Endurance exercise training attenuates morphological signs of cardiac muscle damage induced by doxorubicin in male mice

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Abstract

The purpose of this study was to determine the effect of endurance swimming training (14 wks, 5day/wk, 1h/day) on cardiac muscle tolerance to in vivo doxorubicin (DOX)-induced toxicity, analysing quantitative and qualitative histological signs of muscle damage and plasma cardiac troponin I (cTnI). Thirty-two Charles River CD1 male mice were randomly assigned to non-trained placebo (NT+P, n=8), non-trained DOX (NT+DOX, n=8), trained placebo (T+P, n=8) and trained DOX (T+DOX, n=8). DOX was administered i.p. 24 hours after the last exercise bout, in a single dose of 20mg.kg⁻¹. Twenty-four hours after DOX treatment, the animals were sacrificed and aliquots of plasma were obtained for measuring plasma concentrations of cTnI; cardiac ventricles were extracted for semi quantitative and qualitative morphological analysis of tissue damage using light and electron microscopy. DOX treatment per se elevated (p<0.05) the levels of cTnI, which were significantly attenuated in T+DOX group. Morphological examination revealed that the elevated extension and severity damage scores in sedentary DOX hearts were significantly (p<0.05) reduced in trained group treated with DOX. Moreover, the percentage the total abnormal mitochondria exhibiting extensive loss of cristae, intramitochondrial vacuoles and notorious myelin figures were 0%, 88.3%, 2.9% and 10.1% for NT+P, NT+DOX, T+P and T+DOX, respectively. According to our data, endurance training seems to attenuate the severe morphological and biochemical signs of cardiac muscle injury induced by DOX treatment. These improvements in trained hearts were accompanied by an enhanced mitochondrial protection against DOX side effects.

Key Words: adriamycin, light and electron microscopy, heart, swimming exercise, mitochondria, ultrastructure.

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Introduction

It is generally assumed that endurance exercise training provides myocardial protection against many cardiac insults. When moderately and systematically repeated, exercise could constitute an excellent tool either to prevent and/or to treat several diseases, providing enhanced parallel resistance to the cardiac muscle tissue [25]. Although the exact mechanisms responsible for this protection continue to be debated, it has been argued that they are, at least in part, associated with the decreased ROS and with increased response of the several antioxidant defense systems [see 5, 21]. Accordingly, it has been demonstrated that endurance training up-regulates heat antioxidant enzymes and glutathione content [4], improves mitochondrial respiratory function [32], reduces the formation of lipid peroxidation by-products [32] and induces heat shock proteins (HSP) overexpression [4, 27].

Most of the training-related cross-tolerance cardiac studies used ischemia-reperfusion (I-R) as a model to test cardiac susceptibility to oxidative damage and dysfunction [28]. However, in addition to I-R, other stimuli
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associated with distinct pathways of cellular injury should also be considered in order to better understand all the mechanisms behind training cross-tolerance, which could enlarge its possible beneficial applications.

Doxorubicin (DOX) is a potent and broad-spectrum water-soluble anthracycline antibiotic prescribed for the treatment of a variety of malignancies including leukemia and solid tumors. The successful use of this anti-neoplastic therapeutic agent is limited by the development of a dose-dependent and irreversible cardiac toxicity [34]. The referred toxicity is characterized by various electrocardiographic [30], echocardiographic [6] and evident histomorphological changes [6, 34], seen as loss of myofibrils, distension of sarcoplasmatic reticulum, nuclear pyknosis, interstitial edema, myofibrillar vacuolization and hyalinization with loss of cross-striations [6, 34]. Moreover, mitochondria have also been identified as primary DOX target organelles, and their involvement is evidenced by the results of many studies reporting functional and morphological alterations, such as extensive degeneration or even loss of cristae, intramitochondrial vacuoles and notorious myelin figures, mitochondria swelling and abnormal size and shape [26, 38-40].

