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## Acute Exercise Protects Against Calcium-induced Cardiac Mitochondrial Permeability Transition Pore in Doxorubicin Treated Rats

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Running head: DOX and acute exercise-induced cardioprotection

**Abstract**

Doxorubicin (DOX), an antibiotic used in oncologic treatments, is limited by a dose-related cardiotoxicity, against which acute exercise is protective. However, the mechanisms related to this protection involving mitochondria remain unknown. Therefore, we aimed to determine the effects of an acute endurance exercise bout performed 24h before Doxorubicin (DOX) treatment on heart and liver mitochondrial function.

Twenty adult Wistar-male rats were divided as follows: non-exercised saline (NE+SAL), non-exercised DOX (NE+DOX), exercised-saline (EX+SAL) and exercised-DOX (EX+DOX). The animals performed a 60 min exercise bout on a treadmill or remained sedentary 24h before receiving either a 20 mg.kg<sup>-1</sup> DOX bolus or saline. Heart and liver mitochondrial function (oxygen consumption, membrane potential ( $\Delta\Psi$ ) and cyclosporin-A-sensitive calcium-induced mitochondrial permeability transition pore (MPTP) opening) were evaluated. Respiratory complex, MnSOD, caspase 3 and 9 activities as well as ANT, VDAC, Cyclophylin D, Bax and Bcl-2 contents were also measured.

Acute exercise prevented the decreased cardiac mitochondrial function (state 3, phosphorylative lag-phase, maximal  $\Delta\Psi$  generated both with complex I and II-linked substrates and calcium-induced MPTP opening) induced by DOX treatment. Exercise also prevented DOX-induced decreased cardiac mitochondrial chain complexes I and V, and increased caspase-3 and -9 activities. DOX administration and exercise caused increased cardiac mitochondrial SOD activity. Exercise ameliorated liver mitochondrial complex activities. No alterations were observed in the measured mitochondrial permeability transition pore and apoptosis-related proteins determined in heart and liver mitochondria. Data demonstrate that acute exercise protects against cardiac mitochondrial dysfunction, preserving mitochondrial phosphorylation capacity and attenuating DOX-induced decreased tolerance to MPTP opening.

Keywords: Adriamycin, Exercise, Mitochondrial bioenergetics, Permeability transition, Apoptosis

## Introduction

Doxorubicin (DOX, or adriamycin) is a highly effective antibiotic used to treat several types of cancer. Unfortunately, the clinical use of DOX is limited by the occurrence of a dose-related cardiac toxicity that results in life-threatening cardiomyopathy. It has been described that DOX-induced cardiomyocyte dysfunction is associated with increased levels of oxidative damage and apoptosis, involving mitochondria in the process [1,2].

The myocardial toxicity of DOX is related to a redox-cycling process at mitochondrial Complex I, which leads to increased oxidative stress, depletion of cellular reducing equivalents, inhibition of mitochondrial respiration and phosphorylation and interference with cellular calcium homeostasis [2,3]. Since the cardiomyocyte is an energy-demanding tissue, mitochondria are important players in cardiac bioenergetics and hence, it is considered that the toxicity of DOX at the mitochondrial level explains, in part, the development of the cardiotoxicity associated with drug treatment.

As described, one sensitive and early marker of DOX-induced cardiotoxicity is a loss of calcium loading capacity by mitochondria. Rather than inhibiting calcium uptake, DOX stimulates calcium release from the mitochondrial matrix through induction of the mitochondrial permeability transition pore (MPTP) [4-9], a physiological mitochondrial channel dependent on calcium overload and oxidative stress. In fact, the oxidative stress caused by DOX on cardiac mitochondria, associated with disturbances in cellular calcium homeostasis is considered an optimal environment for the formation and opening of the MPTP, contributing to the mitochondrial bioenergetic failure [1,2]. In an opened state, the MPTP is also associated with the release of pro-apoptotic mitochondrial proteins such as cytochrome c or the apoptosis inducing factor to the cytoplasm, contributing to trigger cell death. Depending on the treatment regimen, DOX administration can also cause perturbations in liver mitochondrial bioenergetics, although the severity is doubtless lower than in the heart [4,10].

Physical exercise, particularly endurance training has been importantly advised as a non-pharmacological tool against myocardial injury. In fact, it is well described that endurance exercise training improves myocardial tolerance to deleterious stimuli which cause intracellular oxidative stress and apoptosis [11,12]. Many preventive and therapeutic strategies have been explored to counteract DOX toxicity and dysfunction, such as antioxidant supplementation and exercise. The benefits of chronic aerobic exercise against DOX-induced cardiac toxicity and dysfunction have been established already. Previous work has suggested that the advantage of endurance training on the cardiovascular system of DOX-treated rats include the protection of cardiac tissue and mitochondria against increased oxidative damage and apoptosis [11,13-20]. It is also known that acute exercise is cardioprotective against damaging insults; in fact, studies revealed that a single endurance exercise bout preserved cardiac function and protected the heart against ischemia/reperfusion-induced oxidative damage and reduce infarct size [21-24]. Recently, Wonders et al. [25] reported that an acute exercise bout performed 24-hours before DOX treatment protected against cardiac dysfunction and decreased lipid peroxidation. However, the mechanisms related to this protection, particularly those involving mitochondria, were not described and remain elusive. Ji and Mitchel [26] reported that DOX administration after a single bout of acute exhaustive exercise antagonized the increased heart mitochondrial respiration induced by exercise during both ADP-stimulated and basal respiration. However, the effects of prior acute endurance exercise on the susceptibility of cardiac mitochondria from

DOX-treated rats to undergo the formation of MPTP and apoptosis was never explored, considering that the loss of calcium loading capacity is an early and sensitive marker of DOX-induced cardiotoxicity. Therefore, our present hypothesis is that a bout of acute exercise attenuates the loss of cardiac mitochondrial calcium loading capacity after DOX treatment. Isolated liver mitochondria from the different experimental groups were also used in order to verify if DOX and/or exercise effects were organ specific. The main finding of the present work was that acute exercise protected against DOX-induced cardiac mitochondrial dysfunction by increasing calcium accumulation capacity, thus reducing the DOX-induced increased susceptibility to calcium-induced MPTP opening.

## Methods

### *Animals*

Male Wistar rats (aged 6-8 wks, weighing 200 g at the beginning of the treatments) were obtained from Charles River (Barcelona). During the experimental protocol, animals were housed in collective cages (2 rats *per* cage) and were maintained in a room at normal atmosphere (21-22°C; ~50-60% humidity) receiving food and water *ad libitum* in 12 hours light/dark cycles. Rats were randomly assigned into four groups (n=5 *per* group): non-exercised saline (NE+SAL), non-exercised receiving DOX (NE+DOX), acutely exercised saline (EX+SAL) and acutely exercised treated with DOX (EX+DOX). The sample size of the present study resulted from the low dispersion and high consistency of the data observed during the experiments. The local Institutional Review Board approved the experimental protocol, which was in compliance with the *Guidelines for Care and Use of Laboratory Animals* in research.

