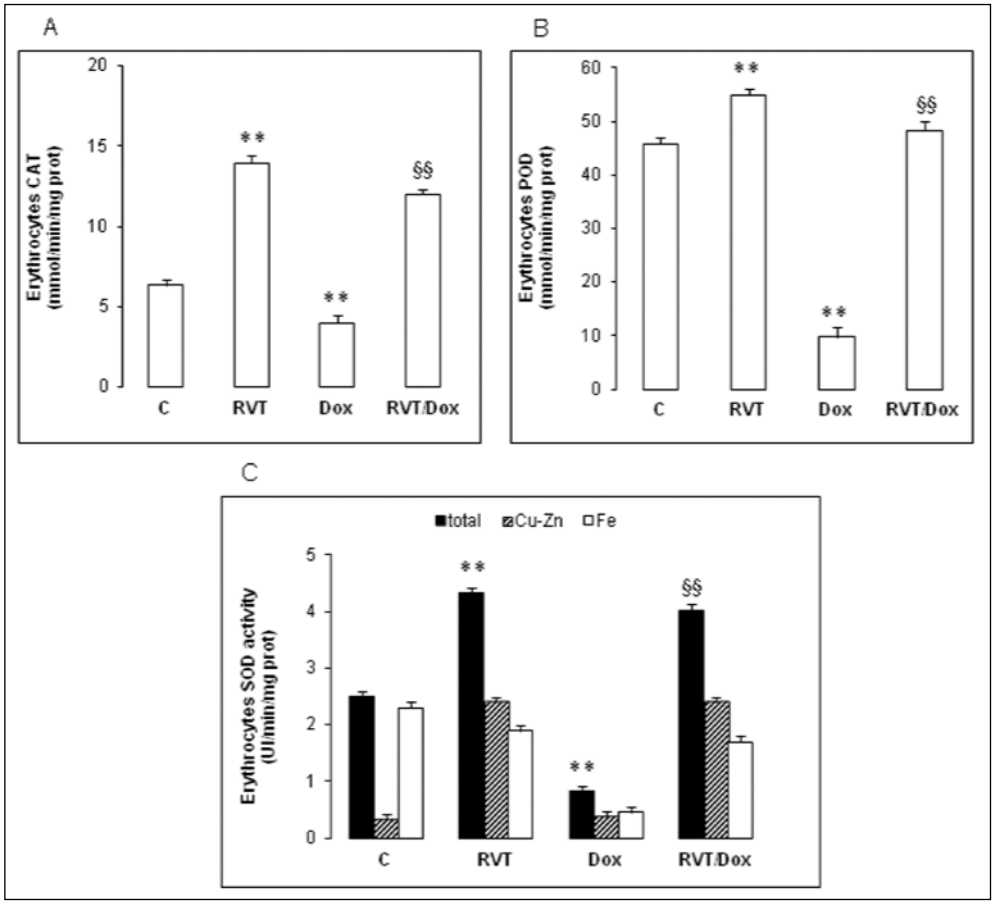

Fig. 3. Effect of Dox and resveratrol on erythrocytes antioxidant enzyme activities. Rats were pre-treated or not with resveratrol during 8 days and challenged with a single dose of Dox at the fourth day. Erythrocytes CAT (*Fig. 3A*), POD (*Fig. 3B*) and SOD (*Fig. 3C*) activities were determined. Results are expressed as mean ±S.E.M. (n=6); ** indicated $p<0.01$ *vs*. C; §§ indicated $p<0.01$ *vs*. Dox.

