Effects of Voluntary Physical Activity and Endurance Training on Cardiac Mitochondrial Function of Rats Sub-Chronically Treated with Doxorubicin

Dissertation submitted to the Faculty of Sports, University of Porto to obtain the 2nd cycle in Physical Activity for Elderly, under the decree-law no. 74/2006 of 24 March

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Porto, June 2013

KEY-WORDS: EXERCISE; HEART; BIOENERGETICS; MITOCHONDRIAL FUNCTION; DOXORUBICIN.
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Ao papá, à mamã, ao Gonçalo e à Ely.
Agora que finalizo o mestrado, gostaria de expressar os meus profundos e sinceros agradecimentos:

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Resumo

O presente estudo teve como objetivo analisar o efeito de dois protocolos de exercício crónico distintos (treino em tapete rolante - TM e atividade física voluntária em roda livre - FW) na disfunção mitocondrial induzida pelo tratamento sub-crónico de doxorubicina (DOX), uma potente droga antineoplásica bastante eficaz cuja principal limitação é a toxicidade cardíaca.

Foram utilizados 32 ratos Sprague-Dawley macho jovens divididos em seis grupos (n = 6 por grupo): salino sedentário (SAL + SED), salino treinado (SAL + TM, 12 semanas de treino em tapete rolante), salino roda-livre (SAL + FW 12 semanas de atividade física em roda livre), tratado com DOX sedentário (DOX + SED [7 semanas de tratamento sub-crónico de DOX (2mg.kg^{-1}.wk^{-1})]), DOX + TM e DOX + FW. Foi analisada a funcionalidade mitocondrial cardíaca in vitro [consumo de oxigênio, potencial transmembranar (ΔΨ) e swelling osmótico], assim como os níveis de MDA e grupos sulfidril.

O tratamento com DOX afetou a funcionalidade mitocondrial cardíaca, alterando o consumo de oxigênio, o potencial transmembranar assim como o swelling osmótico durante a indução do poro de permeabilidade transitória mitocondrial (DOX + SED vs SAL + SED). A disfunção induzida pela administração de DOX no estado 3, no índice de controlo respiratório, ADP/O, no ΔΨ máximo, na repolarização, na lag-phase, amplitude e taxa de swelling, assim como no nível de MDA e no conteúdo grupos sulfidril foram revertidos pelos dois tipos de exercício crónico.

Ambos os protocolos de exercício estudados atenuaram a disfunção bioenergética das mitocôndrias cardíacas associada ao tratamento sub-crónico com DOX. Os nossos resultados são mais um contributo para o estudo dos efeitos cardioprotetores do exercício físico realizado antes, durante e após o tratamento com DOX, não só na população adulta mas também idosa.

PALAVRAS-CHAVE: EXERCÍCIO, CORAÇÃO, BIOENERGÉTICA, FUNCIONALIDADE MITOCONDRIAL, DOXORRUBICINA.
Abstract

The effects of two distinct chronic exercise models (endurance treadmill training – TM and voluntary free-wheel activity - FW) against mitochondrial dysfunction induced by sub-chronic treatment of doxorubicin (DOX), a potent antineoplastic drug known to induce a dose-related cardiac and mitochondrial toxicity, were analyzed.

Male young Sprague-Dawley rats were divided in six groups (n=6 per group): saline sedentary (SAL+SED), saline exercised (SAL+TM; 12-wks treadmill), saline freewheel (SAL+FW, 12-wks voluntary free-wheel), DOX+SED [7-wks sub-chronic DOX treatment (2mg.kg⁻¹.wk⁻¹)], DOX+TM and DOX+FW. In vitro endpoints of heart mitochondrial function [oxygen consumption, membrane potential (ΔΨ) and osmotic swelling], MDA level and sulfhydryl groups content were evaluated.

DOX affected mitochondrial function as seen by oxygen consumption, ΔΨ endpoints and osmotic swelling during MPTP induction (DOX+SED vs. SAL+SED). DOX-induced impairments in state 3, respiratory control ratio, ADP/O, maximal ΔΨ, repolarization, ADP lag-phase, swelling amplitude and average swelling rate as well MDA level and sulfhydryl groups content were reverted by both TM and FW.

Both studied chronic models of physical exercise reestablished heart mitochondrial bioenergetic defects induced by sub-chronic DOX treatment. Our results contribute to the analysis of the cardioprotective effects of exercise performed before, during and after DOX treatment no only on adult but also in older population.

KEY-WORDS: EXERCISE; HEART; BIOENERGETIC; MITCHONDRIAL FUNCTION; DOXORUBICIN.
## Abbreviations and Symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>VO$_{2}^{\text{max}}$</td>
<td>Maximal Oxygen Uptake</td>
</tr>
<tr>
<td>A.M.</td>
<td>Before Midday</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis Inducing Factor</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine Monophosphate-Activated Protein Kinase</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenosine Nucleotide Translocase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium Ion</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CHF</td>
<td>Congestive Heart Failure</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate Synthase</td>
</tr>
<tr>
<td>Cyc D</td>
<td>Cyclophilin D</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>DTNB</td>
<td>Beta Dystrobrevin</td>
</tr>
<tr>
<td>E</td>
<td>Exercise</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FW</td>
<td>Free Wheel</td>
</tr>
<tr>
<td>G</td>
<td>Glutamate</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione Peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat Shock Proteins</td>
</tr>
<tr>
<td>IFM</td>
<td>Intermyofibrillar Mitochondria</td>
</tr>
<tr>
<td>IR</td>
<td>Ischemia Reperfusion</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>Monopotassium Phosphate</td>
</tr>
<tr>
<td>m</td>
<td>Metro</td>
</tr>
<tr>
<td>M</td>
<td>Malate</td>
</tr>
</tbody>
</table>
MDA  Malondialdehyde
MFN  Mitofusin
mg   Milligram
min  Minute
ml   Milliliter
mM   Millimolar
MnSOD  Manganese Superoxide Dismutase
MPTP  Mitochondrial Permeability Transition Pore
mtDNA  Mitochondrial Deoxyribonucleic Acid
MΩ   Megaohm
NaCl  Sodium Chloride
NADH  Reduced Nicotinamide Adenine Dinucleotide
NADPH Reduced Nicotinamide Adenine Dinucleotide Phosphate
nmol  Nanomol
NO   Nitric Oxide
NS   Non-Significant
O₂   Oxygen
O₂⁻  Superoxide Radical
°C   Degree Celsius
OH⁻  Hydroxyl Radical
PGC  Proliferator-Activated Receptor Gamma
RCR  Respiratory Control Ratio
RNA  Ribonucleic Acid
ROS  Reactive Oxygen Species
SAL  Saline
SEM  Standard Error Of The Mean
-SH  Sulfhydryl groups
SOD  Superoxide Dismutase
SSM  Subsarcolemmal Mitochondria
T   Treatment
t   Time
TM  Treadmill
TNF  Tumor Necrosis Factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPP+</td>
<td>Tetraphenylphosphonium</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage Dependent Anion Channel</td>
</tr>
<tr>
<td>(A-VO₂)diff</td>
<td>Arteriovenous oxygen difference</td>
</tr>
<tr>
<td>Δψ</td>
<td>Transmembrane Electrical Potential</td>
</tr>
</tbody>
</table>
1. Introduction

Doxorubicin (DOX, or adriamycin) is a highly effective antibiotic used to treat several types of malignancies. Unfortunately, the clinical use of DOX is limited by the occurrence of a dose-related cardiac toxicity that results in life-threatening cardiomyopathy. DOX-induced cardiomyocyte dysfunction is associated with increased levels of oxidative damage involving mitochondrial bioenergetics collapse in the process (Wallace 2007). Actually, sub-chronic DOX treated rats reveal defects on heart mitochondrial function, which are accompanied by compromised mitochondrial electron transport chain activity and increased oxidative stress and damage (Berthiaume, Oliveira et al. 2005, Santos, Moreno et al. 2002).

Among the strategies advised to counteract the cardiac side effects associated with DOX treatment, physical exercise has been studied and recommended as a non-pharmacological tool against myocardial injury (Ascensao, Ferreira et al. 2007, Ascensao, Lumini-Oliveira et al. 2011, Ascensao, Oliveira et al. 2012). Previous work has suggested that the advantage of both acute (Ascensao, Lumini-Oliveira et al. 2010, Wonders, Hydock et al. 2008) and chronic exercise models (Ascensao, Ferreira et al. 2006, Ascensao, Magalhaes et al. 2005, Ascensao, Magalhaes et al. 2005, Chicco, Hydock et al. 2006, Chicco, Schneider et al. 2005, 2006) on the preconditioning of DOX-treated rats include the protection of cardiac tissue and mitochondria against induced impairments. Moreover, recent studies investigating the effects of exercise performed during and following models of late-onset cardiotoxicity caused by DOX provide evidence of exercise-induced cardioprotection in both adult and juvenile rat models (Hayward, Lien et al. 2012, Hydock, Lien et al. 2012). However, the cellular and molecular mechanisms underlying this protective phenotype induced by exercise are still elusive. In particular, whether perturbations in heart mitochondrial oxidative phosphorylation capacity and pro-oxidant redox modifications associated with cumulative DOX administration are modulated by long-term physical exercise performed during and after treatments is yet unknown.
We therefore aimed to analyze the effects of two types of long-term exercise with distinct characteristics, performed before and during the overall DOX treatment, on cardiac mitochondrial bioenergetics. Heart mitochondrial respiratory parameters associated with oxygen consumption, transmembrane electrical potential and osmotic swelling during mitochondrial permeability transition pore (MPTP) induction as well as markers of oxidative stress (sulphydryl groups (-SH) and malondialdehyde (MDA) contents) were determined.
2. State of art

2.1- Age effects on cardiac function

Aging can be characterized as a time dependent decline of maximal functionality that affects tissues and organs of the whole body (Figueiredo, Mota et al. 2008). The average human life span has markedly increased in modern society, a fact largely attributed to advances in medical and therapeutic sciences that have successfully reduced the severity of several diseased conditions (Chaudhary, El-Sikhry et al. 2011). However, elderly individuals continue to suffer the greatest burden from cardiovascular disease, including coronary heart disease that remains the leading cause of death in industrialized countries (Wei 2004). That can be understood as the result of several morphophysiological, structural and functional alterations, which we will further address in detail (Table 1).

The aging-induced increase on vascular stiffness, septum and myocardium thickness and fibrosis, is associated, among others, with: i) decreased myocyte number; ii) increasing cardiomyocyte size with alterations in calcium (Ca$^{2+}$) homeostasis and iii) increased collagen fibers deposition, leading to cardiac diastolic dysfunction, increased afterload, and loss of arterial and heart compliance (Strait and Lakatta 2012). Importantly, these physiological-related impairments along with unchanged cavity size and increased left ventricle hypertrophy are the major characteristic of aging heart (Chaudhary, El-Sikhry et al. 2011). In fact, Dai et al (2009) reported that aging left ventricle mass index increased by around 75% compared to a young adult group, indicating the increase prevalence of left ventricular pathological hypertrophy with age. It was also reported reduction in diastolic function, as well as worsening of myocardial performance index (Dai, Santana et al. 2009).

Furthermore, the conduction system also undergo some structural alterations leading to heart dysfunction, including fat accumulation around sinoatrial node, which creates a partial or complete separation of the node from atrial tissue (Strait and Lakatta 2012), a marked decreased in peacemakers number cells.
(Wei 2004) and an increased calcification in conduction system leading ultimately to atrioventricular conduction lock. Slow and prolonged systole and diastole are other aging-related heart features usually related to an aberrant Ca\(^{2+}\) handling (Strait and Lakatta 2012). The decreased diastolic function, which falls linearly with aging at a rate of about 6–7% per decade (Gates, Tanaka et al. 2003), is associated to a decrease in diastolic filling reported at rest and under stress conditions, as some daily tasks that promote an acute increase of physical demands. In addition, the degeneration of cardiac valvular apparatus is commonly reported, being valvular annular dilation found in the majority of older persons and is also associated with concomitant coronary artery calcification (Wei 2004).