To the best of our knowledge, there is only one study dealing with the effect of endurance training in DOX treated hearts analysed through morphological alterations [22]. The authors concluded that exercise attenuated the severe toxicity caused by the cumulative administration of the drug, observed in the thin sections examined by light microscopy. However, qualitative and particularly, semi-quantitative information obtained by means of electron microscopy, may provide additional contribution to better understand the protective effect, at subcellular level, of previous endurance training on the toxicity caused by DOX. These new insights would consist in the recognition of the specific cardiomyocyte ultrastructures altered by the coordinated effects of endurance training against DOX treatment. In this sense, the main purpose of this study was to analyse the effect of 14-wk swimming endurance training in cardiac muscle tolerance to in vivo DOX-induced early damage throughout ultrastructural semi-quantitative and qualitative examination. Since mitochondria have been identified as important targets of DOX-induced subcellular damage in the heart, and that endurance training causes important biochemical, morphological and functional mitochondria adaptations [see 3, 16], we hypothesized that these organelles could be a central target of endurance training-induced cardioprotection.

Methods

Sample

32 Charles River CD1 male mice (aged six-eight weeks, weighting 30-35g at the beginning of the experiment) were used. During the experimental protocol, the animals were housed in collective cages (two mice per cage) and were maintained in a room at normal atmosphere (21-22º C; ~ 50-60% humidity) receiving commercial food for rodents and water ad libitum in a 12 h light/dark cycles. The animals were randomly divided into two groups: trained (n=16, trained) and non-trained (n=16, non-trained). Body weights of the mice were monitored carefully throughout the experimental period. Only male animals were used because of the protective effect of estrogen on cardiac tissue in females [33]. The Ethics Committee of the Scientific Board of the Faculty of Sport Sciences approved the experimental protocol, which followed the Guidelines for Care and Use of Laboratory Animals in research.

Endurance training protocol

The trained group was submitted to an endurance swimming training program, while the non-trained was not engaged in any exercise program. All the mice were adapted to water before the beginning of the experiment. The adaptation consisted of keeping the animals in shallow water at 31º C with the purpose of reducing the environment stress without promoting any physical training adaptations. The endurance-training program was performed in the morning (between 9 and 11 a.m.) and consisted of a swimming period 1h/day, 5 days/week for 14 weeks [4]. Swimming was performed in a high filled and deep plastic container (100X100X100cm) with water maintained at a temperature between 31-35ºC. The animals were progressively familiarized with swimming during the first 3 weeks (Table 1), by increasing the swimming time for 20 min every seven days up to the final time of 1 h/day. Exercise sessions lasted 10 min on the first day of the training period and at the 7th day the animals swam continuously for 20 min. According to the protocol, at the end of the 14th day the animals swim 40 min/day and from the 21st day until the end of the training the period of swimming was 60 min/day. In order to optimize endurance-training adaptations, mice supported a 4% body weight load attached to the tail during the swimming periods [13]. All mice were weighed once a week and when necessary the workload was adjusted to body weight changes. During training sessions, mice were allowed to swim at their own pace. Water burbling was produced sparingly to prevent mice floating.
After the end of the endurance-training program, the 16 trained and 16 non-trained animals were again randomly separated into four sub-groups. Thus, trained animals were distributed in trained plus placebo (T+P, n=8) and trained plus DOX (T+DOX, n=8); non-trained animals were also distributed in non-trained plus placebo (NT+P, n=8) and non-trained plus DOX (NT+DOX, n=8). The placebo groups were injected i.p. with a 0.1ml of sterile saline solution (0.9% NaCl). The experimental groups were injected i.p. with a single dose of DOX (20mg.kg\(^{-1}\)) in 0.1ml solution according to others [8]. Both treatments were carried 24 h after the last exercise bout and animals were sacrificed 24 h after DOX and placebo injections.