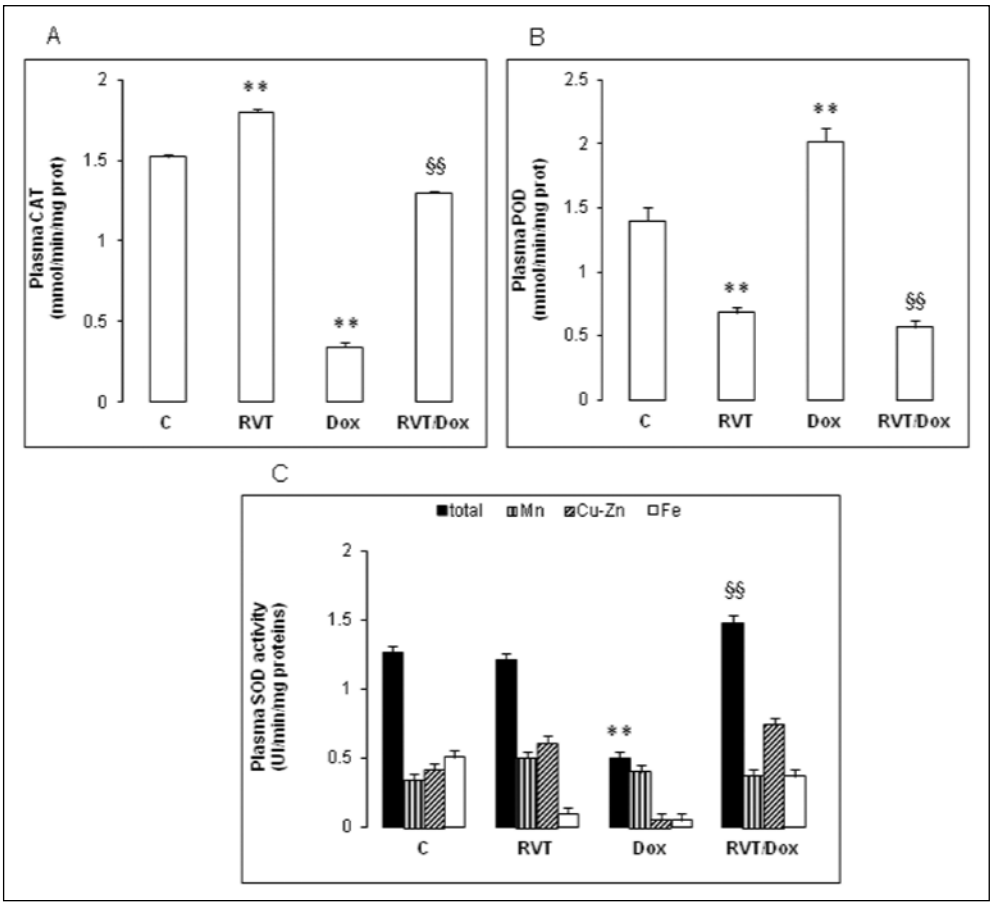

Fig. 4. Effect of Dox and resveratrol on plasma antioxidant enzyme activities. Rats were pre-treated or not with resveratrol during 8 days and challenged with a single dose of Dox at the fourth day. Plasma CAT (*Fig. 4A*), POD (*Fig. 4B*) and SOD (*Fig. 4C*) activities were determined. Results are expressed as mean ±S.E.M. (n=6); ** indicated $p<0.01$ *vs*. C; §§ indicated $p<0.01$ *vs*. Dox.