**Table 1. Effects of aging on cardiovascular system**

<table>
<thead>
<tr>
<th>Morphophysiological changes</th>
<th>Structural Changes</th>
<th>Functional Changes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart and Vascular system</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>↓ Cardiomyocytes number</td>
<td>↑ Septum and Myocardium stiffness</td>
<td>↑ Systolic pressure</td>
<td></td>
</tr>
<tr>
<td>↑ Cardiomyocytes size</td>
<td>↓ Heart compliance</td>
<td>↓ Diastolic function</td>
<td></td>
</tr>
<tr>
<td>↑ Collagen deposition</td>
<td>↑ Vascular thickness</td>
<td>↓ max heart rate</td>
<td></td>
</tr>
<tr>
<td>↑ Fibers deposition</td>
<td>↓ Vascular compliance</td>
<td>↓ max cardiac output</td>
<td></td>
</tr>
<tr>
<td>↓ Cardiomyocytes function</td>
<td></td>
<td>↑ Afterload</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↓ VO2 max</td>
<td></td>
</tr>
</tbody>
</table>

Prolonged systole and diastole

Conduction system

<table>
<thead>
<tr>
<th>↑ Fibrosis and fat accumulation (SA node)</th>
<th>↓ Ventricular compliance</th>
<th>↑ Heart rhythm disturbances</th>
<th>↑ VO2 max</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓ Peacemakers cells number</td>
<td></td>
<td>↑ Risk atrioventricular conduction lock</td>
<td></td>
</tr>
<tr>
<td>↑ Conduction system calcification</td>
<td></td>
<td>↑ Risk atrial arrhythmias,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Risk atrial fibrillation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Risk tachycardia</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Ventricular arrhythmias</td>
<td></td>
</tr>
</tbody>
</table>

Valvular system

<table>
<thead>
<tr>
<th>↑ Valvular annular dilatation</th>
<th>Coronary artery calcification</th>
<th>Reynolds (2004)</th>
</tr>
</thead>
</table>

(↑) – increase; (↓) – decrease
Overall, these aging related heart morphophysiological, structural and functional alterations result in increased systolic pressure, one major risk factors for development of atherosclerosis, hypertension and stroke, and arterial fibrillation (North and Sinclair 2012).

As a consequence of all those age-associated alterations, cardiac function declines maximal functionality, being that, under basal conditions this decline may not be detected (Wessells and Bodmer 2007).

The most used standard index of cardiorespiratory fitness is the maximal oxygen consumption (VO$_2$max), which declines approximately 10% per decade starting at 20 to 30 years old (Souza 2012). VO$_2$max can be estimated by the Fick’s equation:

$$\text{Equation 1: } \text{VO}_2\text{max}=Q \times (A-\text{VO}_2)\text{diff}$$

Q is cardiac output, and is the product of max heart rate and stroke volume, (A-VO$_2$)diff is the arteriovenous difference of oxygen. As it is observed in figure 1, based on Fick’s equation, aerobic exercise capacity depends on cardiac output and arteriovenous difference (Seeley, Stephens et al. 2005).

Reductions in peak heart rate, peripheral oxygen utilization and stroke volume appear to mediate the age-associated decline in VO$_2$max. Actually, impairments in cardiac filling and increased afterload leads to a decrease in heart rate, in a stress situation (Taylor, Cable et al. 2004) being the major responsible for much of the age-associated decrease in maximal cardiac output. Reductions in muscle oxygen delivery and therefore (A-VO$_2$)diff are mainly due to reduced and maldistribution of cardiac output. Also, a decline in skeletal muscle oxidative capacity with aging is reported, due in part to mitochondrial dysfunction, which appears to play a particularly important role in old age, where skeletal muscle VO$_2$max is observed to decline by approximately 50% even under conditions of similar oxygen delivery as young adult muscle (Betik and Hepple 2008).
Although controversial, some reports suggested that left ventricular systolic function remains relatively preserved and without significant alterations in left ventricular stroke volume and cardiac output at rest (for refs see Kappagoda and Amsterdam 2012, Lakatta 2002, Morley and Reese 1989). However, at peak exercise, stroke volume index is reduced in older individuals and is thought to be the consequence of age-related reductions in β-adrenergic stimulation, increases in vascular stiffness, aortic impedance and impaired left ventricular diastolic function (Spina, Turner et al. 1998).

Generally, the morphophysiological and functional alterations related to VO₂max decline with aging are depicted in figure 1.

![Figure 1. Representative scheme of reduced cardiorespiratory fitness (VO₂max) in old adults. (adapted from Oxenham and Sharpe (2003)).](image)

Studies have demonstrated that advancing age is also associated with decreased heart rate variability (O'Brien, O'Hare et al. 1986, Yeragani,
Heart rate variability is a reliable reflection of the many physiological factors modulating the normal rhythm of the heart. In fact, it provides a powerful mean of observing the interplay between the sympathetic and parasympathetic nervous systems and it is thought to reflect the heart’s ability to adapt to changing circumstances by detecting and quickly responding to unpredictable stimuli (Rajendra Acharya, Paul Joseph et al. 2006). It is particularly relevant on daily tasks, as it is important to maintain a normal lifestyle without manifestation of heart injury. Also, it has been suggested that a decreased responsiveness to β-adrenergic receptor in aged hearts might be responsible for decreased heart rate variability in aged people during exercise or stress (Chaudhary, El-Sikhry et al. 2011).

Ischemia-reperfusion (IR) injury is the primary pathological manifestation of coronary artery disease (Lennon, Quindry et al. 2004, Powers, Demirel et al. 1998). The level of IR-induced myocardial injury can range from a small insult, resulting in limited damage, to a large insult, culminating in major cardiac electrical and mechanical dysfunction and ultimately in death (Powers, Quindry et al. 2004). Nowadays, it is well established that numerous age-related cellular and functional changes occur in the heart that could lead to ischemia-reperfusion injury as i) alterations in cardiac gene expression; ii) increased oxidative stress and iii) reduced ability of the heart to response to stress (Powers, Quindry et al. 2004). In this regard, Starnes et al. (1997) reported that immature hearts tolerate and recover from hypoxia better than adult hearts, and that the sarcolemmal membranes of immature rat hearts seem to be less susceptible to damage from hypoxic stress than those of older group.

It is notable that throughout life, there are many impairments originating cardiac injuries and ultimately death. So, it is important to implement countermeasures to attenuate and minimize those consequences, which will enhance old people lifestyle and lifespan. Recently, several approaches have been investigated and physical activity has been shown to be an important countermeasure to protect against myocardial injuries (Ascensao, Ferreira et al. 2007, Kavazis 2009,
Furthermore, among the several mechanisms or causes for cardiac dysfunction, mitochondrial abnormalities have a central role (for refs see Braunwald and Bristow 2000). In fact, due to the key mechanisms to which these organelles are associated such as energy production, ion regulation, pH control, Ca\(^{2+}\) homeostasis, redox reactions, control of cell signaling and apoptosis mitochondria assume a pivotal role in cellular functioning. For these reasons, mitochondria have been suggested as reliable sensors of cellular functionality and (dys)functional mitochondria correlates with (dys)functional heart tissue submitted to a variety of stimuli, including age (Wallace 2010).

Because heart is primarily a postmitotic tissue that exhibits a highly aerobic metabolism due to the abundance of large mitochondria, a dependence on healthy mitochondria for normal organ function is implicated (Judge and Leeuwenburgh 2007).

The following section will address the effects of aging process on the modulation of mitochondrial function and related mechanisms.

### 2.1.1 Aging effects on cardiac mitochondria function

In metabolically active and thus energy-demanding tissues such as the heart, mitochondria, as producers of most of the ATP necessary for metabolism via oxidative phosphorylation, have a very important role in the supply of energy for continuously contracting myocytes (Ascensão 2011). Additionally, mitochondria play other important roles as cellular redox and ion homeostasis, oxygen sensing, signaling and regulation of programmed cell death (for refs see Rabinovitch 2012, Sheu 2009). Therefore, mitochondrial integrity is vital for cellular homeostasis and cardiac performance (Chaudhary, El-Sikhry et al. 2011) being age-related heart mitochondrial dysfunction closely associated to

In 1956, Harman et. al, proposed the free radical theory of aging, postulating that the production of reactive oxygen species (ROS) is a major determinant of lifespan acting as important mediators responsible for the cellular damage seen in aged cells (Harman 1956). Later, it was defined that mitochondria are the main source of ROS and the main target of their injury being ROS produced within mitochondria almost 90% of the total ROS produced in the cell (Hearman 1972); so it was postulated as the “key organelles” initiating cellular processes leading to death (Chaudhary, El-Sikhry et al. 2011). Indeed, evidences suggest that with advanced age, mitochondrial production of ROS significantly increases in heart tissue (Judge, Jang et al. 2005), which leads to development of degenerative diseases (for refs see Rabinovitch 2012). Within cells, ROS are produced in multiple compartments and by multiple enzymes including reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase at the plasma membrane, respiratory chain within mitochondria, lipid oxidation within peroxisomes and by cyclo-oxygenases and xanthine oxidase in the cytoplasm (Dai and Rabinovitch 2009, Dai, Rabinovitch et al. 2012). Although all of these source contribute to the overall oxidative damage, mitochondria are one of the most contributors for ROS generation as a byproduct of electron transfer during oxidative phosphorylation (Dai and Rabinovitch 2009). Most specifically, excess electrons from complex I and III can be transferred directly to oxygen (O$_2$) to generate superoxide anion (O$_2^-$), which is then converted to hydrogen peroxide (H$_2$O$_2$) and ultimately into a hydroxyl radical (OH$^-$), the most reactive ROS species (Dai and Rabinovitch 2009). The unbalance between ROS production and the capacity of antioxidant machinery, favoring ROS production is usually called oxidative stress. Mitochondria have antioxidant enzymes that play a vital role in the protection against oxidative stress (Meng 2007), being manganese superoxide dismutase (MnSOD), catalase (CAT) and glutathione peroxidase (GPX) the most commonly cited (Ascensão 2003). In addition, there are non-enzymatic antioxidant compounds, both endogenous and exogenous, such as glutathione (GSH), vitamins C and E, and lipoic acid that play important roles in
ROS neutralization or in the attenuation of the effects caused by increased ROS production (Ji, Leeuwenburgh et al. 1998). Heat shock proteins (HSPs) are another system of cellular defense against oxidative stress (Ascensao, Ferreira et al. 2007, Hamilton, Staib et al. 2003, Powers, Locke et al. 2001). These “stress-induced proteins” are ubiquitous and highly conserved chaperones, important in the folding of new synthesized, damaged or transported proteins. Moreover, HSPs mediate mitochondrial protection against oxidative stress, namely HSP70 have been associated with myocardial protection (Gunduz, Senturk et al. 2004).

To understand the impact of oxidative stress, which is known to damage proteins, lipids, and deoxyribonucleic acid (DNA) (Shioi and Inuzuka 2012), some oxidative markers are analyzed in mitochondria as carbonil groups (protein oxidation), malondialdehyde (MDA) (lipid peroxidation marker) or thiobarbituric acid reactive substances (TBARS) (nonspecific marker for lipid peroxidation), among others. The accumulation of oxidant-induced damage in mitochondria may be a major contributing factor to the age-related alterations in myocardial function (Chen and Knowlton 2011, Ljubicic, Menzies et al. 2010, Pohjoismaki, Boettger et al. 2012). Nonetheless, some authors have suggested that throughout the aging process, the antioxidant capacity can be adjusted in response to prooxidant exposure (Ji, Leeuwenburgh et al. 1998). Localized oxidative stress in specific organs, tissues, and organelles may stimulate cellular uptake and synthesis of certain antioxidants under complicated genetic, hormonal, and nutritional regulation (Harris 1992). One possibility is that aged mitochondria produce more ROS stimulating antioxidant enzyme gene expression. This scenario is consistent with the finding that mitochondrial antioxidant enzyme activity showed a greater increase in the senescent myocardium (Ji 1993, Ji, Dillon et al. 1991, Rao, Xia et al. 1990, Vertechy, Cooper et al. 1989). Nevertheless, data seems to be controversial as no alteration (Bejma, Ramires et al. 2000, Tian, Cai et al. 1998) or decrease in antioxidant activity (Bagchi, Bagchi et al. 1996, Pritsos and Ma 2000) have also been reported. So, increased antioxidant activity may be interpreted as being beneficial because it provides better protection against oxidant-induced damage.
damage, or it may be viewed as negative because it could indicate a need for enhanced antioxidant defenses due to increased oxidant production (Beckman and Ames 1998).

However, characterization of age-related changes in cardiac mitochondria has been challenged due to the fact there are two distinct populations of mitochondria in the myocardium (Fannin, Lesnefsky et al. 1999). Subsarcolemmal mitochondria (SSM) are located beneath the plasma membrane, whereas interfibrillar mitochondria (IFM) are found in parallel rows between the myofibrils. There are important biochemical and functional differences between SSM and IFM and it has been revealed that IFM are more adversely affected with age (Fannin, Lesnefsky et al. 1999, Judge, Jang et al. 2005), although Judge et al. (2007) suggests that further studies are required to determine the mechanisms contributing to these changes, and to further characterize differential effects of age upon the SSM and IFM populations in the heart.