### Plasma and muscle extraction

Animals were anaesthetized with diethyl ether and placed in supine position. After that, the opening of abdominal cavity exposed the inferior cava vein and a blood sample of approximately 1 ml was collected in a heparinized tube. The blood was immediately centrifuged (5 min at 5000 \(g\), 4ºC) and an aliquot of plasma was obtained and stored at –80ºC for biochemical determination of cardiac troponin I (cTnI). After a quick opening chest, the whole mice hearts were then rapidly excised, rinsed with ice-cold saline, carefully dried and weighted. Both soleus muscles were excised and homogenized in tris buffer (200 mM, tissue:buffer ratio of 100mg/mL, pH 8.0) in a motor-driven Potter-glass homogenizer at 0–4 °C at low speed. The homogenized samples were then centrifuged for two min at 2000 \(g\), the pellet was discharged and the supernatant was used for measuring skeletal muscle oxidative capacity through CS activity. A 25mg of left ventricle was immediately taken and homogenized for cardiac CS activity.

### Tissue preparation for morphological analysis

After heart harvesting the atria and the great vessels were removed, and small pieces of left ventricle were cut into 1 mm cubic pieces and transferred to 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer during two hours. The specimens were post-fixed with 2% osmiumtetroxide, dehydrated in graded alcohol, and embedded in Epon. Semithin sections for light microscopy (Zeiss Axioplan 2 Imaging System) were stained with toluidine blue and ultrathin sections for transmission electron microscopy (Zeiss EM 10A) were contrasted with 0.2% lead citrate and 0.5% uranylacetate. All used reagents were of analytical grade and purchased from acknowledged companies.

### Procedures of morphological analysis

Quantitative analysis of cardiac muscle was performed using a final light microscopic magnification of ×400 on longitudinal and cross sections of heart ventricles. About 120 to 200 fibers from each muscle were analysed for quantification of the severity and the extension of lesions according to the criteria previously established [31] (Table II).

<table>
<thead>
<tr>
<th>Degree</th>
<th>Severity</th>
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<tbody>
<tr>
<td>1</td>
<td>Sarcomplasmatic microvacuolizations and/or interstitial cellular edema</td>
</tr>
<tr>
<td>2</td>
<td>Same as 1 plus sarcoplasmatic macrovaluolizations or atrophy, necrosis, endocardial lesions, and thrombi</td>
</tr>
<tr>
<td>3</td>
<td>Confluent groups of altered myocytes</td>
</tr>
<tr>
<td>4</td>
<td>Most cells damaged</td>
</tr>
<tr>
<td>5</td>
<td>No lesions</td>
</tr>
</tbody>
</table>

The score obtained for each observed section was calculated as follows: severity * extension of damage based on Della Torre et al. [11] with adaptations.

Ultra thin sections were examined using electron microscopy for a qualitative ultrastructural evaluation. The ultra thin sections of heart tissue were also semiquantitatively examined for histopathological evidence of cardiomyopathy, according to severity scores from 0-3, as previously described [26]. Severity of damage was scored using electron microscopy grids: grade 0, no change from normal; grade 1, limited number of isolated cells (less than 5% of the total number of cells per block) exhibiting early myofibrillar loss and/or cytoplasmatic vacuolization; grade 2, groups of cells (5 to
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30% of the total number) exhibiting early myofibrillar loss and/or cytoplasmatic vacuolization; and grade 3, diffuse cell damage (>30% of total number) with the majority of cells exhibiting marked loss of contractile elements, loss of organelles, and mitochondria and nuclear degeneration. All slides were scored independently by two examiners who were blinded to each tissue sample code.

The percentage of abnormal mitochondria (with extensive loss of cristae, intramitochondrial vacuoles and mitochondrial swelling) was evaluated in approximately one hundred random cells from each experimental group as previously described [39].

Biochemical assays

cTnI concentration was quantitatively determined with an established immunoassay using a commercial Abbott kit. Cardiac and soleus CS activities were measured using the method proposed by Coore et al., [9]. The principle of assay was to initiate the reaction of acetyl-CoA with oxaloacetate and link the release of CoA-SH to 5,5-dithiobis (2-nitrobenzoate) at 412nm. Protein contents from both cardiac and soleus muscles homogenates were assayed using bovine serum albumin as standard according to Lowry et al. [24].