### *Acute exercise protocol*

The protocol of animal acclimatization and acute exercise followed that recently used by Wonders et al., [25]. Briefly, animals from the exercised groups were accustomed to treadmill exercise over 2 weeks. During the first week, animals ran 10 min/d, 3 d/wk (10 m/min speed, 0% grade). During the second week, rats ran 10 min/d, 3 d/wk (20 m/min speed, 0% grade). This acclimation protocol has been shown to have minimal effect on mitochondrial enzymes [27]. Twenty-four hours after the acclimation protocol, rats successfully performed an acute single bout of treadmill running of 60 minutes divided into 3 phases. Phase 1 (minutes 0-5): animals ran at 15 m/min and a 0% grade; phase 2 (minutes 5-10): animals ran at 23 m/min and a 0% grade; and phase 3 (minutes 10-60): animals ran at 25 m/min and a 5% grade. The animals from non-exercised groups were placed on a non-moving treadmill for 60 min such that they were exposed to potential handling and environment stresses induced by the treadmill itself.

### *DOX administration*

Twenty-four hours after the cessation of the acute endurance exercise bout, or control non-exercised period, animals from NE+DOX and EX+DOX groups received a single i.p. DOX dose (20 mg.kg<sup>-1</sup>) while animals from NE+SAL and EX+SAL groups were injected with 0.5 mL of vehicle (sterile saline solution).

### *Animal sacrifice, plasma and heart extractions*

Five days after DOX or saline treatments, animals were anaesthetized with 50 mg.kg<sup>-1</sup> sodium pentobarbital and placed in the supine position. After that, the abdominal cavity was opened to expose the inferior cava vein and a blood sample of approximately 2 mL was collected in a EDTA-containing tube. The blood was immediately centrifuged (5 minutes at 5000xg, 4°C) and an aliquot of plasma was obtained and stored at -80°C for biochemical determination of cardiac troponin I (cTnI), glutamate oxaloacetate (GOT) and glutamate pyruvate (GTP) transaminases. After a quick opening of the chest and abdominal cavities, rat hearts and livers were then rapidly excised, rinsed, carefully dried and weighed. A portion of approximately 20-25 mg of tissue was separated, homogenized in homogenization buffer (20 mM Tris HCl, 100 mg/mL, pH 7.4) using a Teflon pestle on a motor-driven Potter-Elvehjem glass homogenizer at 0-4°C (3-5 times for 5 seconds at speed low setting, with a final burst at a higher speed setting). Homogenates were centrifuged 10 min at 3000xg at 4°C and the resulting supernatant was stored at -80°C for later determination of biochemical parameters.

### *Isolation of rat heart and liver mitochondria*

Mitochondria were prepared using conventional methods of differential centrifugation as follows [28]. Briefly, the animals were sacrificed as above stated and the organs were immediately excised and finely minced in an ice-cold isolation medium containing 250 mM sucrose, 0.5 mM EGTA, 10 mM Hepes-KOH (pH 7.4) and 0.1% defatted BSA (Sigma n<sup>o</sup> A-7030). The minced blood-free heart tissue was then resuspended in 40 mL of isolation medium containing 1 mg protease subtilopectidase A Type VIII (Sigma P-5380) *per g* of tissue and homogenized with a tightly fitted homogenizer (Teflon pestle). The suspension was incubated for 1 minute (4°C) and then re-homogenized. The homogenate was then centrifuged at 14,500xg for 10 minutes. The supernatant fluid was decanted and the pellet, essentially devoid of protease, was gently re-suspended in its original volume (40 mL) with a loose-fitting homogenizer. The suspension was centrifuged at 750xg for 10 minutes and the resulting supernatant was centrifuged at 12,000xg for 10 minutes. The pellet was resuspended using a paintbrush and re-pellet at 12,000xg for 10 minutes.

The blood free liver tissue was also resuspended in 40 mL of isolation medium and mechanically homogenized. The homogenate was then centrifuged at 800xg for 10 minutes and the resulting supernatant was centrifuged at 10,000xg for 10 minutes. The mitochondrial pellet was resuspended using a paintbrush and centrifuged twice at 10,000xg for 10 minutes to obtain a final mitochondrial suspension.

For both isolation procedures, EGTA and defatted BSA were omitted from the final washing medium. Mitochondrial and homogenate protein contents were determined by the Biuret method calibrated with BSA [29]. All isolation procedures were performed at 0-4°C. Considering the relatively greater abundance of intermyofibrillar (IMF) (~80%) compared with subsarcolemmal (SS) (~20%) mitochondria within the cells, a potentially dominant role for the IMF subfraction vs. the SS subfraction when studying treatment-based mitochondrial alterations is expected.

An aliquot of heart and liver mitochondrial suspension was taken after isolation and prepared for later semiquantification of proteins by Western Blotting as detailed below. The remaining fresh heart and liver mitochondrial suspensions were used within 4 hours for *in vitro* assays of



mitochondrial oxygen consumption, transmembrane potential, spectrophotometric osmotic swelling and fluorimetric calcium movements were maintained on ice (0-4°C) throughout this period. Isolation procedures yielded well-coupled mitochondria: the respiratory control ratio (RCR) of isolated mitochondria varied from 5-8 (with glutamate/malate) or 3-4 (with succinate plus rotenone) for controls, as determined according to the method of Estabrook [30].

#### *Mitochondrial oxygen consumption assays*

Mitochondrial respiratory function was measured polarographically, at 25°C, using a Biological Oxygen Monitor System (Hansatech Instruments) and a Clark-type oxygen electrode (Hansatech DW 1, Norfolk, UK). Reactions were conducted in 0.75 ml closed thermostatted and magnetically stirred glass chamber containing 0.5 mg of mitochondrial protein in a respiration buffer containing 65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 µM EGTA, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4. After 1-min equilibration period, mitochondrial respiration was initiated by adding glutamate/malate to a final concentration of 10 and 5 mM each, respectively, or succinate (10 mM) plus rotenone (4 µM). State 3 respiration was determined after adding 200 µM ADP; state 4 was measured as the rate of oxygen consumption in the absence of ADP. The RCR (state3/state 4) and the ADP/O ratios, the number of nmol ADP phosphorylated by nmol O<sub>2</sub> consumed, were calculated according to Estabrook [30], using 474 ngatom O/ml as the value for oxygen solubility at 25°C in doubly distilled water.

#### *Mitochondrial Membrane Potential*

Mitochondrial transmembrane potential ( $\Delta\psi$ ) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium (TPP<sup>+</sup>), followed by a TPP<sup>+</sup> selective electrode prepared in our laboratory as described by Kamo et al. [31] using a AgCl reference electrode (Tacussel, Model MI 402). Both the TPP<sup>+</sup> electrode and the reference electrode were inserted into an open vessel with magnetic stirring and connected to a pH meter. The signals were fed to a potentiometric recorder. No correction factor was used to correct the passive binding contribution of TPP<sup>+</sup> to membrane potential as the purpose of this study was to show the relative changes in the potential, rather than absolute values. As a consequence, a slight overestimation of the  $\Delta\psi$  values is anticipated. The  $\Delta\psi$  was estimated from the following equation (at 25°C):  $\Delta\psi = 59 \times \log (v/V) - 59 \times \log (10 \Delta E/59 - 1)$ , where  $v$ ,  $V$ , and  $\Delta E$  stand for mitochondrial volume, volume of the incubation medium, and deflection of the electrode potential from the baseline, respectively.