Despite this fact, some authors report increased H$_2$O$_2$ production from SSM, but not IFM, with age (Judge, Jang et al. 2005, Judge and Leeuwenburgh 2007). This can be a consequence of the lower antioxidant capacity on this subpopulation. Actually, whereas IFM MnSOD, GPX and CAT levels seem to increase with age, in SSM only MnSOD and GPX, but not with CAT, seems to be positively regulated. However, as MnSOD converts O$_2^-$ radicals to H$_2$O$_2$, increased MnSOD activity might also represent higher levels of H$_2$O$_2$. Also, lower glutathione levels and increased oxidative damage have been suggested, supporting that oxidant production within the matrix of “old” IFM is greater than that in “young” IFM (Judge, Jang et al. 2005). Similarly, state 3 respiratory rates were also lower in “old” IFM. This has a particular concern because IFM are likely the major source of ATP production for myosin ATPase (Judge, Jang et al. 2005). Because high levels of ATP are required for both systolic contraction and diastolic relaxation, reduced availability of ATP as a result of IFM dysfunction could contribute to the alterations in cardiac contractility observed with age (Judge, Jang et al. 2005).
Besides nucleus, mitochondria are the only organelles in animal cells that possess their own DNA, the mtDNA, as well as related transcriptional and translational synthesis machinery. mtDNA is localized in matrix with physical proximity to the mitochondrial respiratory chain (Bratic and Trifunovic 2010). However, mitochondria cannot be synthesized de novo, instead they replicate in the cytosolic compartment through a process of division (for refs see Chaudhary, El-Sikhry et al. 2011). Importantly, the proteins that are encoded by mtDNA are vital for normal mitochondrial function and mtDNA does not have the protein protection as nuclear DNA and has less effective repair mechanisms (Desler, Marcker et al. 2011). Therefore, mutations in mtDNA through oxidative stress affect the expression and integrity of oxidative phosphorylation complexes and can cause mitochondrial dysfunction and increased ROS production (Wallace 2010). So, it is notable the interrelationship between oxidative phosphorylation complexes, ROS levels, mtDNA mutations and ultimately cell death. Furthermore, mutations in mtDNA and the resultant decline in mitochondrial activity observed in aged tissues are responsible for the increased generation of ROS, which in turn, will further negatively impact mitochondria causing further mtDNA damage. This “vicious cycle” concept postulated that accumulation of mtDNA mutations is exponential and associated with massive increase in ROS production (Lenaz 1998).

Being mitochondria strategic organelles essential for cell function and homeostasis, providing energy to the cell (Chaudhary, El-Sikhry et al. 2011), they must undergo some dynamic mechanisms. Mitochondrial turnover and death can occur via several processes that are suggested to be interrelated namely apoptosis, necrosis, autophagy and related dynamics of organelles. In this dynamic network biogenesis, fusion and fission are closely associated mechanisms (Chen and Knowlton 2011).

In fact, apoptosis is mediated by two pathways: the extrinsic and the intrinsic pathways, and both have been described in cardiac myocytes (Whelan, Kaplinskiy et al. 2010). The extrinsic apoptotic pathway can be triggered by Fas ligand or tumor necrosis factor (TNF)-α, which are expressed in cardiac
myocytes and have been implicated in cardiovascular pathology (Whelan, Kaplinskiy et al. 2010).

Intrinsic pathways involve the participation of endoplasmic reticulum (ER) and/or mitochondria (Kroemer, Galluzzi et al. 2007). Impairment in mitochondrial integrity, dynamics or metabolic activity may result in a range of deleterious effects to the cell, such as reduced ATP production, elevated cytosolic Ca\(^{2+}\), increased ROS release, release of proapoptotic factors as cytocrome c or caspases activation and Bax translocation, triggering cell death (Chen and Knowlton 2011). In mammalian cells, apoptosis is regulated by a variety of factors that are essentially either pro-life or pro-death (Goldspink, Burniston et al. 2003). Quantitation of the expression of genes involved in the apoptotic pathway might represent a good index of the probability for a cell to undergo apoptosis. As the number of Bax-expressing cells dramatically increases in left ventricular hypertrophy and left ventricular dysfunction (Condorelli, Morisco et al. 1999, Green and Reed 1998), it is suggested that mitochondrion is the primary organelle mediating the intrinsic apoptotic pathway in these conditions (Chiong, Wang et al. 2011)

Also, if excessive Ca\(^{2+}\) enters to mitochondria and enhanced oxidative stress conditions are present, a phenomenon known as permeability transition may occur (Ascensao, Lumini-Oliveira et al. 2011). The mitochondrial permeability transition is characterized by the loss of the impermeability of the mitochondrial membranes and it is suggested that this condition is mediated by the formation and opening of protein complex-like pores in the inner mitochondrial membrane, the mitochondrial permeability transition pore (MPTP) (for refs see Ascensao, Lumini-Oliveira et al. 2011). Increased pro-oxidant generation causing oxidative stress is one condition that augmented the susceptibility for the opening of these pores and the release of pro-apoptotic proteins within mitochondria as cytochrome c, SMAC/DIABLO and the apoptosis inducing factor (AIF), which will activate the caspase-related apoptotic pathways. It is suggested that the release of these proteins is dependent on the formation and opening of MPTP that cross the inner and the outer membranes leading to the loss of
mitochondrial membrane potential ($\Delta \psi$), increased mitochondrial osmotic swelling and rupture of the outer mitochondrial membrane, which leads to death (Ascensao, Lumini-Oliveira et al. 2011). It is believed that the structure and regulation of this multi-protein complex comprises the outer membrane voltage-dependent anion channel (VDAC) as well as the inner membrane adenine nucleotide translocator (ANT) and cyclophilin D (Cyc D). Myocyte loss has been shown to occur in the aged rat heart and to precede the occurrence of ventricular dysfunction (Anversa, Hiler et al. 1986), being apoptotic cardiomyocyte death present under different conditions in humans (Haunstetter and Izumo 1998, Narula, Haider et al. 1996, Olivetti, Abbi et al. 1997).

Among the excess of biological phenomena affected by aging, the malfunction and decrease of biogenesis of mitochondrial biogenesis seems to exert some of the most potent effects on the organism (Lopez-Lluch, Irusta et al. 2008). If biogenesis is affected, it is reasonable to expect that mitochondrial turnover must be slower and the accumulation of modified lipids, proteins and DNA must also increase, further aggravating the conditions resulting on deficient activity of aged mitochondria (Lopez-Lluch, Irusta et al. 2008). The precise reason for the decrease in the rate of mitochondrial biogenesis during aging is currently unknown. However, it seems that both, extra- and intra-cellular regulatory factors of mitochondrial biogenesis are implicated. Specifically, peroxisome proliferator-activated receptor gamma coactivator (PGC1-α) has been shown to act as a common intracellular mediator during mitochondrial biogenesis induced by hormonal factors (Weitzel, Iwen et al. 2003), and adenosine monophosphate-activated protein kinase (AMPK) an intracellular regulator of mitochondrial biogenesis, which activity appears to be one of the main factors associated with deficient mitochondrial biogenesis (Reznick, Zong et al. 2007). PGC family members have gained particular interest because of their ability to drive virtually all mechanisms of mitochondrial biogenesis in the heart, including mitochondrial number, mitochondrial respiration, expression of oxidative phosphorylation and fatty acids oxidation genes, and ROS levels (Lehman, Barger et al. 2000). Decreased PGC-1α expression has been linked to the development of heart failure in mouse models (for refs see Moslehi, DePinho et
al. 2012), and decline in mitochondrial biogenesis and mitochondrial protein quality control in cardiac muscle was found in aging (Koltai, Hart et al. 2012).

In addition, it is well known that mitochondria are dynamic organelles that constantly undergo fission and fusion and it has been found to be vibrant organelles that continuously divide and fuse within the cell and have functions extending beyond energy production, including cell signaling (Liesa, Palacin et al. 2009). Disruption of fission and/or fusion can also lead to cellular dysfunction and to apoptosis. This dynamic mechanism is regulated by proteins controlling fission, such as hFis1 and Drp1, and fusion, such as mitofusin 1 and 2 (MFN1 and MFN2) and OPA1. The correct function of these proteins seems to be critical for normal mitochondrial activity, and their deregulation is associated with several pathologic conditions (Lopez-Lluch, Irusta et al. 2008). Indeed, the impairments on fission-related protein hFis1 has been associated with the process of senescence in mammalian cell cultures (Lee, Jeong et al. 2007). Moreover, depletion of hFis1 by RNA interference (RNAi) induces dramatic changes in mitochondrial structure, including the enlargement and flattening of the organelle. Furthermore, elimination of any of the mitochondrial fusion proteins as MFN1, MFN2 or OPA1, induces mitochondrial fragmentation, as expected, being that down-regulation of Opa1 expression in cells by RNAi results in spontaneous apoptosis (for refs see Chen and Knowlton 2011). Overall, defects in the mitochondrial fission/fusion machinery and the loss of symmetry between fusion and fission (Hoppins, Edlich et al. 2011) may contribute to the decline in mitochondrial function during aging. However, several fundamental questions remain to be answered (Bossy-Wetzel, Barsoum et al. 2003).

In the next section, the roles of physical exercise as a strategy to improve cardiac function in adult and old subjects as well as the mitochondrial-mediated mechanisms associated with exercise-induced cardioprotection will be addressed.
2.2. Exercise and cardioprotection

Cardiac damage is a major contributor to morbidity and mortality in industrialized countries; so it becomes important to develop strategies that result in cardioprotective phenotype. In this regard, several approaches have been investigated and physical activity has been shown to be an important countermeasure to protect against myocardial injuries (Bowles, Farrar et al. 1992, Harris and Starnes 2001, Powers, Demirel et al. 1998, Powers, Quindry et al. 2004). In fact, cardiorespiratory fitness is inversely related to cardiovascular and all-cause mortality and it has crucial role preventing heart injury (Kokkinos, Myers et al. 2010). Some reports had postulated exercise-induced benefits based on decreases of some risk factors to develop cardiac and myocardium impairments such as body mass index, body weight, waist circumference, abdominal and visceral fat and consequently insulin resistance, triglyceride levels, blood pressure and, in general, metabolic syndrome-related parameters (for refs see Golbidi and Laher 2012). Therefore, chronic aerobic exercise is able, not only, to improve cardiovascular function in young healthy subjects, but also, and most importantly, in older people and those with cardiovascular risk factors (Hambrecht, Fiehn et al. 1998).

The study of the mechanisms responsible for exercise-induced cardioprotection has been ongoing for over decades and morphological and biochemical/molecular alterations have been considered as putative mechanisms of exercise-induced cardioprotection. Those include morphological adaptations of heart and coronary arteries, induction of myocardial HSPs, increased myocardial cyclooxygenase-2 activity, elevated ER stress proteins, nitric oxide production, improved function of sarcolemmal and/or mitochondrial adenosine triphosphate (ATP)-sensitive potassium channels and increased myocardial antioxidant capacity (for refs see Kavazis 2009).

For instance, exercise induces vascular remodeling and so, morphological alterations in coronary arteries through angiogenesis and arteriogenesis (Leung, Yung et al. 2008). Here, nitric oxide (NO) assumes important roles due its anti-inflammatory, vasodilator and platelet inhibitory effects (Landmesser and
Drexler 2005). Also, NO protects against ischemia-represhufusion (IR) injury in such a way that the heart responds to ischemia using nitric oxide species in a harmonized manner and these mechanisms could be based on inhibition of Ca\textsuperscript{2+} influx into myocytes, antagonism of \(\beta\)-adrenergic stimulation, reduction in cardiac oxygen consumption and ability to increase the expression of HSP70 (for refs see Golbidi and Laher 2011). HSPs protect cell against oxidative injury and apoptosis (Poll, Kantengwa et al. 1996) and furthermore, enhance recovery from acute myocardial cellular injury protecting heart for subsequent injury (for refs see Powers, Locke et al. 2001) by promoting restoration of dysfunctional enzymes and preventing aggregation of severely denatured proteins. The majority of evidences indicate that members of the 70-kDa family are the cytoprotective proteins most responsible for cell protection (for refs see Powers, Locke et al. 2001). The expression of HSP70 in cardiomyocytes is associated with increased cell survival and protection against ischemic damage and its now well established that acute and chronic exercise are able to induce increases in the expression of HSP70 (Kregel and Moseley 1996, Powers, Demirel et al. 1998), although in a temperature-dependent “fashion”.

The improved function of sarcolemmal ATP sensitive potassium channels (Powers, Quindry et al. 2008) and elevated ER stress proteins are other important exercise-induced cardioprotection-related alterations and both have special relevance during a cardiac insult (Golbidi and Laher 2012). The ER stress proteins help cellular homeostasis by maintaining intracellular Ca\textsuperscript{2+} regulation and protein folding during IR injury (Logue, Gustafsson et al. 2005).