Statistical analysis

Mean and mean standard errors were calculated for all variables in each of the experimental groups. One-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 the analysis. The significance level was set at 5%.

Results

Mice body weights, absolute and relative heart weights are expressed in Table III. In accordance with the well-described body mass and cardiac adaptations induced by endurance training, the 14 weeks of swimming training decreased mice weight and increased the relative heart weight (p<0.05).

Training program resulted in a significant (p<0.05) improvement in skeletal muscle oxidative capacity as evidenced by CS activity in soleus muscle, whereas no changes were observed in cardiac CS activity among groups (see Table III). The improved enzymatic activity in soleus reflects that endurance swimming training was an efficient chronic stimulus to ameliorate muscle oxidative metabolism.

As can be depicted from Fig 1, DOX induced a significant increase in plasma levels of cTnI. However, endurance training resulted in a significant reversal (p<0.05) of DOX-induced increase in that leaked cardiac protein (NT+DOX vs. T+DOX).

Table 3. Effect of endurance swimming training and DOX treatment on mice weights, absolute and relative heart weights. All values are mean and SD * NT+P vs. T+P and T+DOX; # NT+DOX vs. T+P and T+DOX. (p<0.05).

<table>
<thead>
<tr>
<th></th>
<th>NT+P</th>
<th>NT+DOX</th>
<th>T+P</th>
<th>T+DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice weight (g)</td>
<td>50.3 ± 3.5*</td>
<td>52.1 ± 2.5</td>
<td>44.3 ± 4.1</td>
<td>43.7±3.3</td>
</tr>
<tr>
<td>Heart weight (mg)</td>
<td>212.2 ± 13.4*</td>
<td>214.6±13.5</td>
<td>229.6 ± 15.8</td>
<td>224.8 ± 10.2</td>
</tr>
<tr>
<td>Heart weight/mice weight (mg.g⁻¹)</td>
<td>4.4 ± 0.35*</td>
<td>4.3 ± 0.33 #</td>
<td>5.2 ± 0.21</td>
<td>5.36±0.48</td>
</tr>
<tr>
<td>Skeletal muscle CS (µmol.mg⁻¹.min⁻¹)</td>
<td>0.019±0.001*</td>
<td>0.020±0.001 #</td>
<td>0.035±0.001</td>
<td>0.031±0.002</td>
</tr>
<tr>
<td>Cardiac CS (µmol.mg⁻¹.min⁻¹)</td>
<td>0.038±0.0004</td>
<td>0.036±0.003</td>
<td>0.035±0.002</td>
<td>0.037±0.001</td>
</tr>
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</table>
Figure 1. Effect of endurance swimming training and DOX treatment on plasma cTnI content. Values represent mean and SEM and are expressed as percentage of control (NT+P). * NT+DOX vs. all other groups; # T+DOX vs. all other groups (p<0.05).

Morphological changes under light microscopy can be depicted from table IV. Briefly, in contrast with the normal appearance of NT+P group, the myocardium from NT+DOX was characterized by prominent and consistent vacuolization affecting a large number of cells with interstitial edema. In contrast, only vacuolar morphological changes were noted in a few small areas in the sections from T+DOX mouse hearts (Fig. 2). The most elevated damage score recorded in NT+DOX group were clearly attenuated in the T+DOX group (table IV).

Table 4. Cardiomyopathy scores recorded from hearts of all experimental groups seen under light microscopy.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animals</th>
<th>Cardiomyopathy score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>NT+P</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T+P</td>
<td>0</td>
</tr>
<tr>
<td>DOX</td>
<td>NT+DOX</td>
<td>2.72*</td>
</tr>
<tr>
<td></td>
<td>T+DOX</td>
<td>0.31**</td>
</tr>
</tbody>
</table>

* p<0.05 NT+DOX vs. all other groups; ** p<0.05 T+DOX vs. all other groups.