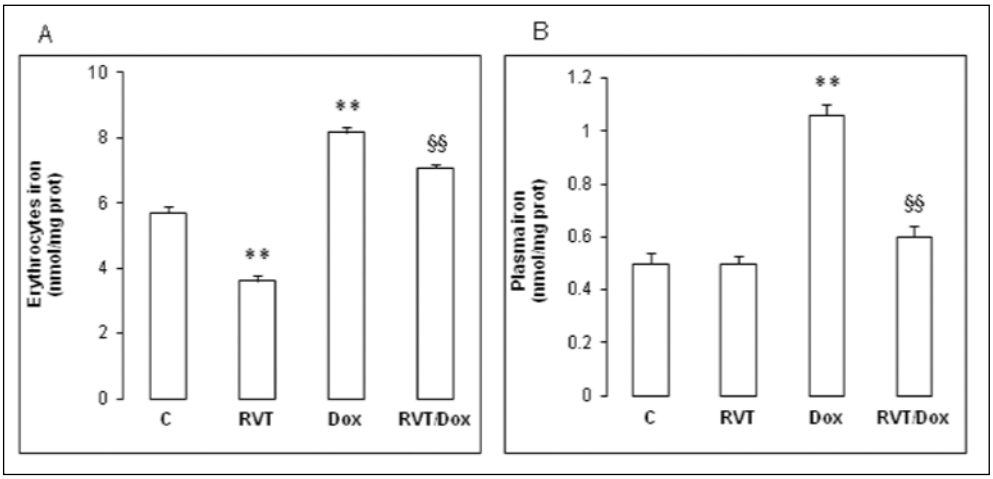

Fig. 5. Effect of Dox and resveratrol on erythrocytes and plasma iron. Rats were pre-treated or not with resveratrol during 8 days and challenged with a single dose of Dox at the fourth day. Erythrocytes (*Fig. 5A*) and plasma (*Fig. 5B*) free iron were determined. Results are expressed as mean ±S.E.M. (n=6); ** indicated $p<0.01$ *vs*. C; §§ indicated $p<0.01$ *vs*. Dox.

Erythrocytes and plasma free iron

We further sought to determine the putative involvement of iron in Dox effect (*Fig. 5*). Dox increased erythrocytes (*Fig. 5A*) and plasma (*Fig. 5B*) free iron. Resveratrol per se decreased erythrocytes iron without affecting it into the plasma. Co-treatment with resveratrol counteracted the Dox-induced free iron elevation till control level.

Erythrocytes and plasma H_2O_2

We reported in *Fig. 6* the effect of Dox on erythrocytes (*Fig. 6A*) and plasma (*Fig. 6B*) H_2O_2. Dox increased erythrocytes and plasma H_2O_2. Resveratrol per se decreased H_2O_2 into plasma but not into erythrocytes. Co-treatment with resveratrol counteracted Dox-induced H_2O_2 elevation to near control level.

Erythrocytes and plasma calcium

Fig. 7 dealt with the effect of Dox and resveratrol on erythrocytes and plasma calcium levels. Dox decreased calcium into erythrocytes (*Fig. 7A*) and plasma (*Fig. 7B*), whereas resveratrol per se had no effect. Co-treatment with resveratrol counteracted Dox-induced calcium depletion to control level.

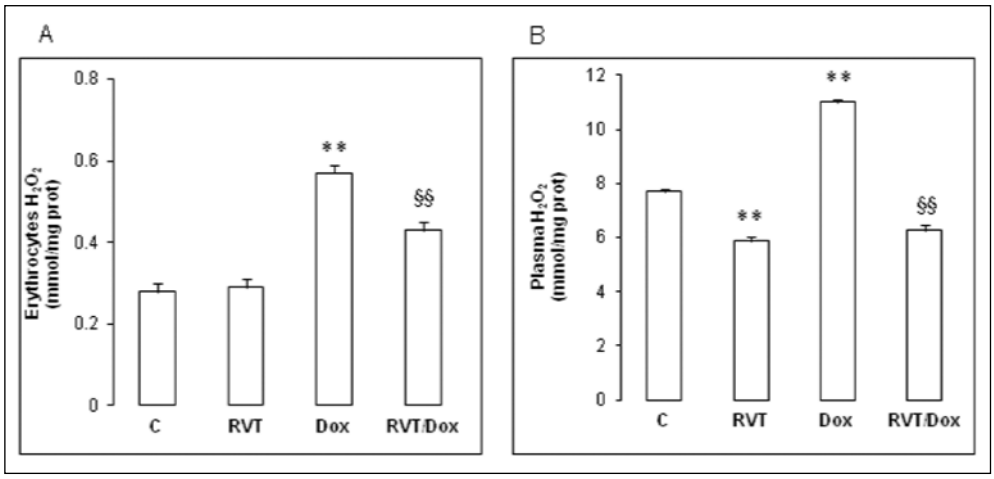

Fig. 6. Effect of Dox and resveratrol on erythrocytes and plasma H_2O_2. Rats were pre-treated or not with resveratrol during 8 days and challenged with a single dose of Dox at the fourth day. Erythrocytes (*Fig. 6A*) and plasma (*Fig. 6B*) H_2O_2 were determined. Results are expressed as mean ±S.E.M. (n=6); ** indicated $p<0.01$ *vs*. C; §§ indicated $p<0.01$ *vs*. Dox.