Considering the importance of mitochondrial machinery in the maintenance of cardiac function, mitochondrial-mediated mechanisms have also been associated with exercise induced cardioprotection phenomenon. This topic will be further discussed in the next sections.
2.2.1 Exercise and cardiac mitochondrial adaptations

As previously mentioned, mitochondrial adaptations may play a critical role in exercise-induced protection against cardiovascular impairments. The mechanisms behind this phenomenon remain unclear, however it may be related to morphological and biochemical adaptations including biogenesis, antioxidant production or resistance to cell death pathways (Ascensao, Ferreira et al. 2007, Kavazis, Alvarez et al. 2009, Kavazis, McClung et al. 2008). Those will be briefly addressed in the following section.

2.2.1.1. Morphological and biochemical adaptations

Heart is a highly oxidative organ, with a low rate of cell growth and slow turnover of proteins; so, it would be expected that heart might have a limited ability to adapt to acute and/or chronic conditions.

Although there are scarcely addressed outcomes about exercise-induced cardiac mitochondrial morphological adaptations in healthy hearts, the same cannot be stated regarding hearts under deleterious conditions. In fact, under deleterious conditions as DOX treatment, diabetes, or aging, morphological impairments on heart mitochondria have been reported (Ascensao, Oliveira et al. 2012, Searls, Smirnova et al. 2004). Despite this fact, in a study where trained animals were submitted to an endurance swimming training program, while the non-trained were not engaged in any exercise program Ascensao et al. (2006) reported that endurance swimming training per se caused notable changes in myocardial structure seen as an apparent increased glycogen 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. Also, mitochondria division, mild and focal loss of cristae density and organization as minimal degradation by-products, were also present in non-treated trained hearts. Another study developed by Searls et al. (2004)
with type I diabetes rats found in type I diabetes rats that 9 weeks of moderate exercise is able to reverse some of the phenotype of diabetic cardiomyopathy. Specifically, the mitochondrial quality, cytoplasmic area, and collagen cross-sectional area returned toward non-diabetic values with exercise.

Mitochondrial biochemical adaptations to exercise were also reported and several approaches have been trying to prove the exercise-induced modifications of some parameters involved in mitochondrial bioenergetics. Judge et al. (2005) reported that wheel running had no effect on mitochondrial protein yield, rates of oxygen consumption (states 4 and 3) or RCR. This was not entirely surprising given that other studies have shown that, unlike skeletal muscle, oxidative capacity of cardiac muscle is not increased in response to treadmill training, an exercise protocol that is typically much more intense than voluntary wheel running (for refs see Judge, Jang et al. 2005). Also, Starnes et al. (2007) reported that endurance training has no impact on mitochondrial oxidative phosphorylation, being that all oxidative phosphorylation parameters were similar in endurance trained and sedentary rats. Because the heart is highly oxidative, it is not expected to be as responsive to exercise-induced increases in oxidative capacity as skeletal muscle. Although, several reports suggested that exercise *per se* is able to improve mitochondrial protein yield, rates of oxygen consumption as well as cardiac function (for refs see Ascensao, Ferreira et al. 2007, Ascensao, Magalhaes et al. 2006, Chicco, Schneider et al. 2006, Powers, Lennon et al. 2002). Moreover, Kavasis et al. (2009) reported that the abundance of several proteins involved in bioenergetics was altered following endurance exercise in both SS and IMF mitochondria. Most specifically, the protein levels of several proteins involved in β-oxidation of fatty acids were increased following repeated bouts of endurance exercise. It is of especial concern as during the development of heart disease or genetic defects in mitochondrial fatty acid β-oxidation the myocardial energy source switches from fatty acid β-oxidation to glycolysis. Also, there are some controversial reports about endurance exercise benefits against Ca\(^{2+}\)-induced mitochondrial dysfunction. In fact, while Starnes et al. (2007) found that endurance exercise training had no influence when mitochondria was challenged with identical Ca\(^{2+}\)
concentrations, being that mitochondria from trained and sedentary animals displayed similar declines in ATP production, French et al. (2008) found that exercise-induced protection against IR injury, in part, by attenuating IR-induced oxidative modification of important Ca\(^{2+}\)-handling proteins and preventing subsequent calpain activation. In this study, MnSOD may have had an important role as, the results support that exercise-induced increases in myocardial MnSOD activity attenuate the oxidation and degradation of Ca\(^{2+}\)-handling proteins, preventing calpain activation during IR. It has special concern because calpain activation within the myocardium has been directly linked with IR-induced necrotic and apoptotic cardiac myocyte death (French, Hamilton et al. 2008). Furthermore, several reports suggest that exercise *per se* is able to enhance cardiac mitochondria Ca\(^{2+}\) uptake capacity without MPTP induction (Ascensao, Luminí-Oliveira et al. 2011, Kavazis, McClung et al. 2008, Marcil, Bourduas et al. 2006). Therefore, exercise may induce beneficial morphological and biochemical adaptations in mitochondria, which can be translated in a cardiac phenotype more functional and protected against deleterious stimuli.

**2.2.1.2. Mitochondrial biogenesis**

Due to the high-energy demand of the heart, a decline in mitochondrial function can result in a deterioration of cardiac performance (Li, Muhlfeld et al. 2011) and can lead to a wide variety of pathophysiological conditions. According to some reports, the malfunction of mitochondria and the decrease of mitochondrial biogenesis, together with increased oxidative damage, seem to exert some of the most deleterious effects on the organism (Guarente 2008, Lopez-Lluch, Irusta et al. 2008). Fortunately, although controversial, heart seems to have the ability to alter some gene expression profile and phenotype to produce new and more functional mitochondria (Lee and Wei 2007). Regular exercise or increased energy demand are important stimuli that lead to increased mitochondria biogenesis (for refs see Lopez-Lluch, Irusta et al. 2008). In fact, increases in cytosolic Ca\(^{2+}\) levels induced by exercise stimulates
calmodulin kinase which then promotes PGC1-α expression (Wu, Kanatous et al. 2002) that has been shown to be a major regulator of mitochondrial biogenesis (Rodgers, Lerin et al. 2005). Also, Geng et al. (2010) showed that in skeletal muscle-specific PGC-1α knockout mice, exercise-induced mitochondrial biogenesis and angiogenesis were significantly attenuated. However, exercise-induced mitochondrial biogenesis on cardiac muscle is still not fully illusive. In fact, although a significant amount of experimental data on mechanisms involved in exercise-induced mitochondrial biogenesis has been obtained in skeletal muscle, less information is available about the heart. In this regard, Li et al. (2011) reported no changes in mitochondrial biogenesis on left ventricle after 3 months of endurance exercise. Actually, in contrast to skeletal muscle in which adaptive changes in volume fraction of mitochondria can readily occur, this is not so frequent in cardiac muscle (Hood, Balaban et al. 1994). So, the exercise-induced benefits on heart mitochondria biogenesis are still controversial.

### 2.2.1.3. Oxidative stress and antioxidant capacity

With exercise performance, increased O$_2$ consumption creates favorable conditions for increased generation of ROS, an inevitable consequence that may increase oxidative stress at the organelle, cell, and tissue (Ji, Leeuwenburgh et al. 1998). However, if these stimuli are repeated over time, it may have a strong modulating effect on various defense systems in cardiac cells (Powers, Lennon et al. 2002). Also, in some deleterious conditions that lead to ROS production, as IR-induced myocardial injury, DOX administration or aging, physical exercise as been reported to induce benefic countermeasures. In fact, IR-induced myocardial injury is manifested due to the complex interaction of numerous factors but ROS generated by mitochondria during IR injury are believed to play key role in this process (for refs see Honda, Korge et al. 2005). During IR, mitochondrial ROS generation can lead to increased general oxidative stress and consequently Ca$^{2+}$ overload, which could be of
special concern due its close relation with MPTP opening (Ascensao, Lumini-Oliveira et al. 2011), resulting in the release of proapoptotic proteins and subsequent activation of programmed cell death (Adhihetty, Ljubicic et al. 2007). Also, an additional production of ROS has been related with aging process and it has been also reported that ROS expression is upregulated during cardiac hypertrophy and heart failure (Gustafsson and Gottlieb 2009), being that, increased mitochondrial oxidant production is accepted as a cause of myocardial cell loss via apoptosis and necrosis (Judge, Jang et al. 2005). In this regard, exercise has been shown to provide intrinsic protection (Bowles, Farrar, & Starnes, 1992; M. B. Harris & Starnes, 2001; Powers et al., 1998; Powers et al., 2004) and mitochondria play an important role on this cardioprotective phenotype (Bejma, Ramires et al. 2000, Zhu, Zuo et al. 2007). In fact, the decreased mitochondrial oxidant damage (Judge, Jang et al. 2005) is an important adaptive strategy within mitochondria that contribute to cardioprotection.

Decreased oxidant damage can be understood as a result of both reduction of oxidant production or increased of antioxidant enzymes. Several reports had postulate that chronic endurance exercise results in a reduction of mitochondrial oxidant production (Judge, Jang et al. 2005, Starnes, Barnes et al. 2007) and enhanced mitochondrial antioxidant enzyme activity (Judge, Jang et al. 2005, Kavazis, McClung et al. 2008, Starnes, Barnes et al. 2007) which could reduce oxidative stress induced by different stress stimulators (Ascensao, Magalhaes et al. 2006, Lennon, Quindry et al. 2004, Powers, Locke et al. 2001). In general, most studies designed to investigate the influence of regular exercise on cardiac antioxidant activity focus on endurance training programs, and there are little available reports on the effect of sprint training in the modulation of antioxidant defenses (for refs see Ascensao, Ferreira et al. 2007). However, it seems clear that cardiac muscle tissue, when stimulated by acute exercise, reveals increased signs of cell damage due to oxidative stress. Despite this fact, Ascensao et al. (2011) reveal that a single bout of exercise increased SOD activity, and so, decreased oxidative stress.
As previously described, GPX, SOD and CAT are included on primary enzymatic antioxidant defenses and the relation of those with exercise has been hardly studied. Interestingly, current data have reported increases (Lennon, Quindry et al. 2004, Powers, Demirel et al. 1998), no changes (Ascensao, Magalhaes et al. 2005), or even decreases (Chicco, Schneider et al. 2005, Hong and Johnson 1995) in those antioxidant enzyme activities following endurance exercise training. These differences can be a consequence of some methodological differences, as characteristics of the animals and/or training protocols; biochemical procedures or type of markers and enzymes considered (Ascensao, Ferreira et al. 2007). However, the general approaches indicate that chronic exercise is responsible for either improved or unchanged levels of antioxidant enzymes activity.

Also, lipid peroxidation and protein oxidation following endurance training had been hardly studied and results are still controversial. In fact, both heart lipid peroxidation and protein oxidation have demonstrated increases (Aydin, Ince et al. 2007), no changes (Ascensao, Magalhaes et al. 2005, Chicco, Schneider et al. 2005) or decreases (Ascensao, Magalhaes et al. 2005) in response to endurance training. However, major tendencies may be indicative that, in general, endurance exercise training has some positive modulator effects on some enzymatic and non-enzymatic antioxidant systems (for refs see Ascensao, Ferreira et al. 2007). In addition, the rate of H₂O₂ production during exercise performance has been studied, in order to analyze oxidative stress. Judge et al. (2005) showed that exercise could decrease H₂O₂ production in myocardial mitochondria of male rats. More recently, Starnes et al. (2007) also reported a H₂O₂ decrease following an endurance exercise protocol. Importantly, they found similar decreases in both IFM and SSM mitochondrial populations. However, different results are under apparently conflict since Marcil et al. (2006) did not find differences on H₂O₂ levels following an endurance exercise protocol. It is possible that differences in sex or rat strain are responsible for the different results (Starnes, Barnes et al. 2007). However, it is generally accepted that endurance training induces adaptations within myocardial mitochondria, resulting in a decreased oxidative stress.
Furthermore, Navarro et al. (2004) reported that moderate treadmill exercise significantly decreased the aging-associated development of heart oxidative stress preventing the increase in protein carbonyls and TBARS contents of submitochondrial membranes and the decrease in antioxidant enzyme activities.

In general, it is accepted that endurance exercise induces cardioprotection against oxidative stress possible through an increased antioxidant enzyme activity and/or decreased oxidant production.