Ultrastructural analysis was performed in heart ventricles from animals of all experimental groups. As demonstrated in figure 3, non-trained DOX-treated mice (Figs. 3C and 3D) evidenced marked myocardial damage when compared to the normal appearance of their control (Fig. 3A) counterparts (NT+P vs. NT+DOX). These changes consisted of mitochondria damage with extensive degeneration or even loss of cristae, intramitochondrial vacuoles and notorious myelin figures that probably resulted in the formation of secondary lysosomes, mitochondria swelling and abnormal size and shape. Moreover, the accumulation of intracytoplasmic vacuoles, suggestive of sarcoplasmic reticulum dilatation, and myofilament disarray were also evident, compared to the normal histological appearance of non-trained control hearts. Endurance swimming training per se (NT+P vs. T+P) caused notable changes in myocardial structure seen as an apparent increased glycolcogen content, intercalated discs showing a notorious scalloped appearance and evident signs of mitochondria biogenesis with elevated number of encroached mitochondria per fiber area, probably resulting in an increased volume/density of mitochondria. It is important to note that mitochondria division, mild and focal loss of cristae density and organization within mitochondria and minimal degradation by-products, probably secondary lysosomes, were also present in non-treated trained hearts (Fig. 3B). Regarding hearts harvested from trained animals treated with DOX (T+DOX), although maintained ultrastructural alterations described for T+P group, the above-referred signs of morphologic damage seen in NT+DOX hearts were notoriously attenuated in T+DOX mouse hearts (Figs. 3E and 3F).
Figure 3. Representative electron micrographs of cardiac tissue from all the groups. (A) NT+P group (magnification: x16,000); (B) T+P group (magnification: x10,000); (C and D) NT+DOX group (magnifications: x10,000 and x12,500, respectively); (E and F) T+DOX group (magnifications: x6,500 and x10,000, respectively); Notice the cytoplasmatic vacuolation, myofibrillar disorganization, and the severe mitochondria damage with extensive degeneration or even loss of cristae, intramitochondrial vacuoles and notorious myelin figures in the NT+DOX group (C and D) that were partially attenuated in T+DOX group (E and F).

In accordance, the semiquantitative analysis of these histopathological changes confirmed that the severe ultrastructural abnormalities induced by DOX treatment in sedentary hearts were significantly attenuated in trained DOX treated group (Table V).

Table 5. Effects of endurance running training and DOX treatment on ultrastructural histological cell damage scores

<table>
<thead>
<tr>
<th>Severity of cell damage</th>
<th>Treatment</th>
<th>Non-trained</th>
<th>Trained</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>0.00±0.00</td>
<td>0.69±0.28#</td>
</tr>
<tr>
<td></td>
<td>Doxorubicin</td>
<td>2.81±0.37*</td>
<td>1.49±0.27**</td>
</tr>
</tbody>
</table>

* p<0.05 NT+DOX vs. all other groups; ** p<0.05 T+DOX vs. NT+P; # T+P vs. T+DOX.

Semiquantitative analysis of the relative amount of abnormal mitochondria indicated that mitochondria from T+P group had a low frequency of mild alterations in mitochondrial morphology compared with NT+P, while most all mitochondria were markedly abnormal in hearts from NT+DOX. Most importantly, mitochondria from T+DOX hearts were distinctly protected from DOX-induced cardiac damage (table VI).

Table 6. Semiquantitative analysis of mitochondrial damage in mouse hearts from all experimental groups.

<table>
<thead>
<tr>
<th>Percent abnormal mitochondria</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-trained</td>
</tr>
<tr>
<td>Saline</td>
<td>0.0%</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>88.3% #</td>
</tr>
</tbody>
</table>

* Mitochondria were considered as abnormal only if presented mild focal loss of cristae density.
# Mitochondria evidencing extensive degeneration or even loss of cristae, intramitochondrial vacuoles and notorious myelin figures that probably resulted in the formation of secondary lysosomes and mitochondria swelling.