A mitochondrial matrix volume of 1.1 µl/mg of protein was assumed. Reactions were carried out in 1 ml of reaction buffer containing 65 mM KCl, 125 mM sucrose, 10 mM Tris, 20 µM EGTA, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4, supplemented with 3 µM TPP<sup>+</sup> and 0.5 mg/ml of protein with the temperature maintained at 25°C. For measurements of  $\Delta\psi$  with complex I substrates, energization was carried out with 10 mM of glutamate and 5 mM of malate and ADP-induced phosphorylation was achieved by adding 200 µM ADP. For measurements of  $\Delta\psi$  with complex II substrates, 10 mM succinate supplemented with 4 µM rotenone were added to the medium containing 3 µM TPP<sup>+</sup> and mitochondria. The lag phase, which reflects the time needed to phosphorylate the added ADP, was also measured for both substrates.

### *Determination of Mitochondrial Calcium Accumulation and PTP Induction*

Mitochondrial calcium accumulation capacity was determined by adding small pulses of calcium (100 and 20 nmol each, for heart and liver mitochondria, respectively until MPTP opening was observed as an irreversible drop in  $\Delta\psi$ ). The reaction was continuously stirred and the temperature was maintained at 25°C. The assays were performed in 1 ml of reaction medium containing 200 mM sucrose, 10 mM Tris, 10  $\mu$ M EGTA, 5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4, supplemented with 4  $\mu$ M rotenone, 10 mM succinate with 0.5 mg/ml protein. Control trials were made by using 1  $\mu$ M of cyclosporin-A, the selective MPTP inhibitor [32].

### *cTnl, GOT and GTP*

cTnl concentration was quantitatively determined with an established immunoassay using commercial Abbott kit (Amadora, Portugal).

Plasma activities of GOT and GTP were determined spectrophotometrically using commercial ABX Diagnostics kits (Mompellier, France).

### *Respiratory complex activities*

Mitochondrial membranes were disrupted by a combination of freeze-thawing cycles to allow free access to substrates. All the assays were performed at 30°C in a final volume of 500  $\mu$ L. Complex I activity was measured by following the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm for 4 minutes, after which rotenone was added and the absorbance was measured again for 4 minutes [33]. Complex II activity was determined according to Birch-Machin et al. [34]. Briefly, the catalyzed reduction of DCIP by the protein complex was followed at 600 nm for 3 minutes after addition of 65  $\mu$ M ubiquinone. Complex V ATP synthase activity was measured according to Simon et al. [35]. Phosphate produced by hydrolysis of ATP reacts with ammonium molybdate in the presence of reducing agents to form a blue-colour complex, the intensity of which is proportional to the concentration of phosphate in solution. Oligomycin was used as an inhibitor of mitochondrial ATPase activity.

### *Analysis of ANT, VDAC, Cyclophilin D, Bcl-2 and Bax Protein Levels*

Equivalent amounts of proteins obtained from heart and liver mitochondrial suspensions were electrophoresed on a 15% SDS-PAGE gel as described by Laemmli [36], followed by blotting on a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech) according to Locke et al. [37]. Membranes were stained with Ponceau S to verify quality of transfer and equal protein loading. After blotting, non-specific binding was blocked with 5% non fat dry milk in TTBS (Tris-buffered saline (TBS) with Tween 20) and the membrane was incubated with either anti-Bcl-2 (1:500; sc-7382 mouse monoclonal IgG; Santa Cruz Biotechnology) or anti-Bax (1:500; sc-493 rabbit polyclonal IgG; Santa Cruz Biotechnology), or anti-ANTQ18 (1:1000; sc-9300 goat polyclonal IgG; Santa Cruz Biotechnology) or anti-VDAC/porin (1:1000; ab34726 rabbit polyclonal, IgG; abcam) or anti-Cyp D (1:1000; sc-33068 goat polyclonal IgG; Santa Cruz Biotechnology) antibodies for 2 hours at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies (1:1000; Amersham Pharmacia Biotech) or anti-goat IgG antibodies (1:1000; 705-035-147 Jackson ImmunoResearch Laboratories) for 2 hours.



Protein bands were visualized by treating the immunoblots with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshire, UK), according to the supplier's instructions, followed by exposure to X-ray films (Sigma, Kodak Biomax Light Film, St. Louis, USA). The films were analyzed with QuantityOne Software (BioRad). Optical density results were expressed as percentage variation of control values.

#### *Caspase Activity Assay*

To measure caspase 3 and 9 activities, aliquots of heart ventricles homogenate were incubated in a reaction buffer containing 25 mM Hepes (pH 7.4), 10% sucrose; 10 mM DTT, 0.1% CHAPS and 100  $\mu$ M caspase substrate Ac-LEHD-*p*NA (235400, Calbiochem) for 2 h at 37°C. Caspase activity was determined by following the detection of the chromophore *p*-nitroanilide after cleavage from the labeled substrate Ac-LEHD-*p*-nitroanilide. The method was calibrated with known concentrations of *p*-nitroanilide (Calbiochem).

#### *MnSOD Activity*

Heart and liver MnSOD activity was determined spectrophotometrically according to Marklund and Marklund [38] by monitoring the rate of autoxidation of pyrogallol at 420 nm. The reaction buffer (25°C) consisted of 1 mM *N,N*-bis(2-(bis(carboxymethyl)amino)-ethyl) glycine (DTPA) and 50 mM Tris-HCl, pH 8.2, in 1 mL final volume. Pyrogallol (0.3 mM) was added to the cuvette to start the reaction and the rate of absorbance increase was measured. One unit of SOD activity was defined as the amount of sample required to inhibit the autoxidation of pyrogallol by 50%, and the activity is expressed as Units per mg of protein.

#### *Statistical analysis*

Mean and standard error of mean were calculated for all variables in each group. Two-way ANOVA followed by the Bonferroni post-hoc test was used to compare groups. Statistical Package for the Social Sciences (SPSS Inc, version 10.0) was used for all analyses. The significance level was set at 5%.

### **Results**

As can be seen from table 1, there were no differences in the mean body mass of the animals among the experimental groups at the time of injection. Significant decreases were observed in the variation of body mass 5 days after DOX treatment, although not independently of the exercise status as  $\Delta$  body mass in EX+DOX group was also lower than in the NE+SAL group. Absolute heart mass as well as heart to body mass ratio in NE+DOX and EX+DOX groups were significantly lower than NE+SAL.

Significant increases in the plasma content of cardiac troponin I were observed in NE+DOX and EX+DOX when compared to NE+SAL group (Table 1). Plasma levels of GOT and GTP were determined as combined markers of liver damage. DOX treatment induced an increase in GOT (NE+SAL vs. NE+DOX and EX+DOX).

Considering the high acute bolus of DOX administered to the animals and the 5 days between injection and the sacrifice, some level of mortality could likely be expected. However, the percentage of rat survival was 100%, as no animal had died before the scheduled sacrifice.