### 2.2.1.4. Cell death pathways

As reviewed in previous sections, apoptosis is one of the pathways of cell death and it is also the most studied phenomenon in which exercise induces protection. Kavazis et al. (2008) reported that exercise induces a cardiac mitochondrial phenotype that resists to apoptotic stimuli in both SSM and IMF. In this study, cytochrome c was described with primary role in oxidative adaptation within mitochondria and following an exercise training protocol cytochrome c release susceptibility after ROS challenge was decreased. Also, Cyc D and ANT levels decreased following exercise training, being that, ANT decreased in both SSM and IMF. Furthermore, exercise induces increased antioxidant (Ascensao, Ferreira et al. 2007, Powers, Locke et al. 2001) and exercise is related to attenuation of the oxidation and degradation of Ca\(^{2+}\)-handling proteins, which can lead to caspase-3 and -9 activation, and prevention of calpain activation (French, Hamilton et al. 2008). These reports suggest that both subfractions of mitochondria undergo biochemical adaptations in response to endurance exercise leading to decreased apoptotic susceptibility.

In addition to IR, the cardiomyopathy-related to and STZ-induced type I diabetes and aging, the chronic endurance exercise also provide protection against tissue and mitochondrial deleterious effects of DOX treatment, a topic of special interest in the present dissertation. In the following section, the
mechanisms and consequences of the selective toxicity of this anti-cancer drug for the heart will be addressed.

2.3. Doxorubicin: therapeutic agent vs. cardiotoxicity

One of the mostly used chemotherapeutic drugs is the highly effective anthracycline DOX. However, its clinical use is limited by the dose-related and cumulative cardiotoxicity and consequent dysfunction that can lead to apoptosis (Ascensao, Magalhaes et al. 2005, Carvalho, Santos et al. 2009, Wallace 2003). In fact, DOX is an anthracycline prescribed alone or in combination with other chemotherapeutics for the treatment of various neoplasms such as leukemias, lymphomas, thyroid and lung carcinomas, several sarcomas, stomach, breast, bone and ovarian cancers (Wallace 2003). It has been shown that DOX antineoplastic activity is attributed to its ability to intercalate into the DNA double helix and/or to bind covalently to proteins involved in DNA replication and transcription, leading ultimately to cellular death through the inhibition of DNA, ribonucleic acid (RNA) and protein synthesis (Carvalho, Santos et al. 2009). More specifically, DOX can be classified as a topoisomerase II poison (Fortune and Osheroff 2000). Topoisomerase II is an essential enzyme that plays a role in virtually every cellular DNA process and as a result of this action DOX generate high levels of enzyme-mediated breaks in the genetic material of treated cells and ultimately trigger cell death pathways (Fortune and Osheroff 2000). It has been shown that DOX, by simple diffusion, enters cancer cells and binds with high affinity to a proteasome in cytoplasm forming a DOX proteasome complex that translocates into the nucleus in an ATP-dependent process facilitated by nuclear localization signals. Finally, DOX dissociates from the proteasome and binds to DNA due to its higher affinity for DNA than for the proteasome (Kiyomiya, Matsuo et al. 2001). This process is pictured on figure 2.
Unfortunately, the clinical use of DOX is limited due the occurrence of a multidirectional cytotoxic effects, being cardiotoxicity the most known (Berthiaume and Wallace 2007, Sardao, Pereira et al. 2008). Actually, the clinical use of DOX soon proved to be hampered by serious problems such as the development of resistance in tumor cells or toxicity in healthy tissues and heart seems to be a particularly susceptible organ to DOX-induced toxicity (Carvalho, Santos et al. 2009). Several mechanisms, in which mitochondria are involved, are proposed to be responsible for the increased heart sensitivity to DOX-induced toxicity including: i) a special affinity of DOX by cardiolipin, which is a major phospholipid component of the inner mitochondrial membrane in the heart; ii) a higher mitochondrial density per unit volume in cardiomyocytes when compared to other tissues; iii) the possible but controversial existence of a specific and external reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase able to initiate DOX redox cycling and, consequently, increased formation of ROS and iv) the fact that the heart has low levels of antioxidant defenses when compared with other tissues (Lebrecht, Setzer et al. 2003, Sardao, Pereira et al. 2008, Wallace 2003).
However, despite the clinical manifestations of DOX-induced cardiomyopathy and the knowledge that DOX induces several cardiac ultrastructural changes, the mechanisms responsible for DOX-induced cardiac toxicity remain elusive. Nevertheless, clinical manifestations of DOX-induced cardiotoxicity can be acute and chronic but there is a wide variation in the frequency of clinical DOX-induced cardiotoxicity (Carvalho, Santos et al. 2009). Following some reports, acute effects in the heart, including arrhythmias, hypotension and several electrocardiographic alterations can be clinically controlled and frequently reversible, disappearing once the treatment ceases (van Acker, Kramer et al. 1996). On the other hand, chronic cardiotoxic effects induced by DOX are dose-dependent and culminate in CHF (Swain, Whaley et al. 2003). In both cases, mitochondria play an important role being classified as a primary target organelle of DOX-induced cardiotoxicity, evidenced by morphological and biochemical alterations. Interestingly, Hayward et al. (2012) suggested that all patients treated with anthracyclines are at greater risk of cardiotoxicity, particularly those individuals treated with these drugs at an early age. While it is still unclear why children are at a greater risk, it have been proposed that exposure to anthracyclines at a young age leads to chronically elevated hemodynamic stress and an eventual stunting of normal cardiac growth. Also, Huang et al. (2010) suggested that DOX exposure in juvenile rats decreased the ability of the heart to adapt to increases in workload as adults or olds.

### 2.3.1 Cardiac mitochondrial toxicity induced by DOX

Mitochondria are the producers of most of the ATP necessary for metabolism via oxidative phosphorylation. Also, they play other important roles as cellular redox and ion homeostasis, oxygen sensing, signaling and regulation of programmed cell death (for refs see Rabinovitch 2012, Sheu 2009). Therefore, mitochondrial integrity is vital for cellular homeostasis and cardiac performance (Chaudhary, El-Sikhry et al. 2011).
Unfortunately, mitochondria have been identified as primary DOX target organelles, and their involvement is evidenced by the results of many studies reporting functional and morphological alterations, as will be briefly described. Indeed, it has been suggested that cardiomyocyte dysfunction induced by DOX treatment is related to increased levels of ROS-induced damage and apoptotic cellular death, involving mitochondria in the process (Jung and Reszka 2001) culminating in the disruption of major functions (Jung and Reszka 2001).

The next sections will briefly describe some DOX-induced mitochondrial dysfunctions such as morphological evidences, increased oxidative stress and susceptibility to apoptosis.

### 2.3.1.1 Morphological evidences

As a consequence of acute or chronic DOX treatment, ultrastructural alterations in rat cardiomyocytes have been reported (Ascensao, Ferreira et al. 2007, Ascensao, Oliveira et al. 2012, Yilmaz, Atessahin et al. 2006, Zhou, Starkov et al. 2001). These include nuclear swelling associated with disruption of nuclear membrane structure, a marked interstitial and cellular edema, perinuclear vacuolation, disorganization and degeneration of the myocardium, loss of myofibrils, distension of the sarcoplasmic reticulum, slight enlargement of the T-tubules and myofibrillar damage and loss (for refs see Carvalho, Santos et al. 2009, Zhou, Starkov et al. 2001). Also, in mice cardiomyocytes, DOX-induced acute alterations in mitochondria were observed, such as vacuolization, myelin deposition, disruption of membrane and organelle degeneration with cristae degeneration, intramitochondrial vacuoles as well as myelin figures (Ascensao, Ferreira et al. 2007, Ascensao, Oliveira et al. 2012, Sardao, Oliveira et al. 2009).

These ultrastructural alterations are dose-dependent being that higher DOX concentrations promote more profound cellular alterations (Carvalho, Santos et
al. 2009) and those ultrastructural injuries are not repaired after cessation of DOX treatment becoming even more extensive.

### 2.3.1.2 Increased oxidative stress

One of the most known DOX-induced side effects is the increased oxidative stress and it seems to be an event that occurs both acutely and chronically (Ascensao, Oliveira et al. 2012, Wallace 2003). It has been suggested that DOX accumulation in mitochondria leads to a redox cycling by complex I of the mitochondrial respiratory chain, where single electrons are transferred to DOX (Ascensao, Oliveira et al. 2012). As previously mentioned, the possible but controversial existence of a specific NADH dehydrogenase able to start this redox cycling may have an important role. DOX enters mitochondria and reacts with mitochondrial complex I to form semiquinone radical intermediates, which is a short-lived metabolite and can react with O₂ producing ROS (for refs see Carvalho, Santos et al. 2009). Then, ROS can react with mitochondrial biomolecules in the vicinity, which include lipids, proteins and nucleic acids. Furthermore, increased cardiac oxidative stress associated with DOX toxicity leads to the depletion of reducing equivalents, impairment in oxidative phosphorylation with consequent decline in ATP, and interference with cellular Ca²⁺ homeostasis (Wallace 2003). Also, DOX is known to react with mitochondrial mtDNA forming adducts that interfere with proteins expression, lipid oxidation and normal mitochondrial function, which in turn further increases ROS production (Sardao, Pereira et al. 2008). This “vicious cycle” postulated that accumulation of mtDNA mutations is exponential and associated with massive increase in ROS production (Lenaz 1998). As previously described, heart mitochondria are important target of DOX, accumulating the drug at relatively high concentrations. So, it is not surprising that these organelles are especially susceptible to DOX-induced oxidative damage, and at the same time, they are also important sources of DOX-induced ROS (Ascensao, Oliveira et al. 2012). On other hand, studies show an upregulation of antioxidant defenses,
suggesting an adaptive response of cells to oxidative unbalance promoted by DOX (Yilmaz, Atessahin et al. 2006). Interestingly, the antioxidant capacity seems to increase significantly following DOX treatment in young but not in old Fischer suggesting an increase in DOX-induced oxidative damage with age (Pritsos and Ma 2000). Despite this fact, it is notable that, although the heart has relatively low antioxidant capacity, it shows an upregulation as an adaptive response to this oxidative unbalance. Moreover, as a response to this cellular stress, HSP 60 and HSP 70 increased in hearts and mitochondria from DOX treated animals and cells (Ascensao, Magalhaes et al. 2005, Kavazis, Smuder et al. 2010).

### 2.3.1.3 Increased susceptibility to apoptosis

Another important DOX-induced alteration on cell is the increased susceptibility to trigger apoptosis. DOX toxicity leads to impairments on cellular Ca$^{2+}$ homeostasis, which leads to increased susceptibility to the MPTP opening (Oliveira and Wallace 2006) that is characterized by the loss of the impermeability that characterizes the inner mitochondrial membrane, as it was previously described. This complex process is mediated by the formation and opening of protein complex-like pores, the MPTP (Ascensao, Lumini-Oliveira et al. 2011, Lumini-Oliveira, Magalhaes et al. 2011). As already mentioned, one of the consequences of the MPTP induction, besides the disturbance of cell and mitochondrial Ca$^{2+}$ homeostasis, is the release of cytochrome c and some others pro-apoptotic proteins, with consequent initiation of apoptotic cascades. Also, the increased oxidative stress induced by DOX, is able to interfere with mitochondrial functionality, which leads to apoptosis. Indeed, DOX-induced cardiomyocytes apoptosis has been suggested to occur both acutely and chronically (Carvalho, Santos et al. 2009).

As previously mentioned, it is important to develop a strategy that results in a cardioprotective phenotype against DOX-induced impairments. In this regard, physical exercise in its various forms has been shown to be an effective
intervention that can counteract the acute and chronic deleterious insults for the myocardium, which includes DOX treatment. The following sections will briefly discuss this issue.

2.4. Exercise as a therapeutic and preventive strategy against DOX-induced cardiotoxicity.

Exercise has been considered the most effective strategy to promote a healthy lifestyle and its benefits against DOX-related impairments are evident (Ascensao, Lumini-Oliveira et al. 2011, Ascensao, Magalhaes et al. 2005, Ascensao, Oliveira et al. 2012, Chicco, Schneider et al. 2005, Emter and Bowles 2008, Hayward, Lien et al. 2012, Kavazis, Smuder et al. 2010, Powers, Lennon et al. 2002, Wonders, Hydock et al. 2008). Although the most studied form of exercise in DOX treated animals is the chronic exercise, acute exercise also seems to provide beneficial effects. Mitochondrial adaptations may play critical role in exercise-induced protection against DOX-induced cardiac impairments (Ascensao, Ferreira et al. 2007, Ascensao, Oliveira et al. 2012). The effects of exercise on DOX-treated mitochondria are highlighted in table 2.

2.4.1 Acute exercise

Acute exercise broadly refers to a single bout of exercise performed only once and it was already proved to be effective against DOX-induced impairments. In fact, Wonders et al. (2008) reported that an acute bout of treadmill running performed 24 hours prior to DOX injection attenuated the hemodynamic impairment observed after acute DOX administration and reduced left ventricular lipid peroxidation. In addition, Ascensao et al. (2011) showed that an acute bout of treadmill exercise protects against cardiac mitochondrial dysfunction, preserving mitochondrial phosphorylation capacity and attenuating DOX-induced decreased tolerance to MPTP induction. In the same study, it was
observed that acute exercise prevented the decreased cardiac mitochondrial function detected as impaired state 3, phosphorylative lag-phase and maximal transmembrane potential. Also, acute exercise prevented the inhibitory effects of DOX treatment on the activity of cardiac mitochondrial respiratory chain complexes I and V, and on increased caspase-3 and -9 activities.