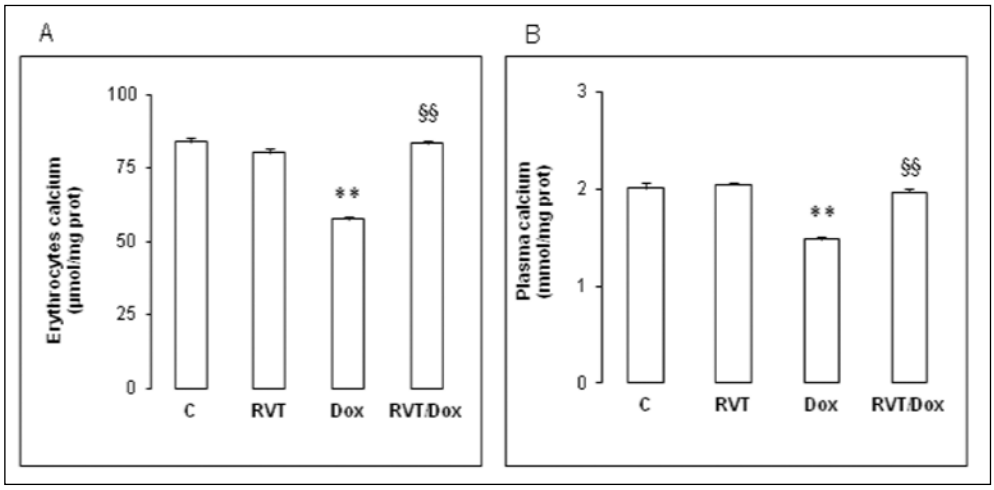

Fig. 7. Effect of Dox and resveratrol on erythrocytes and plasma calcium. Rats were pre-treated or not with resveratrol during 8 days and challenged with a single dose of Dox at the fourth day. Erythrocytes (*Fig. 7A*) and plasma (*Fig. 7B*) calcium were determined. Results are expressed as mean ±S.E.M. (n=6); ** indicated $p<0.01$ *vs*. C; §§ indicated $p<0.01$ *vs*. Dox.

DISCUSSION

In the present study we analyzed the toxicity of Dox into the blood compartment. We found that Dox provoked a drastic oxidative-stress status within erythrocytes and plasma as assessed by high MDA and carbonyl protein, elevated AST and ALT within plasma and a drastic depression in some of the antioxidant enzymes in both compartments. Dox also increased free iron and H_2O_2 and depressed Ca^{2+} within erythrocytes as well as into plasma. As Dox simultaneously increased free iron and H_2O_2, it likely induced the highly toxic hydroxyl radical which in turn affected Ca^{2+} homeostasis (34). Several previous studies have shown the implication of Ca^{2+} disturbances in Dox-induced cardiotoxicity (35). To our knowledge our data are the first to describe such a phenomenon into erythrocytes which could be considered as a useful biomarker of Dox-induced cellular damages and of clinical relevance.

Furthermore Dox treatment depressed CAT, POD and SOD activities within erythrocytes. Among SOD, the Fe-SOD was the only affected isoform, although we noticed in the same time that Dox increased both free iron and H_2O_2. Consequently Dox induced a drastic oxidative stress within erythrocytes that lead to hydroxyl radical generation which has been implicated in H_2O_2-induced calcium activation and cell death (34). However in the present case we found Dox to depress erythrocytes Ca^{2+} (36) whereas most studies described Dox-induced Ca^{2+} overloading (37). Differential experimental conditions as Dox administration at a single high dose of 20 mg/kg could explain this discrepancy. Further studies should investigate the putative involvement of calcium channels in the mechanism of action of Dox using calcium channel blockers as verapamil or nifedipine.

Overall Dox-induced oxidative stress within erythrocytes appeared much more drastic than into the heart as demonstrated by some of us (38). Because of the high polyunsaturated fatty acid content of their membranes and the high cellular concentration of oxygen, erythrocytes are highly susceptible to oxidative damage. Increased MDA level is known to affect the fluidity of the membrane lipid bilayer (39) and high MDA is generally correlated to stress-induced pathological conditions including aging (40).