\*\*\*Insert Table 1 \*\*\*

### *Oxygen consumption and $\Delta\Psi$*

#### Heart mitochondria

To elucidate if a single acute running bout performed 24 hours before DOX treatment could prevent the DOX-associated cardiac mitochondrial dysfunction, oxygen consumption (Table 2) and transmembrane potential ( $\Delta\Psi$ ) (Table 3) were determined with substrates for both Complex I and II.

Using complex I and II substrates, DOX treatment caused a significant decrease in heart mitochondrial state 3 respiratory rates in the non-exercised groups (NE+SAL vs. NE+DOX), which was prevented by exercise. Acute running exercise *per se* (NE+SAL vs. EX+SAL) increased the RCR when glutamate-malate was used as substrate. When compared to non-exercised DOX treated group, an increased state 4 respiratory rate was observed in the exercised DOX treated (NE+DOX vs. EX+DOX) when succinate was used.

As seen in Table 3, DOX treatment caused significant decrease in maximal  $\Delta\Psi$  developed and in the repolarization after ADP phosphorylation in the non-exercised group (NE+SAL vs. NE+DOX). The decrease in both parameters was counteracted by previous exercise. Also, an important result is that DOX increased the time necessary for the phosphorylation of the added ADP, a negative effect which was antagonized by acute exercise.

\*\*\*Insert Table 2 \*\*\*

\*\*\*Insert Table 3 \*\*\*

#### Liver mitochondria

DOX and exercise induced fewer alterations in the functional endpoints evaluated in liver mitochondria during oxygen consumption and  $\Delta\Psi$  experiments, when compared to what was observed in heart mitochondria, in which the effects were more evident. Nevertheless, an unexpected increase in state 3 respiration caused by DOX treatment (NE+SAL vs. NE+DOX) was observed when succinate was used as substrate.

### *Calcium accumulation and MPTP induction*

#### Heart mitochondria

Calcium-induced  $\Delta\Psi$  depolarization is a typical phenomenon that is associated with MPTP induction. Figure 1 and table 4 show the alterations of heart mitochondrial electric potential induced by DOX and exercise during the addition of several calcium pulses until the threshold for MPTP opening was reached.

Regarding heart mitochondria, DOX treatment induced a decrease in both the  $\Delta\Psi$  developed after the addition of 5 calcium pulses and in the total amount of calcium pulses accumulated before MPTP opening, when compared to all other groups. Therefore, heart mitochondria from exercised rats were able to accumulate more calcium than their non-exercised counterparts, when later treated with DOX (NE+DOX vs. EX+DOX).

### Liver mitochondria

Exercise *per se* (NE+SAL vs. EX+SAL) increased the capacity of liver mitochondria to maintain  $\Delta\psi$  after 3 calcium pulses, although no differences between groups were found regarding the total number of calcium pulses added before MPTP opening (Table 4).

\*\*\* Insert Figure 1 \*\*\*

\*\*\* Insert Table 4 \*\*\*

### *Respiratory complex activities*

#### Heart mitochondria

The activities of respiratory complex I, II and V were also determined in heart and liver mitochondria (Table 5). As seen in the table, acute exercise prevented the impairment of heart mitochondrial complex I activity and attenuated the decreased complex V activity caused by DOX. Interestingly, exercise *per se* (NE+SAL vs. EX+SAL) increased heart mitochondrial ATP synthase (complex V) activity.

#### Liver mitochondria

Although no significant effects of DOX were observed in the activities of the analyzed complexes in the livers of non-exercised rats (NE+SAL vs. NE+DOX), the combination treatment (EX+DOX) resulted in an increased activity of the complexes compared to the non-exercised treated group (NE+DOX vs. EX+DOX). Exercise alone also increased liver mitochondrial complex I, II and V activities.

\*\*\* Insert Table 5 \*\*\*

### *Apoptotic signaling and MPTP-related proteins*

#### Heart and liver

As markers for apoptotic signaling, we measured heart tissue caspase 3 and 9 activities. As can be observed in figure 2, DOX increased both caspase 3 and 9 activities in non-exercised (NE+SAL vs. NE+DOX) animals, an effect which was prevented by the previous single bout of running.

\*\*\* Insert Figure 2 \*\*\*

Immunoblots were then performed in order to semiquantify heart and liver mitochondrial proteins that are known to be involved in mitochondrial apoptotic signalling including the pro and anti-apoptotic Bcl-2 family proteins, Bax and Bcl-2, respectively as well as MPTP component and/or sensitizing proteins such as the adenine nucleotide translocator (ANT), the voltage dependent anion channel (VDAC) and Cyclophylin D (Cyp D), (Figures 3 and 4). However, none of the stimuli either isolated or combined caused significant changes in any of these proteins.

\*\*\* Insert Figure 3 \*\*\*

\*\*\* Insert Figure 4 \*\*\*

### *MnSOD activity*

#### Heart and liver

In order to measure an end-point for mitochondrial antioxidant enzymatic protection, the activity of the mitochondrial specific isoform of SOD in liver and heart was determined (Figure 5). Both DOX (NE+SAL vs. NE+DOX) and exercise (NE+SAL vs. EX+SAL) *per se* induced an increase of SOD activity in heart mitochondria. In liver mitochondria, the only observed increase on SOD activity was induced by exercise alone.

\*\*\* Insert Figure 5 \*\*\*

### **Discussion**

In the present work, we tested the hypothesis that impairment in mitochondrial bioenergetics, including increased susceptibility to calcium-induced MPTP opening in isolated heart mitochondria after acute DOX treatment are prevented by 60 minutes of treadmill running performed 24 hours prior to DOX treatment. Our hypothesis was confirmed as mitochondrial function including calcium accumulation capacity that were affected by DOX, was prevented by exercise. However, no large body of conclusive data were obtained regarding the mechanisms associated with decreased apoptotic signaling provided by exercise on DOX-treated rats, as seen by the data on caspase-3 and -9 activities. In fact, MPTP regulatory and/or structural components such as Bax, Bcl-2, ANT, Cyp D and VDAC were unchanged in all experimental groups.

Data on mitochondrial function obtained in the present work confirms, at least in part, the cardiac protection afforded by acute exercise previously described against deleterious stress stimuli [21-24] particularly acute single dose of DOX treatment [25].

Cardiac dysfunction associated with defective mitochondrial function in DOX treated animals has previously been studied [2,3]. The present work confirmed that DOX administration results in decreased heart mitochondrial respiration, maximal developed  $\Delta\Psi$  and increased phosphorylative lag phase (Tables 2 and 3). Moreover, DOX decreased the ability of heart mitochondria from non-exercised rats to accumulate calcium before MPTP opening (Table 4 and Fig 1). The acute bout of running performed 24h before DOX treatment resulted in attenuation or complete prevention of the heart mitochondrial impairments induced by DOX.

Alterations in mitochondrial oxidative phosphorylation induced by DOX can be originated by several factors: 1) inactivation of dehydrogenases, providing limited amounts of reducing equivalents to the mitochondrial electron transport chain, 2) decreased aconitase activity and 3) decreased activity/content/organization of electron transport chain complexes or proteins of the phosphorylation system.