Furthermore, it has also been described that acute exercise may contribute to diminished free radical production (for refs see Ascensao, Oliveira et al. 2012). However, further research is needed to clarify the exact mechanisms by which an acute exercise induces a protective phenotype in DOX-treated cardiac mitochondria.

### 2.4.2 Chronic exercise

Unlike acute exercise, chronic exercise has been hardly studied and the results consistently demonstrate that is able to antagonize DOX-induced cardiac impairments (Ascensao, Magalhaes et al. 2005, Chicco, Schneider et al. 2005, Hayward, Lien et al. 2012, Hydock, Lien et al. 2008, Kavazis, Smuder et al. 2010). Those alterations induced by chronic exercise can be seen at morphological, functional and biochemical levels and the role of mitochondria is pivotal in this process (Ascensao, Oliveira et al. 2012, Kavazis, Smuder et al. 2010). Ascensao et al. (2005) reported the involvement of mitochondria in cardioprotection afforded by endurance training against DOX treatment, demonstrating prevention of acute DOX-induced mitochondrial alterations regarding oxidative stress, respiration, and Ca\(^{2+}\) loading capacity.

At ultrastructural level, it has been reported that cardiac alterations induced by DOX treatment are also attenuated by previous chronic exercise (Ascensao, Magalhaes et al. 2005). In fact, when compared with saline (SAL) group, sedentary (SED) DOX-treated animals showed myocardial damage (Ascensão, Magalhães et al. 2006). The observed morphological alterations consisted of mitochondrial damage with extensive degeneration and loss of cristae, swelling
and abnormal size and shape, intramitochondrial vacuoles and notorious myelin figures that probably resulted in the formation of secondary lysosomes. All of these alterations were attenuated in trained animals treated with DOX (Ascensão, Magalhães et al. 2006).

At functional level, different authors reported the protective effects of chronic exercise. In fact, Hydock et al. (2008) suggested that exercise training in rats before DOX treatment attenuated DOX-induced cardiac dysfunction, through the maintenance of fractional shortening, developed pressure and contractility. Also, Chicco et al. (2005) reported that both low intensity exercise training and an endurance training protocol with gradually increased intensity attenuated the adverse effects of DOX by preventing DOX-induced decline in cardiac function through maintenance of left ventricular diastolic pressure, rate of left ventricular pressure development and rate of left ventricular relaxation.

At biochemical level, as depicted on table 2, several authors had proved the beneficial effects of chronic exercise. Importantly, some of the most studied biochemical parameters associated with exercise and DOX are the antioxidant capacity, oxidative stress markers and apoptotic susceptibility (for refs see Ascensao, Oliveira et al. 2012). As previous referred, HSP have an important role as antioxidants molecules contributing to normal cellular integrity and are overexpressed after endurance training. However, Chicco et al (2006) and Kavasis et al. (2010) also showed that exercise had no influence on HSP or that it is not determinant on cardioprotection being that exercise in cold vs. normal temperatures may also display other types of differences regarding alteration of mitochondrial physiology, besides alteration in the expression of HSPs. Furthermore, the upregulation of mitochondrial manganese superoxide dismutase (MnSOD) seems contribute for cardioprotection. In fact, as mitochondrial DOX toxicity has been largely attributed to increased oxidative stress, increased antioxidant activity may be important to explain how endurance training counteracts some of DOX-induced myocardial damage. Chicco et al. (2006) associated low-intensity treadmill exercise training-induced cardioprotection to the inhibition of apoptotic signaling and the increased activity
of GPX. Also, the effect of training on preventing activation of cardiac apoptotic pathways has been described being that, training decreased the susceptibility of appearance of apoptotic markers in the hearts of DOX-treated animals, as increased mitochondrial Bax, Bax-to-Bcl2 ratio and tissue caspase 3 activity (Ascensao, Magalhaes et al. 2005). In fact, it has been suggested that chronic exercise stimulation may also afford protection against the increased susceptibility to the MPTP as the deleterious effects of Ca\(^{2+}\) on heart mitochondrial respiration of DOX-treated animals were attenuated in trained group treated with DOX (Ascensao, Magalhaes et al. 2005). As previous described, MPTP is related to oxidative damage and therefore, it is possible that increased resistance of cardiac mitochondria from trained animals to the MPTP can be related to increased antioxidant defenses. Accordingly, the higher levels of reduced sulphydryl groups in trained mitochondria than in sedentary groups may be indicative of enhanced antioxidant capacity and/or of more elevated sulphydryl-donors, such as GSH, in mitochondria from trained animals (for refs see Ascensao, Oliveira et al. 2012). However, further studies are necessary in order to better understand this issue.

Table 2. Summary of some described mitochondrial-related alterations associated with DOX-induced cardiotoxicity and the modulation effect afforded by physical exercise against DOX (adapted from Ascensao, Oliveira et al. 2012).

<table>
<thead>
<tr>
<th></th>
<th>DOX effect</th>
<th>Exercise effect against DOX</th>
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<tbody>
<tr>
<td><strong>ROS production</strong></td>
<td>↑</td>
<td>↓</td>
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<tr>
<td><strong>Oxidative damage markers</strong></td>
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<tr>
<td>Lipid peroxidation</td>
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<tr>
<td>Protein oxidation</td>
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<tr>
<td>DNA oxidation</td>
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<tr>
<td>Aconitase activity</td>
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<td><strong>Apoptotic signaling</strong></td>
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<tr>
<td>Bax-Bcl-2 ratio</td>
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<tr>
<td>Cytochrome c release</td>
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<td>Caspase 9 activation</td>
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<tr>
<td><strong>Respiratory endpoints</strong></td>
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<td>State 3</td>
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<tr>
<td>State 4</td>
<td>↑ or = or ↓</td>
<td>= or ↓</td>
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<tr>
<td>RCR</td>
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<td>= or ↑</td>
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<tr>
<td>ADP/O ratio</td>
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<tr>
<td>Uncoupled respiration</td>
<td>↓</td>
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<tr>
<td>Creatine-stimulated respiration</td>
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<tr>
<td>Maximal ΔΨ</td>
<td>↓ or =</td>
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<tr>
<td><strong>Ca²⁺-induced MTPT</strong></td>
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<tr>
<td><strong>ANT content and functioning</strong></td>
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<tr>
<td><strong>Mitochondrial chaperones</strong></td>
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<td><strong>Mitochondrial antioxidants</strong></td>
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<tr>
<td>Thiols</td>
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<td>Vitamin E</td>
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<tr>
<td>Enzymes</td>
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<tr>
<td>Coenzyme Q isoenzymes</td>
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<tr>
<td><strong>ETC complex activity</strong></td>
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<td>Complex I</td>
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<td>Complex II</td>
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<td>Complex III</td>
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<td>Complex IV</td>
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<tr>
<td>Complex V</td>
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</tbody>
</table>

(↑) – increase; (↓) – decrease; (=) – no alterations

Despite the extensive number of studies on this topic, the effects of both endurance treadmill training and voluntary free-wheel running activity performed
before and during sub-chronic DOX treatment schedule on cardiac mitochondrial bioenergetics are yet to be elucidated.

This is of particular importance in the context of exercise-induced protection against DOX-related cardiac mitochondriopathy, as cancer patients undergoing DOX treatment may be advised to exercise for many reasons including to counteract physical fatigue and to improve performance, and also to mitigate cardiac damage as a result of chemotherapy.

This master sports science course in which this work is inserted is the context of physical activity and elderly. Despite developed with adult rats, the present work can contribute to extend the knowledge in this particular area representing preliminary findings in adult population and considering that DOX-based chemotherapeutic treatments against several types of malignances are more prevalent with increasing age.
3. Aim

The aim of the present study was to analyze the effect of two types of physical exercise (treadmill endurance training (TM) and free-wheel voluntary physical activity (FW)) against heart mitochondrial dysfunction induced by sub-chronic treatment of DOX.

We can define as specific purposes of this work the analysis of the adaptations induced by both types of exercise on heart mitochondria from DOX treated animals on:

- Mitochondrial respiratory function;
- Mitochondrial electrical transmembrane potential;
- MPTP susceptibility;
- Mitochondrial oxidative damage.
4. Materials and methods

4.1 Reagents

Deionized water (18.7 MΩ) from an arium®611VF system (Sartorius, Göttingen, Deutschland) was used. Doxorubicin hydrochloride, commercial/clinic use, was obtained from Ferrer Farma (Barcelona, Spain), prepared in a sterile saline solution, sodium chloride (NaCl) 0.9% (pH 3.0) and stored at 4ºC for no longer than five days upon rehydration. All other chemicals were purchased from Sigma Aldrich (Sintra, Portugal).

4.2 Animals

All experiments involving animals were conducted in accordance with the European Convention for the Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes (CETS no. 123 of 18 march 1986 and 2005 revision) and the Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (C (2007) 2525). Supervisors of this work are accredited by the Federation of Laboratory Animal Science Associations (FELASA) for animal experimentation. The Ethics Committee of the Faculty of Sport approved this experimental protocol.

Thirty-six male Sprague-Dawley rats (aged 2 weeks old) were obtained from Charles River Laboratories (L'Arbresle, France) and were randomly divided into six groups (n=6 per group): Saline sedentary (SAL+SED), saline treadmill endurance training (SAL+TM), saline free wheel voluntary physical activity (SAL+FW), doxorubicin sedentary (DOX+SED) doxorubicin treadmill endurance training (DOX+TM) and doxorubicin free wheel voluntary physical activity (DOX+FW). Only male rats were used to avoid hormone-dependent influence in drug mitochondrial function/toxicity. During the experimental protocol, animals were housed in collective cages (two rats per cage) and were maintained in a
room at normal atmosphere (21–22 °C; 50–60% humidity) receiving food (Scientific Animal Food and Engineering, A04) and water \textit{ad libitum} in 12-h light/dark cycles.

4.3 Exercise protocols

4.3.1 Endurance training protocol

The animals from TM groups were exercised 5 days/week (Monday–Friday) in the morning (between 10:00 and 12:00 A.M.), for 12 weeks on a LE8700 motor driven treadmill (Panlab, Harvard, U.S.A). The treadmill speed was gradually increased over the course of the 12-week training period. The protocol included 5 days of habituation to the treadmill with 10 min of running at 15 m/min, with daily increases of 5-10 minutes (min) until 30 min was achieved (week 0). Habituation was followed by one consecutive week of continuous running (30 min/day) at 15 m/min and was gradually increased until 60 min/day on the week 1. The velocity increased gradually from 18 m/min to 27 m/min (week 7). SAL+TM continue to increase velocity to 30 m/min while DOX+TM animals gradually decreased running velocity to 20 m/min (Table 3).

<table>
<thead>
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<th>Week</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
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</thead>
<tbody>
<tr>
<td>SAL+ TM</td>
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<tr>
<td>TM Velocity (m/min)</td>
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<td>20</td>
<td>22</td>
<td>24</td>
<td>25</td>
<td>25</td>
<td>27</td>
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<td>28</td>
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<td>30</td>
<td>30</td>
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<tr>
<td>DOX + TM</td>
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<tr>
<td>TM Velocity (m/min)</td>
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<td>25</td>
<td>22</td>
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<tr>
<td>Exercise Duration (min/day)</td>
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</tr>
</tbody>
</table>
4.3.2 Voluntary physical activity

The animals from FW groups were housed in a polyethylene cage equipped with a running wheel [perimeter=1.05 m, Type 304 Stainless steel (2154F0106-1284L0106) Technicplast, Casale Litta, Italy)]. The rats were allowed to exercise *ad libitum* with an unlimited access to the running wheel 24h/day. Running distance was recorded using ECO 701 from Hengstler (Lancashire, U.K.).

4.4 Doxorubicin treatment

After the 5\textsuperscript{th} week of endurance training or free wheel exercise, the animals were sub-chronically treated with DOX (2 mg/Kg of body weight) or sterile saline solution NaCl 0.9\% (SAL, 2 mg/kg of body weight) intraperitoneal injection/week during 7 weeks. The animals assigned to the TM groups received DOX or SAL injections during the weekend in a day-off training.