In our opinion the most important result drawn from the present study is the ability of resveratrol to alleviate Dox-induced oxidative stress in blood compartment. Resveratrol is safe, devoid of any toxicity and could exert ROS scavenging activity (41). Thus, owing to its antioxidant properties (42), resveratrol counteracted Dox-induced lipoperoxidation, carbonylation and transaminases in erythrocytes and plasma.

Pre-treatment of gastric epithelial cells with red wine prevented from xanthine induced oxidative stress cell damage (43). Furthermore, grape seed extract protected the skin from damages induced by UV radiations (44). Resveratrol was also found to up-regulate antioxidant enzyme activities in the blood compartment as reported by some of us in the brain (45), heart (18, 46) and kidney (47). Our data corroborates a tremendous literature dealing with antioxidant and anti-inflammatory effects of resveratrol that have been observed *in vitro* or *in vivo* and in various experimental settings (48). Resveratrol efficiently protected the liver (49), the brain (50) and the blood

compartment (51) from oxidative stress induced by lipopolysaccharide, and from acetaminophen (21), cadmium (52), alcohol (23), CCl4 (53), naphthalene (54), pyrogallol (55) and ischemia/reperfusion injury (56).

Our data gave some new insight into Dox as well as resveratrol mode of action. Dox simultaneously increased free iron and H_2O_2 and resveratrol counteracted these effects. Consumption of resveratrol is protective against cardiovascular diseases in humans (57) and for human erythrocytes *in vitro* (58). A possible mechanism by which resveratrol exerted its beneficial effect on erythrocytes is its ability to chelate free iron and to scavenge H_2O_2. Free iron is a well established catalyst of auto-oxidation and iron-mediated oxidations of cysteine residues represent a common mechanism through which H_2O_2 exerts its second messenger role in signal transduction pathways (59). Further studies should analyze the implication of specific proteins involved in free iron metabolism as hepcidin (60). Moreover H_2O_2 is able to induce dual roles in both survival and cell death pathways, largely depending on its concentration and also on the cell type. H_2O_2 exerted a prolongevity effect in several species including rat by induction of SOD activity (61).

However, our results also raised several discrepancies. In particular Dox appeared in the same time as pro-oxidant in erythrocytes and plasma, being able to increase MDA, carbonyl proteins, free iron and H_2O_2 and also antioxidant by its ability to increase plasma POD activity. Besides, Dox decreased plasma CAT and SOD activities. In this latter case, Dox mainly down-regulated the Cu/Zn and the Fe isoforms. With regards to erythrocytes, Dox decreased CAT, POD and SOD activities especially the Fe isoform. Resveratrol per se was able to increase CAT, POD and SOD activities mainly the Cu/Zn isoform into erythrocytes. Concerning plasma, resveratrol up-regulated CAT, decreased POD but had no effect on SOD activity.

Modulation of antioxidant enzyme could correspond to post-translational modification as phosphorylation inducing some gain or loss of function. Thus, Borchi *et al.* (62) reported increased CAT and GPx activities after tyrosine phosphorylation in a case of human heart failure. Similarly, resveratrol could induce phosphorylation of CAT, POD and Cu/Zn-SOD and by this way enhanced their activities without modifying their expression nor abundance. At the opposite, resveratrol was shown to reduce endothelial oxidative stress by modulating the gene expression of SOD1 and GPx1 (63), which is unlikely to occur in our present case because of the enucleated status of erythrocytes.

Moreover we can't exclude a positive effect of resveratrol on Cu or Zn accumulation into erythrocytes leading to increased activity of the corresponding SOD isoform. Our data are consistent with those of Danz *et al.* (64) who reported cardioprotective effect of resveratrol against Dox-induced oxidative damage partly by its effect on Mn-SOD activity but not on protein expression. They further highlighted the use of erythrocytes as a convenient system for the study of resveratrol mode of action by means of post-translational regulation of antioxidative enzymes.

Conflict of interests: None declared.

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