Sequestration of excess cytosolic calcium by mitochondria represents an important cytoprotective mechanism in the latter stages of cell injury [39]. The interplay between mitochondrial fidelity and cell calcium homeostasis is exemplified by the fact that calcium enters the mitochondrial matrix via an electrophoretic mechanism [39]. Consequently, the inward flux of calcium represents a depolarizing current, the strength of which is determined by the  $\Delta\Psi$ , which itself is a function of the fidelity of the mitochondrial electron transport chain. The significant decrease in mitochondrial  $\Delta\Psi$  observed in the present study agrees with the impairment reported in cardiomyocytes incubated with DOX [40]. It is however important to

note that the  $\Delta\Psi$  values above -200 mV in all experimental groups do not seem to compromise ATP synthase flow or the transports of ions and metabolites. In fact, the range of the  $\Delta\Psi$  is -120 to -220 mV. For instance, regarding the driving force for ATP generation, it has been shown that the kinetics of the ATP synthase follow a sigmoid pattern in response to  $\Delta\Psi$ , reaching saturation at approximately -100 mV [41].

One possible explanation for the observed protective effect of exercise may be associated with the preservation of mitochondrial complex activity, namely complex I and V in EX+DOX in opposition to the decreased activity observed in NE+DOX group (Table 5). In fact, mitochondrial complex I of the respiratory chain catalyses the futile redox cycling of DOX with consequent generation of free radicals [42,43] being the activity of this complex affected in the process [4]. Others have also reported DOX-induced impairments in mitochondrial ATPase activity or in the redox state of respiratory carriers [44]. As opposed to the increment in heart mitochondrial complex V observed in the present study, in which animals were sacrificed 6 days after 60 minutes of endurance exercise, Bo et al. [45] described no alterations in heart mitochondrial ATP synthase immediately after acute exercise. Possible differences in the time between the end of exercise and mitochondrial sampling may justify the discrepancies.

As mentioned, one early and sensitive marker of DOX toxicity is related to the increased susceptibility to MPTP opening, although not exclusively in heart mitochondria. Previous data showed that heart mitochondria isolated from sedentary animals treated during 7 weeks with cumulative DOX doses revealed a decreased ability to accumulate calcium caused by enhanced MPTP induction [4-6,9]. In addition, liver mitochondria from DOX treated rats also show increased sensitivity to MPTP induction [4,46]. Several important pharmacological and non-pharmacological countermeasures have been successfully used against DOX-induced mitochondrial toxicity, including antioxidants and physical exercise [5,11]. In fact, physical exercise, particularly endurance training has proven to positively modulate heart MPTP dynamics [47-49].

Results of the present study provide for the first time evidence that 60 minutes of endurance treadmill running afforded protection against the increased sensitivity of the heart to MPTP opening caused by DOX administration (Table 4 and Fig 1). Calcium accumulation capacity of heart and liver mitochondria from each of the four experimental groups was determined by following the effect of known amounts of calcium (pulses of 100 and 20 nmol  $\text{CaCl}_2$  each for heart and liver mitochondria, respectively) on mitochondrial  $\Delta\Psi$  (Table 4 and Fig 1). Mitochondria possess a finite capacity for accumulating calcium before undergoing the calcium-dependent MPTP in a phosphate-rich medium, resulting in irreversible apoptosis. Our results showed that heart mitochondria from NE+DOX rats have lower calcium tolerance than all other groups including EX+DOX (Fig 1). These results show that acute exercise activated cellular and/or mitochondrial defense mechanisms that probably contributed to prevent the increased MPTP opening susceptibility caused by DOX. Regarding liver mitochondria, no cross tolerance effect was observed as DOX did not alter calcium accumulation capacity and only EX+SAL group demonstrated a higher  $\Delta\Psi$  developed after 3 pulses of 20 nmol calcium when compared with NE+SAL.

In an attempt to better understand the above referred mechanisms that might contribute to the observed functional phenotypes, we quantified the levels of several potential proteins



known to be involved in the modulation of the pore, either as components and/or sensitizers, including ANT, Cyp D, VDAC, Bax and Bcl-2 (Figs 3 and 4).

Calcium-induced MPTP is modulated by a variety of physiological effectors. It is increasingly recognized that the molecular composition of the pore is likely variable [50,51]; despite this, the prevailing hypothesis is that the ANT, VDAC and the regulatory matrix Cyp D are the most common proteins forming the MPTP complex [52,53]. In fact, despite some conflicting results [54,55], variations in the level of expression of the MPTP component/regulatory proteins ANT and Cyp D were reported to correlate with the susceptibility of isolated mitochondria to undergo PTP opening and decisively contribute to mitochondrial dysfunction [56,57]. In fact, it was previously shown that the toxicity caused by DOX on cardiac tissue involved a decrease in ANT levels [9]. However, this was not observed in the present study as none of these important proteins was significantly altered by DOX and/or exercise (Fig 3). It is possible that the functional results obtained are related to the coordinated modulation of other mechanisms involving for instance stress chaperones, antioxidants or other defense systems. In fact, it is known that in response to endurance exercise cardiac tissue up-regulates the levels of heat shock proteins [58], which act as molecular chaperones possibly contributing to the preservation of integrity and activity of mitochondrial complexes. The protective role of heat shock proteins may be accomplished through facilitation of nuclear-encoded protein importation and assembly in the mitochondrial matrix and through the improved assisted folding of proteins within mitochondria. Moreover, acute endurance exercise also induces an increase in the content of cardiac uncoupling proteins [45], which are suggested to contribute to a decrease in free radical production by causing a slight dissipation of  $\Delta\Psi$  (mild uncoupling) [59] and also by translocating fatty acid peroxides from the inner to the outer membrane leaflet [60].

Interestingly, the levels of MnSOD activity of heart mitochondria from NE+DOX and EX+SAL and of liver mitochondria from EX+SAL were increased when compared to NE+SAL, which can be interpreted as a sign of adaptation of both stimuli to the increased oxidative stress imposed. Intriguing was the fact that although MnSOD activity from NE+DOX and EX+SAL had increased, no up-regulation in activity was detected when exercise and DOX were combined (EX+DOX). One possible explanation is that both stimuli combined may lead to up-regulation of other antioxidant defense mechanisms, which would make an increased expression of MnSOD not necessary to counteract any particular oxidative stress. Further work in this regard is warranted.

Mitochondrial (dys)function is increasingly considered a key event in a variety of forms of cell death, including apoptosis. Therefore, we also analyzed the effects of DOX and exercise on caspase-3 and -9 activities (Fig 2) and, as mentioned, on the expression of the pro- and antiapoptotic Bcl-2 family proteins Bax and Bcl-2 (Fig 3). The relative expression of the later in the mitochondrial outer membrane is thought to decide the fate of the cell by regulating outer membrane integrity and the consequent release of mitochondrial apoptotic proteins such as cytochrome c or the apoptosis-inducing factor [61]. Moreover, data suggest that Bax, and perhaps its homologs Bak and Bid, may regulate MPTP opening, considering that these effects are antagonized by anti-apoptotic Bcl-2 family members [50]. Unexpectedly, our results do not confirm previous findings reporting increased Bax/Bcl-2 4-5 days after acute DOX treatment [15,62]. However, the observed increased activities of heart tissue caspase-3 and -9 with DOX in



non-exercise group was prevented by exercise, which suggest that DOX-induced *in vivo* increased downstream cellular and mitochondrial-related apoptotic events which were prevented by exercise.