4.5 Animal sacrifice, heart and soleus extraction

Forty-eight hours after the last TM exercise bout, non-fasted rats were euthanized by cervical dislocation between 9:00 and 10:00 AM to eliminate possible effects due to diurnal variation. After quickly opening the chest cavity, rat hearts were rapidly excised, rinsed, carefully dried, and weighed. Right *soleus* muscle was also rapidly extracted and weighed. Portions of approximately 50 milligrams (mg) of one *soleus* muscle were homogenized in homogenization buffer (200 minimolar (mM) Tris, 137 mM NaCl, 0.2 mM EDTA, 0.5 mM EGTA, 1\% triton X-100, tissue: buffer ratio of 100 mg/mL, pH 7.4) using a Teflon pestle on a motor driven Potter-Elvehjem glass homogenizer at 0–4°C three to five times for 5 s at speed low setting, with a final burst at a higher speed setting. Homogenates were centrifuged (2 min at 3000 xg, 4°C, in order to eliminate cellular debris) and the resulting supernatants were stored at −80°C.
for later determinations, as detailed below. Protein content from *soleus* homogenates were spectrophotometrically determined using the biuret method and bovine serum albumin as standard (Gornall, Bardawill et al. 1949).

### 4.6 Isolation of heart mitochondria

Heart mitochondria were daily prepared using conventional methods of differential centrifugation (Bhattacharya, Thakar et al. 1991) as follows. Briefly, the animals were sacrificed as stated above and the heart was immediately excised and finely minced in an ice-cold isolation medium containing 250 mM sucrose, 0.5 mM EGTA, 10 mM HEPES (pH 7.4), and 0.1% defatted bovine serum albumin (BSA, Sigma, cat. no. A-7030). The minced blood-free tissue was then resuspended in 40 mL of isolation medium containing 0.75 mg/mL protease subtilopeptidase A Type III (Sigma P-5380) and homogenized with a tightly fitted homogenizer (Teflon: glass pestle). The suspension was incubated for 1 min (4°C) and then re-homogenized. The homogenate was then centrifuged at 13,000 xg for 10 min. The supernatant was decanted and the pellet, essentially devoid of protease, was gently re-suspended with a loose-fitting homogenizer. The suspension was centrifuged at 750 xg for 10 min and the resulting supernatant was centrifuged at 12,000 xg for 10 min. The pellet was re-suspended using a paintbrush and re-pellet at 12,000 xg for 10 min. EGTA and defatted BSA were omitted from the final washing medium. Mitochondrial protein content was determined by the Biuret method calibrated with BSA (Gornall, Bardawill et al. 1949). The isolation procedures were performed within approximately 1 h at 0–4°C. Aliquots of isolated mitochondria were separated and frozen at −80°C for later determination of oxidative damage. The remaining mitochondrial were used within 2–3 h after the excision of the heart and was maintained on ice (0–4°C) throughout this period.
4.7 Mitochondrial respiratory activity

Mitochondrial respiratory function was measured polarographically at 30°C using a Biological Oxygen Monitor System (Hansatech Instruments) and a Clarktype oxygen electrode (Hansatech DW1, Norfolk, UK). The reactions were conducted in a 0.75 mL closed, thermostatted and magnetically stirred glass chamber containing 0.5 mg/mL of mitochondrial protein in a respiration buffer containing 50 mM KCl, 130 mM sucrose, 2.5 mM KH$_2$PO$_4$, and 0.5 mM Hepes, pH 7.4. After 1-min equilibration period, mitochondrial respiration was initiated by adding glutamate/malate (G/M) to a final concentration of 5 and 2.5 mM, respectively. State 3 respiration was determined after adding ADP (150 nmol); state 4 was measured as the rate of oxygen consumption after ADP phosphorylation. The RCR (state 3/state 4) and the ADP/O (the number of nmol ADP phosphorylated by atom of oxygen consumed) ratios were calculated according to Estabrook (1967).

4.8 Mitochondrial transmembrane electric potential

Mitochondrial transmembrane electric potential ($\Delta \psi$) was monitored indirectly based on the activity of the lipophilic cation tetr phenyl phosphonium (TPP+) using a TPP+ selective electrode prepared in our laboratory as previously described (Ascensao, Lumini-Oliveira et al. 2011). Reactions were carried out in 1 mL of reaction buffer containing 50 mM KCl, 130 mM sucrose, 2.5 mM KH$_2$PO$_4$, and 0.5 mM Hepes, pH 7.4 supplemented with 3 µM TPP+ and 0.5 mg/mL of mitochondrial protein. For the measurements of $\Delta \psi$ with complex I-linked substrates, energization was carried out with G/M (5 mM and 2.5 mM, respectively) and ADP phosphorylation was achieved by adding 150 nmol ADP. The lag phase, which reflects the time needed to phosphorylate the added ADP, was also measured during experiments.
4.9 Mitochondrial osmotic swelling during MPTP induction

Mitochondrial osmotic volume changes were followed by monitoring the classic decrease of absorbance at 540 nm with a Jasco V-630 spectrophotometer. Swelling amplitude and rate of decreased absorbance upon Ca\(^{2+}\) addition were considered as MPTP susceptibility indexes. 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 HEPES, 5 mM KH\(_2\)PO\(_4\), 10 µM EGTA, pH 7.4, supplemented with 1.5 µM rotenone, 8 mM succinate and a single pulse of 80 nmol of Ca\(^{2+}\) with 0.5 mg/ml protein. Control trials were performed by using 1 µM of cyclosporin-A, the selective MPTP inhibitor (Broekemeier, Dempsey et al. 1989).

4.10 Mitochondrial oxidative damage

Before analysis, mitochondrial membranes were disrupted by several freeze-thawing cycles to allow free access to substrates. The extent of lipid peroxidation in heart mitochondria was determined by measuring MDA contents by colorimetric assay, according to a modified procedure described previously (Buege and Aust 1978). Suspended mitochondria were centrifuged at 12,000 xg for 10 min and re-suspended in 150 µL of a medium containing 175 mM KCl and 10 mM Tris-HCl, pH 7.4. Subsequently, mitochondria from the six groups were mixed with 2 volumes of trichloroacetic acid (10%) and 2 volumes of thiobarbituric acid (1%). The mixtures were heated at 80–90 °C for 10 min and re-cooled in ice for 10 min before centrifugation (4,000 xg for 10 min). The supernatants were collected and the absorbance measured at 535 nm. The amount of MDA content formed was expressed as nanomoles of MDA per milligram of protein (ε\(_{535}=1.56 \times 10^{-5} \text{ M}^{-1} \text{ cm}^{-1}\))

The basal mitochondrial content of oxidative modified -SH groups, including GSH and other -SH containing proteins, was quantified by spectrophotometric measurement according to Hu (1990). Briefly, a mitochondrial suspension
containing 5 mg/mL protein was mixed with 0.25 M Tris buffer pH 8.2 and 10 mM DTNB and the volume was adjusted to 1 mL with absolute methanol. Subsequently, the samples were incubated for 30 min in the dark at room temperature and centrifuged at 3000 xg for 10 min. The colorimetric assay of supernatant was performed at 414 nm against a blank test. Total -SH content was expressed in nanomoles per milligrams of mitochondrial protein (ε414=13.6 mM⁻¹ cm⁻¹).

4.11 Soleus citrate synthase activity

Soleus CS activity was measured using the method proposed by Coore et al. (1971). 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 412 nm.

4.12 Statistical analysis

All data are expressed as the mean±SEM (Standard Error of the Mean). Statistical analyses were performed using GraphPad Prism (version 6.0) or Statistical Package for the Social Sciences (SPSS version 21.0). Three-way repeated-measures ANOVA for body weight and distance cover by exercised groups to verify the effect of exercise and treatment over time. Two-way analysis of variance ANOVA were used to examine possible effect of treatment and/or exercise. To determine specific group differences, the two-way ANOVA were followed by Bonferroni post-hoc tests. In all cases, the significance level was set at p≤0.05.
5. Results

5.1. Characterization of animals and exercise protocols

Body weight alterations and distances covered by the animals during the entire protocol are shown in figure 3. No significant differences in the mean body weight of the animals from the beginning of the protocol until the 5th week, when sub-chronical DOX treatment was initiated, were found. Body weights of DOX treated animals were lower than SAL counterparts at the end of the protocol (DOX+SED vs. SAL+SED; DOX+TM vs. SAL+TM; DOX+FW vs. SAL+FW; p≤0.05). No differences in body weight between exercised groups, were found. TM and FW decreased body weight at 12th and 9th week, respectively (SAL+TM and SAL+FW vs. SAL+SED; p≤0.05). DOX treatment combined with TM decreased body weight from the 5th week (DOX+TM vs. DOX+SED; p≤0.05), whereas no significant differences were found between FW and SED treated groups (Figure 3A). After the 5th week DOX treated groups consumed less food than their SAL counterparts (data non shown, p≤0.05). Water consumption increased in FW groups (SAL and DOX) compared with SAL+SED and DOX+SED groups (data non shown, p≤0.05).

Figure 3. Effect of exercise and DOX treatment on (A) body mass over time and (B) distance covered per day by TM and FW groups during the 12 wks of protocol. Significant differences (p≤0.05) are mentioned in the text. Significant (p≤0.05) effects of Exercise (E), Treatment (T), time (t) or their interaction (E x T x t) are shown; Non Significant (NS, p>0.05).
As can be seen in Figure 3B, voluntary running distance decreased significantly in DOX+FW after the 5th week and remained lower until the end of the protocol (p≤0.05). Animals from TM group ran at the same velocity throughout the 8 weeks of the protocol. Running velocity and distance covered diminished in DOX+TM group at 11th and 12th week compared to SED+TM (p≤0.05).

Body, heart absolute weights, heart weight and femur length to body weight ratios, mitochondrial protein yielding as well as the activity of soleus citrate synthase in the six groups are shown in Table 4. Final body, heart weight and ratio of heart weight to body weight significantly decreased with DOX treatment (SAL+SED vs DOX+SED). Both chronic exercise types decreased final body weight, increased heart weight and the heart to body ratio (SAL+TM and SAL+FW vs SAL+SED). DOX treatment combined with TM and FW exercise induced a significant increase in heart weight and heart weight to body weight ratio compared with their DOX+SED counterparts. No significant differences were observed between groups regarding the Initial body weight, femur length to body weight ratio and yield of mitochondria isolation. TM induced a significant increase in the activity of soleus citrate synthase in both SAL and DOX treated animals (SAL+SED vs SAL+TM and DOX+SED vs DOX+TM).
<table>
<thead>
<tr>
<th></th>
<th>SAL+SED</th>
<th>SAL+TM</th>
<th>SAL+FW</th>
<th>DOX+SED</th>
<th>DOX+TM</th>
<th>DOX+FW</th>
<th>P*</th>
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<tr>
<td><strong>Initial body weight (g)</strong></td>
<td>207±3.91a</td>
<td>214±3.76a</td>
<td>212±1.55a</td>
<td>209±4.68a</td>
<td>207±4.63a</td>
<td>209±2.60a</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Final body weight (g)</strong></td>
<td>598±10.58a</td>
<td>522±9.87b</td>
<td>498±5.98b</td>
<td>438±6.22c</td>
<td>426±10.79c</td>
<td>429±16.95c</td>
<td>ExT</td>
</tr>
<tr>
<td><strong>Heart weight (g)</strong></td>
<td>1.44±0.03a</td>
<td>1.92±0.08b</td>
<td>1.85±0.10b</td>
<td>1.10±0.03c</td>
<td>1.45±0.05a</td>
<td>1.55±0.11a</td>
<td>E, T</td>
</tr>
<tr>
<td>Heart weight/body weight (mg.g⁻¹)</td>
<td>2.32±0.08a</td>
<td>3.53±0.10bc</td>
<td>3.68±0.12c</td>
<td>2.62±0.11d</td>
<td>3.34±0.08b</td>
<td>3.34±0.12b</td>
<td>E,T</td>
</tr>
<tr>
<td>Femur length/body weight (mm.g⁻¹)</td>
<td>0.08±0.00a</td>
<td>0.08±0.00a</td>
<td>0.09±0.00a</td>
<td>0.08±0.01a</td>
<td>0.09±0.00a</td>
<td>0.09±0.00a</td>
<td>E, T</td>
</tr>
<tr>
<td>Mitochondrial protein yielding (mg protein/g tissue)</td>
<td>18.58±0.62a</td>
<td>15.15±1.22a</td>
<td>15.01±1.50a</td>
<td>16.96±0.83a</td>
<td>18.60±4.68a</td>
<td>17.84±1.57a</td>
<td>NS</td>
</tr>
<tr>
<td>soleus citrate synthase activity (nmol. min⁻¹.mg⁻¹)</td>
<td>10.94±2.07a</td>
<td>23.34±1.79b</td>
<td>11.22±2.65a</td>
<td>8.69±1.15a</td>
<td>22.22±1.97b</td>
<td>10.27±1.32a</td>
<td>E, T</td>
</tr>
</tbody>
</table>