Discussion

The present study provides biochemical and histological evidence that endurance training attenuates the effects of high single DOX dose-induced early cardiotoxicity. It was demonstrated that the early deleterious and severe ultrastructural changes to the cardiac morphology induced by DOX treatment, particularly those affecting mitochondria, were largely attenuated by previous endurance swimming training.

An alternative approach for the detection of cardiac injury involves measurements of plasma concentrations of cardi-specific proteins that are released from damaged myocytes. The plasma content of the highly sensitive cTnI, one of the components of the troponin complex of the muscle cells, has been widely recommended as a clinical parameter for the diagnosis of cardiac disease in various conditions [7, 15, 29], including DOX-induced cardiac damage [6]. The data from the present study showed that DOX administration induced a lower rise in cardiac cTnI release to the plasma in the T+DOX group compared to NT+DOX. Given that cTnI was used as a systemic marker of myocardial damage and considering that plasma content of this protein correlates with loss of cardiac cell membrane integrity, these results suggest that cardiomyocytes from T+DOX animals suffered from less extent of membrane disturbances caused by DOX treatment compared with their sedentary NT+DOX counterparts.

Under light microscopy, the obtained morphological lesions in NT+DOX hearts demonstrated that the cardiac myocytes exhibited an evident interstitial edema, suggestive of an inflammatory reaction. This is in
agreement with other reports in which histopathological findings were consistent with increased mieloperoxidase (MPO) activation [14, 37] as well as with the attenuation of the cardiotoxic effects of DOX in mice treated with the anti-inflammatory agent ibuprofen [19]. In fact, despite the importance of mitochondrial electron transport chain [34], other source of ROS that may contribute to cardiac injury include neutrophils activation [1], which migrate to the tissue during tissue injury and have a role in oxidative damage mechanisms through the action of either NADPH oxidase or MPO systems. In fact, stimulated neutrophils can increase production of large amounts of hypochlorous acid and superoxide radicals oxidizing other molecules, including proteins, lipids and nucleic acids, contributing to cause secondary damage by degrading the surrounding tissue and thus aggravating the injury [17]. Nevertheless, the thin sections of hearts harvested from trained mice treated with DOX exhibited less extensive interstitial edema and cell vacuolization, with fewer, smaller and more sparsely distributed vacuoles when compared to their sedentary counterparts. Despite the indirect signs of inflammatory reaction induced by DOX administration, no evidence of infiltrative leukocytes was found in the analyzed sections of NT+DOX group.

The examination of ultrathin sections revealed that extensive sarcoplasmic vacuolization, mainly resulting from mitochondrial swelling/degeneration and sarcoplasmic reticulum distension, accompanied by other ultrastructural alterations, including myofilament disarray and fine-structure disruption predominates in the DOX-treated myocardium. However, all of these changes were dramatically attenuated in the hearts extracted from trained mice also treated with DOX, presenting less extensive swollen cardiac mitochondria and a lower intracellular edema evidenced by a less sarcoplasmic reticulum distension. Actually, the reduction of damage by endurance swimming training occurred at general cell level as demonstrated by the lower damage scores of T+DOX hearts compared with NT+DOX (Tables IV and V). This protective effect was accompanied by the diminished percentage of abnormal mitochondria with an apparent condensed chromatin at the periphery, no further apoptotic signals were found in the hearts harvested from DOX-treated mice of the present study. However, and despite the existence of some nucleus with an apparent condensed chromatin at the periphery, no further apoptotic signals were found in the hearts harvested from DOX-treated mice of the present study. However, and despite the existence of some nucleus with an apparent condensed chromatin at the periphery, no further apoptotic signals were found in the hearts harvested from DOX-treated mice of the present study.

In summary, the data from the present study provides evidence that endurance swimming training improves myocardial tolerance to in vivo DOX-induced early morphologic signs of damage. It is possible that these improvements can be related, at least partially, to train-
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from DOX side effects.

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