In conclusion, our results indicate that the respiratory dysfunction and the increased susceptibility to PTP gating of heart mitochondria isolated from DOX-treated rats can be prevented by an acute bout of endurance treadmill running. These mitochondrial adaptations could potentially be beneficial to the cardiac tissue in the setting of the DOX cardiomyopathy, a condition in which acute exercise was shown to be protective [25]. Considering that DOX can result in a mitochondrial disease with consequent loss of organelle and tissue functions, non-pharmacological countermeasures such as physical exercise that can afford protection against this mitochondrionopathy should be taken into account, due to its positive effects.

### Grants

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## Figure legends

**Figure 1.** Calcium accumulation capacity of freshly heart mitochondria isolated from each animal: calcium accumulation capacity, measured with a TPP+ selective electrode at 25°C in a total volume of 1 mL, was assessed by adding several pulses of CaCl<sub>2</sub> (100 nmol/mg protein each), in order to induce MPTP. The opening of MPTP was determined as a drop in  $\Delta\psi$ . In the presence of cyclosporin A, a specific inhibitor of MPTP induction, the number of pulses of calcium needed to induce a similar decrease membrane potential is much higher for all mitochondrial preparations from each group (not shown). The traces are representative of experiments performed with different mitochondrial preparations.

**Figure 2.** Effect of DOX treatment and acute exercise on caspase 3 and caspase 9 activities of heart tissue. Data are means $\pm$ SEM, # vs. all other groups.

**Figure 3.** Semiquantification of heart and liver mitochondrial Bax (upper panel) and Bcl-2 (lower panel) protein expression for each experimental condition. Typical immunoblots of Bax and Bcl-2 are presented.

**Figure 4.** Semiquantification of heart and liver mitochondrial ANT (upper panel), Cyp D (middle panel) and VDAC (lower panel) protein expression for each experimental condition. Typical immunoblots of VDAC, Cyp D and ANT are presented.

**Figure 5.** Effect of DOX treatment and acute exercise on heart and liver mitochondrial SOD activities. Data are means $\pm$ SEM, \* vs. NE+SAL.

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**Table 1** Animal data and mitochondrial protein isolation yielding

	NE+SAL	NE+DOX	EX+SAL	EX+DOX
Body mass at injection (g)	241.0±24.4	261.5±29.5	277.8±12.3	278.8±9.4
Body mass at sacrifice (g)	268.3±25.7	233.8±27.9	293.3±12.1	243.5±7.3
Δ body mass (g)	27.3±3.5	-27.7±1.7*	15.5±1.1*	-34.5±2.9*
Heart mass (g)	0.78±0.04	0.53±0.06*	0.85±0.01	0.64±0.03
Heart mass/body mass (mg.g <sup>-1</sup> )	2.99±0.15	2.31±0.08*	2.94±0.09	2.65±0.16*
Plasma cardiac troponin I (ng.mL <sup>-1</sup> )	0.0±0.0	1.20±0.31*	0.1±0.01	1.14±0.21*
Heart mitochondrial yielding (mg.g <sup>-1</sup> )	20.7±2.3	22.8±6.4	18.6±2.6	23.5±1.9
Liver mass (g)	9.6±1.0	7.3±0.7	9.4±0.6	7.6±0.2
Liver mass/body mass (mg.g <sup>-1</sup> )	3.6±0.8	3.2±1.5	3.2±0.9	3.2±1.8
Plasma GOT (U.L <sup>-1</sup> )	162.5±13.3	244.5±25.8*	166.1±19.0	230.1±17.6*
Plasma GPT (U.L <sup>-1</sup> )	49.3±7.4	44.3±3.5	54.2±4.6	50.7±2.9
Liver mitochondrial yielding (mg.g <sup>-1</sup> )	6.51±0.8	9.7±0.4	5.8±0.4	6.7±0.5

Note: Values are mean ± SEM. ND – not determined, NE+SAL – non-exercised saline, NE+DOX – non-exercised doxorubicin, EX+SAL – exercised saline, EX+DOX – exercised doxorubicin, \* vs. NE+SAL, p<0.05

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**Table 2** Respiratory parameters (state 3 and state 4 rates, respiratory control ratio- RCR and ADP/O ratio) of glutamate-malate and succinate energized heart mitochondria isolated from each experimental group.

Glutamate-Malate	NE+SAL	NE+DOX	EX+SAL	EX+DOX
State 3 (natomO.min <sup>-1</sup> .mg prot <sup>-1</sup> )	414.0±18.5	334.0±20.7*	404.4±10.3	401.6±22.9
State 4 (natomO.min <sup>-1</sup> .mg prot <sup>-1</sup> )	81.3±9.5	72.1±6.5	64.3±12.6	88.1±10.9
RCR	5.9±0.1	4.7±0.23	7.8±0.8#	5.4±0.1
Succinate				
State 3 (natomO.min <sup>-1</sup> .mg prot <sup>-1</sup> )	498.2±34.2	383.8±12.1#	508.0±32.2	494.8±12.9
State 4 (natomO.min <sup>-1</sup> .mg prot <sup>-1</sup> )	218.5±17.4	158.0±14.9	243.2±19.4	261.6±13.5†
RCR	2.6±0.1	2.8±0.9	3.4±0.4	2.4±0.1

Note: Data are means ± SEM for heart mitochondria (0.5mg/ml protein) obtained from different mitochondrial preparations for each experimental group energized with glutamate (10mM)-malate (5 mM) and succinate (10 mM). Oxidative phosphorylation was polarographically measured at 25°C in a total volume of 0.75 mL. RCR – respiratory control ratio (state 3/state 4); ADP/O - number of nmol ADP phosphorylated by natom O consumed. \* vs. NE+SAL, # vs. others, † vs. NE+DOX (p<0.05).

**Table 3** Mitochondrial transmembrane potential ( $\Delta\Psi$ ) fluctuations (maximal energization, ADP-induced depolarization, and repolarisation) and ADP phosphorylation lag phase of glutamate-malate and succinate energized heart mitochondria isolated from each experimental group.

Glutamate-Malate	NE+SAL	NE+DOX	EX+SAL	EX+DOX
Maximal energization $\Delta\Psi$ (-mV)	222.4±1.9	214.2±1.4#	224.0±1.1	223.0±1.7
ADP depolarization $\Delta\Psi$ (-mV)	41.5±2.5	39.3±0.3	43.2±4.5	36.6±1.2
Repolarization $\Delta\Psi$ (-mV)	219.6±2.0	210.5±1.5#	221.6±1.2	223.0±1.9
Lag phase (s)	32.8±2.7	58.2±4.5#	29.3±1.1	29.3±3.6
Succinate				
Maximal energization $\Delta\Psi$ (-mV)	226.2±1.0	217.7±2.6#	230.9±0.1	227.1±2.4
ADP depolarization $\Delta\Psi$ (-mV)	66.8±4.6	53.3±2.6	61.4±3.3	48.7±1.5
Repolarization $\Delta\Psi$ (-mV)	225.6±0.9	217.5±2.5#	230.6±0.1	228.4±2.1
Lag phase (s)	46.8±2.8	64.9±1.9#	39.0±1.9	44.3±3.6

Note: Data are means  $\pm$  SEM for heart mitochondria (1 mg/ml protein) obtained from different mitochondrial preparations for each experimental group. Table shows the average response of mitochondrial membrane potential developed with glutamate (10 mM) - malate (5 mM) or succinate (10 mM) (energization), the decrease in membrane potential after ADP addition (depolarization), the repolarization value after ADP phosphorylation. Mitochondrial transmembrane potential was measured using a TPP<sup>+</sup>-selective electrode at 25°C in a total volume of 1 mL. # vs. all others (p<0.05).

**Table 4** Calcium accumulation capacities of freshly isolated heart and liver mitochondria energized with succinate (10 mM) obtained from each group.

<b>Heart</b>	NE+SAL	NE+DOX	EX+SAL	EX+DOX
$\Delta\Psi$ after 5 pulses of 100 nmol calcium (-mV)	214.3±6.8	199.1±8.3#	222.6±3.8	216.4±3.4
Calcium pulses (n)	9.3±1.7	5.8±0.7#	9.0±1.1	8.5±0.4
<b>Liver</b>				
$\Delta\Psi$ after 3 pulses of 20 nmol calcium (-mV)	196.1±7.8	208.3±5.5	218.5±0.8*	210.9±7.8
Calcium pulses (n)	3.3±0.4	4.5±0.8	3.0±1.1	4.0±0.6

Note: Data are means  $\pm$  SEM for heart (1 mg/ml protein) and liver mitochondria (0.5 mg/ml protein). Table shows the average response of mitochondrial membrane potential developed after 5 calcium pulses of 100 nmol (heart) and after 3 pulses of 20 nmol (liver) and the number of calcium pulses before MPTP-induced  $\Delta\Psi$  drop. Mitochondrial transmembrane potential was measured using a TPP<sup>+</sup>-selective electrode at 25°C in a total volume of 1 mL. # vs. all other experimental groups, \* vs. NE+SAL (p<0.05).

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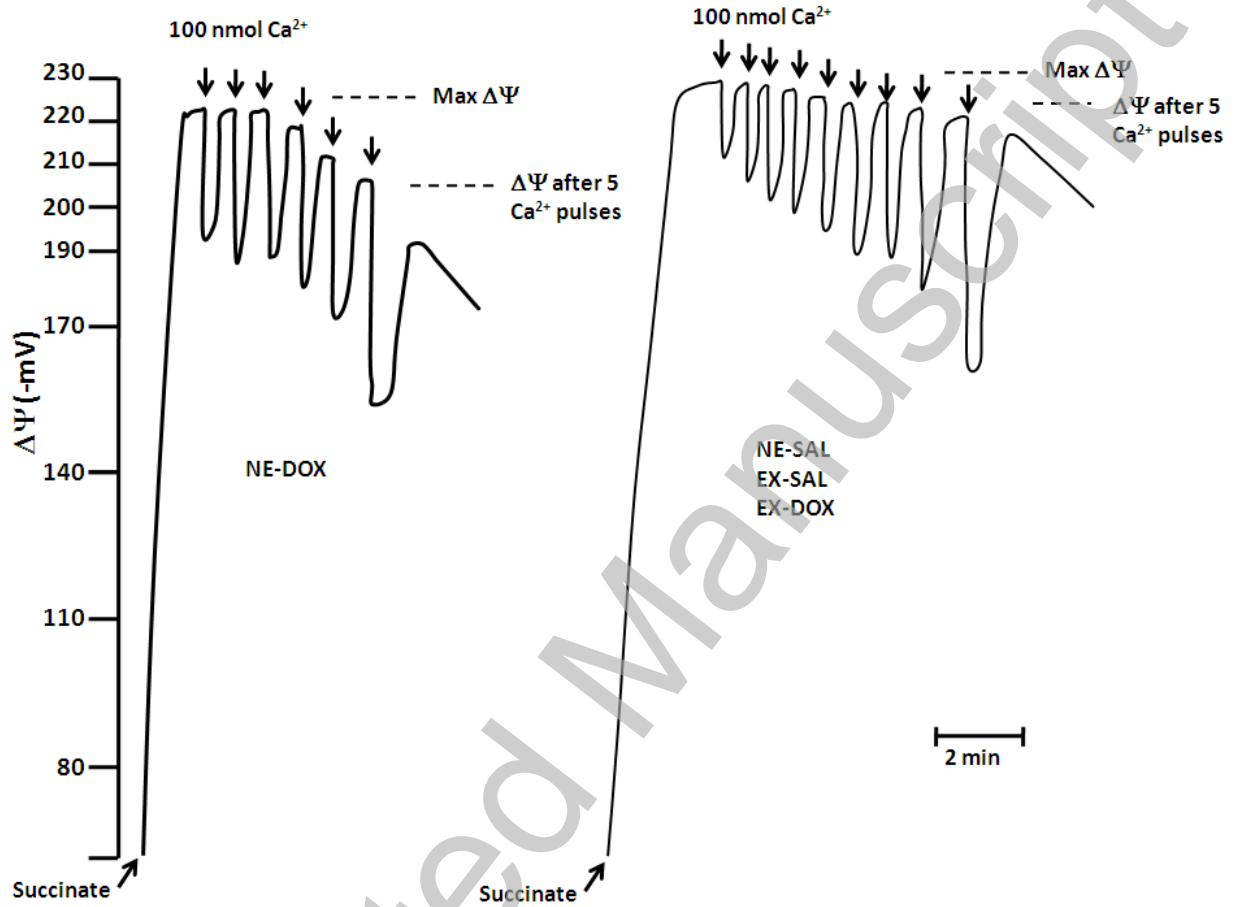
**Table 5** Heart and liver mitochondrial chain complex activities

<b>Heart</b>	NE+SAL	NE+DOX	EX+SAL	EX+DOX
Complex I (U/g)	1.17±0.08	0.57±0.07#	1.15±0.13	1.84±0.15
Complex II (µmol/g/min)	3.06±0.26	3.58±0.71	3.80±0.30	4.76±0.43
Complex V (µmol Pi/min/mg)	3.39±0.06	1.19±0.02*	4.94±0.07#	2.43±0.05
<b>Liver</b>				
Complex I (U/g)	0.54±0.10	0.69±0.05	1.04±0.15*	1.21±0.05†
Complex II (µmol/g/min)	1.03±0.08	0.89±0.08	1.42±0.02#	1.05±0.04
Complex V (µmol Pi/min/mg)	0.78±0.07	0.75±0.02	1.18±0.08*	1.15±0.06†

Note: Data are means ± SEM. # vs. all others, \* vs. NE+SAL, † vs. NE+DOX (p<0.05).

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Figure 1

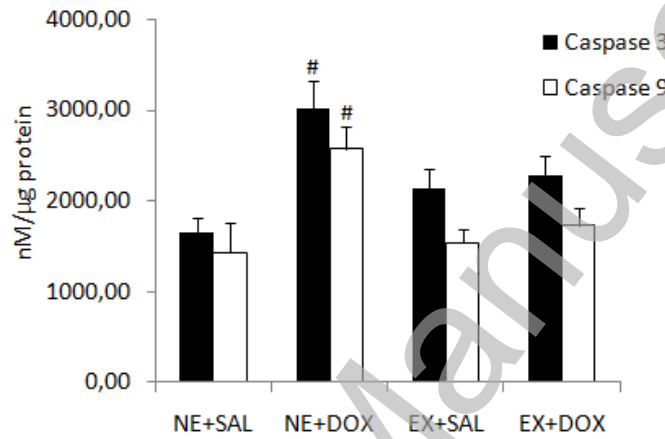


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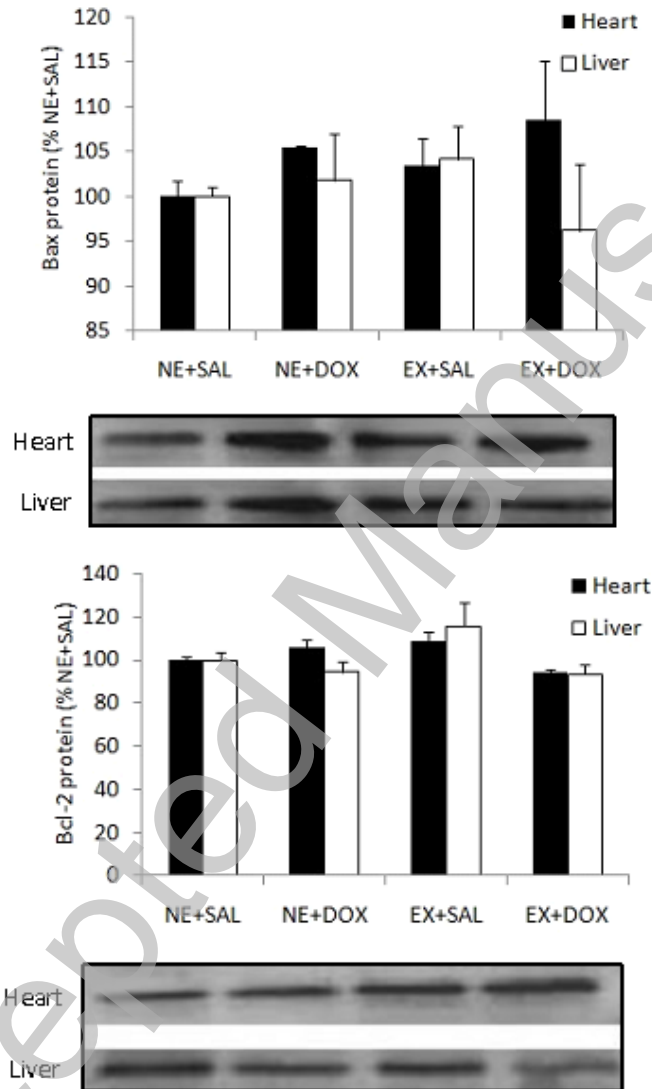
Figure 2



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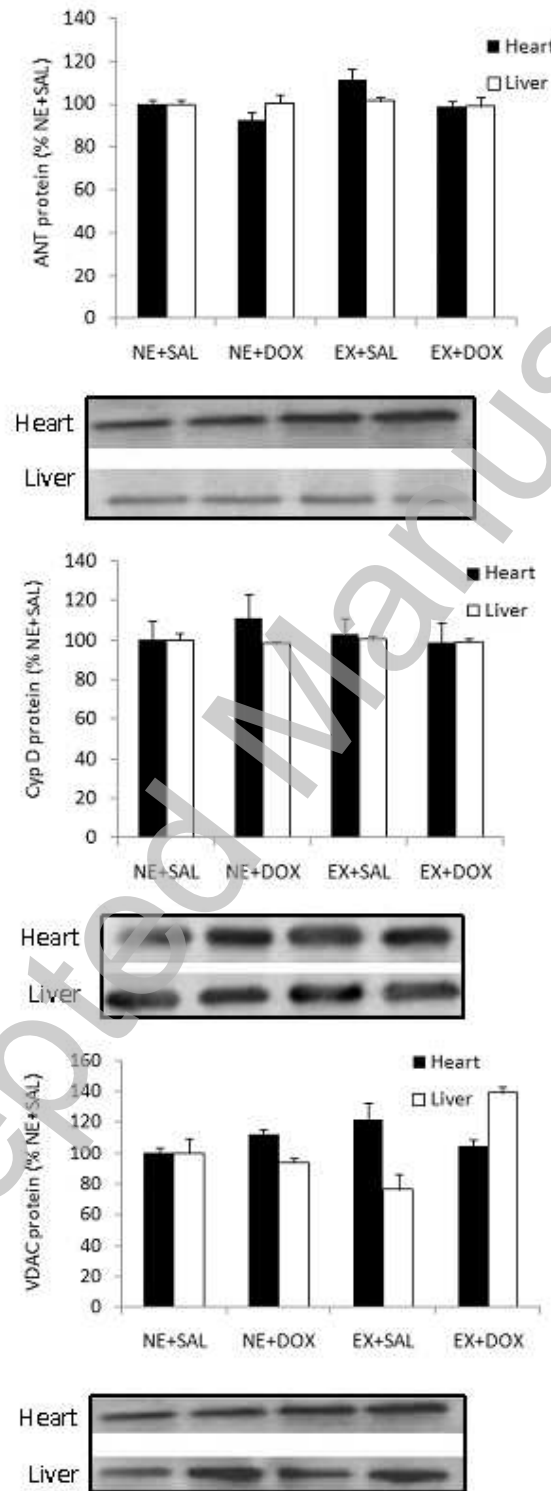
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Figure 3



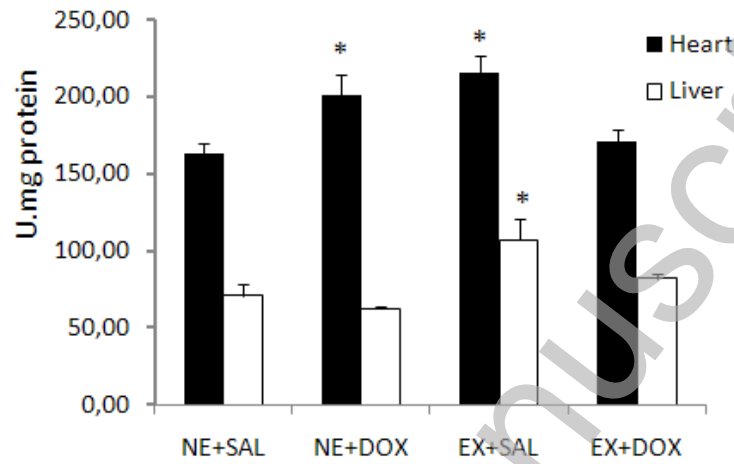
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Figure 4



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Figure 5



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