Values (mean ± SEM). Different letters are significantly different (p<0.05). * Significant (p≤0.05) effects of Exercise (E), Treatment (T), or their interaction (E x T) are shown; Non Significant (NS, p> 0.05).
5.2 Heart mitochondrial oxygen consumption

Mitochondrial respiratory activity in both SAL and DOX treated groups was measured to identify exercise-dependent effects (Figure 4). DOX treatment decreased heart mitochondrial respiration during state 3 and increased state 4 in SED animals (DOX+SED vs SAL+SED). Importantly, TM and FW exercise per se increased State 3 respiration in both SAL and DOX (SAL+TM and SAL+FW vs. SAL+DOX; DOX+TM and DOX+FW vs. DOX+SED). The coupling between oxygen consumption and ADP phosphorylation (RCR) was significantly affected by DOX treatment (DOX+SED vs. SAL+SED). TM significantly increased RCR in both SAL and DOX groups (SAL+TM vs. SAL+SED; DOX+TM vs. DOX+SED). FW increased RCR in DOX treated animals (DOX+FW vs. DOX+SED). Also, both TM and FW increased ADP/O in DOX group (DOX+SED vs. DOX+TM and DOX+FW).
Figure 4. Effect of exercise and DOX treatment on (A) state 3 of heart mitochondrial respiration, (B) state 4 of heart mitochondria respiration, (C) RCR and (D) ADP/O. Data are means±SEM for heart mitochondria (0.5 mg/mL protein) obtained from different mitochondrial preparations for each experimental group. Oxidative phosphorylation was measured polarographically at 30°C in a total volume of 0.75 mL. Respiration medium and other experimental details are provided in methods. RCR, respiratory control ratio (state 3/state 4); ADP/O, number of nmol ADP phosphorylated by atom of oxygen consumed. Different letters are significantly different (P≤0.05). Significant (p≤0.05) effects of Exercise (E), Treatment (T), or their interaction (E x T) are shown; Non Significant (NS, p>0.05).

5.3 Heart mitochondrial transmembrane electric potential

Heart mitochondrial variations in maximal ∆ψ and during ADP phosphorylation were determined using G/M as substrates. DOX treatment significantly affected the maximal ∆ψ, repolarization and ADP lag-phase (Figure 5). FW but not TM, increased maximal ∆ψ and repolarization, whereas both types of exercise decreased the ADP lag phase (SAL+TM and SAL+FW vs. SAL+SED). Both exercise protocols were able to counteract the DOX harmful effect normalizing maximal ∆ψ, repolarization and ADP lag phase.
Figure 5. Effect of exercise and DOX treatment on heart mitochondria $\Delta \psi$ fluctuations (A) maximal energization, (B) ADP-induced depolarization, (C) repolarization and (D) ADP phosphorylation lag phase. Data are mean±SEM for heart mitochondria (0.5 mg/mL protein) obtained from different mitochondrial preparations for each experimental group. Figure shows the average response of maximal mitochondrial membrane potential developed with glutamate (5 mM) plus malate (2.5 mM), the decrease in membrane potential after ADP addition (depolarization), the repolarization value after ADP phosphorylation, and the lag phase. Mitochondrial transmembrane potential was measured using a TPP$^+$-selective electrode at 30ºC in a total volume of 1 mL. Reaction medium and other experimental details are provided in methods. Different letters are significantly different (p≤0.05). * Significant (p≤0.05) effects of Exercise (E), Treatment (T), or their interaction (E x T) are shown; Non Significant (NS, p>0.05)

5.4 Mitochondrial osmotic swelling during MPTP induction

The effects of both types of exercise training and DOX treatment on in vitro susceptibility to Ca$^{2+}$-induced MPTP opening were investigated. The addition of Ca$^{2+}$ on mitochondria suspension resulted in a decrease in absorbance with three distinct phases. Initially, an increase in absorbance was observed, which most likely results from the formation of opaque Ca$^{2+}$ crystals inside mitochondria (Andreyev, Fahy et al. 1998). Upon MPTP opening, a decrease of
absorbance with a slow followed by a fast kinetic rate is usually observed in cardiac mitochondria. Incubation of mitochondrial suspension with cyclosporine A, a specific MPTP inhibitor (Broekemeier, Dempsey et al. 1989), limits the absorbance decrease after Ca$^{2+}$ addition, which demonstrate the association with MPTP opening.

Figure 6 shows different end-points measured from the recordings obtained, namely (A) swelling amplitude (the difference between the initial and the final absorbance value) and (B) the average swelling rate. The results demonstrate that DOX treatment significantly increased susceptibility to Ca$^{2+}$-induced MPTP opening (DOX + SED vs. SAL + SED). Heart mitochondria isolated from SAL+TM group, but not SAL+FW were less susceptible to Ca$^{2+}$-induced MPTP opening (SAL + TM vs. SAL + SED). Both types of exercise were able to mitigate DOX-induced increased susceptibility to MPTP opening (DOX + TM and DOX + FW vs. DOX + SED).

Figure 6. Effect of exercise and DOX treatment on heart mitochondria to Ca$^{2+}$-induced MPTP (A) Swelling amplitude; (B) Average swelling rate. Data are mean ± SEM. The absorbance of mitochondrial suspension was followed at 540 nm. Mitochondria were incubated as described in methods. A 80 nmol of Ca$^{2+}$ pulse (160 nmol/mg protein) was added to 0.5 mg of mitochondrial protein in order to attain the cyclosporin A-sensitive swelling, indicating that the decreased optical density corresponding to the increased swelling was due to MPTP opening. Different letters are significantly different (p≤0.05). * Significant (p≤0.05) effects of Exercise (E), Treatment (T), or their interaction (E x T) are shown; Non Significant (NS, p>0.05)
5.5 Oxidative stress markers

The next step was to ascertain whether exercise and DOX treatment-modulated mitochondrial oxidative stress markers. According to the protective phenotype seen in Fig.7 DOX treatment increased MDA levels and decreased –SH content (SAL + SED vs. DOX + SED). Exercise, particularly TM decreased mitochondrial MDA levels (SAL + TM vs. SAL + SED). Both types of exercise were able to revert DOX-induced alterations in MDA level and –SH content (DOX + SAL vs. DOX + TM and DOX + FW).

Figure 7. Heart mitochondrial (A) MDA and (B) reduced sulfhydryl contents. Data are means ± SEM for heart mitochondria obtained from different mitochondrial preparations for each experimental group. Different letters are significantly different (p≤0.05). Significant (p<0.05) effects of Exercise (E), Treatment (T), or their interaction (E x T) are shown; Non Significant (NS, p> 0.05).
6. Discussion

The current study provided additional support to understand the effects of both endurance treadmill training and voluntary wheel running activity performed before, during and after sub-chronic DOX treatment schedule on cardiac mitochondrial bioenergetics. Only male rats were used to avoid hormone-dependent influence in drug-induced mitochondrial toxicity (Lagranha, Deschamps et al. 2010). Rats were sub-chronically treated with DOX in an attempt to mimic human’s treatment, a protocol previously used by others (Pereira, Pereira et al. 2012, Santos, Moreno et al. 2002). Moreover, in the present study 1st DOX injection was administrated 5 weeks after the beginning of the exercise protocol with subsequent weekly injection until the end of protocol. This set up can be understood with both a preconditioning (preventive) and a therapeutic strategy against DOX treatment schedules. Two different types of exercise were analyzed: voluntary free wheel run and treadmill run. Considerable attention has been focused on the efficacy of exercise protocols since it has been speculated that further stress induced by exercise could be potentially detrimental, exacerbating the impairments induced by DOX (Emter and Bowles 2008). In fact, patients undergoing chemotherapy experience severe fatigue or exercise intolerance. Consequently, the intensity and duration of exercise they are able to tolerate is likely to be severely limited (Emter and Bowles 2008). For these reason, and because we wanted to analyze the response on heart mitochondria of DOX treated rats at distinct intensity and duration, both free wheel and run treadmill were performed.

The results on mitochondrial function obtained in the present study confirm at least in part, the cardiac protection afforded by both endurance treadmill training and voluntary free wheel running (for refs see Ascensao, Oliveira et al. 2012) against DOX toxicity. Cardiac dysfunction and defective mitochondrial function in DOX-treated animals has been studied previously (Ascensao, Lumini-Oliveira et al. 2011, Ascensão, Magalhães et al. 2006, Chicco, Schneider et al. 2005, Kavazis, Smuder et al. 2010, Sardao, Pereira et al. 2008, Wallace 2003). The present study confirmed that sub-chronic DOX administration in heart
mitochondria results in: (i) worsening heart mitochondrial respiration; (ii) decreased maximally developed ΔΨ, repolarization and increased phosphorylative lag phase; (iii) decreased ability of heart mitochondria to accumulate Ca²⁺ before MPTP induction and (iv) increased MDA levels and decreased -SH content. Both types of exercise performed before and during DOX treatment resulted in attenuation or complete prevention of the heart mitochondrial impairments induced by DOX.

6.1 Heart mitochondrial oxygen consumption and transmembrane electric potential

Previous studies have shown that exercise attenuates DOX-induced cardiac damage, diminishing the increased biochemical and morphological signs of toxicity induced (Ascensao, Magalhaes et al. 2005, Ascensao, Oliveira et al. 2012, Chicco, Schneider et al. 2006, Hydock, Lien et al. 2008, Kanter, Hamlin et al. 1985, Kavazis, Smuder et al. 2010); however, no data are available concerning the cross-tolerance effect of both voluntary free wheel running and endurance training on sub-chronic DOX treatment mitochondrial malfunction. Present results demonstrate that sub-chronic treatment of DOX induces impairments on mitochondrial respiration, and that 12 weeks of endurance running training and voluntary free wheel running prevented the inhibition of mitochondrial respiration. Alterations in mitochondrial oxidative phosphorylation induced by DOX relies in several factors including the decreased aconitase activity; increased ROS production and activity; and the decreased activity, content or organization of the electron transport chain complexes or proteins of the phosphorylation system (Ascensao, Lumini-Oliveira et al. 2011). Also, the depressed activity of mitochondrial complexes I and II caused by DOX (Santos, Moreno et al. 2002) could partially justify the diminished electron transport through electron transport chain (ETC) in the SAL+DOX group. Thus, the unaltered state 3 respiration observed in exercised groups suggest that, among other possible effects, training probably prevented the inactivation of complex I
and II in DOX-treated heart mitochondria. The enhanced ETC functionality could also be due to an up regulation of oxido-reductase activity or increased availability of reduced equivalents formation, consequently increasing the supply of electrons to the ETC (Nulton-Persson and Szweda 2001) or even to enhanced capability of phosphorylative system due an upregulation of Krebs cycle enzymes (Holloszy, Oscai et al. 1970). Furthermore, our experiments reveal that mitochondria isolated from hearts of animals treated with DOX exhibited impaired coupling (i.e., lower RCR). RCR is known as a respiratory parameter associated with mitochondrial functionality and structural integrity (Brand and Nicholls 2011). As exercise training prevent DOX-induced uncoupled cardiac mitochondrial respiration, this might suggest that exercise training enhance mitochondrial respiratory activity due increased phosphorylative system functionality. Interestingly, regarding RCR, FW running protocol was more effective at counteracting DOX-induced impairments, which may be consequence of a slight decreased in sate 4 observed in DOX+FW group. Concerning ADP/O, both TM and FW groups reverted the values of DOX+SED group, which suggest that training prevented the heart mitochondrial impairments in oxidative phosphorylation capacity in DOX rats.

Because mitochondrial complexes rely on enzymatic machinery (Bernstein, Bucher et al. 1978), they can become prone to impairments induced by ROS, resulting in accumulation of products of protein oxidation. DOX-induced impairments in oxidative damage markers, such as MDA level and -SH content were consistent with alterations in mitochondrial respiratory function, which can suggest that exercise may counteract DOX-induced impairments in redox homeostasis. One possible justification for these alterations is that exercise induced up-regulation of mitochondrial defenses including HSPs or SOD contributing to the up-regulation of mitochondrial tolerance against DOX effects (Ascensao, Ferreira et al. 2007). The up-regulation of other antioxidants such as GSH and CAT has also been described with exercise and DOX (Ascensao, Magalhaes et al. 2005, Kavazis, Smuder et al. 2010).
The complementary study of Δψ is indispensable for a complete analysis of mitochondrial function being that it reflects the basic energetic relation to cellular homeostasis maintenance. In fact, the electrochemical gradient due the pumping of protons through the inner membrane (Murphy and Brand 1988) is indispensable to ADP phosphorylation (Stock, Leslie et al. 1999). Moreover, when cytosolic concentration of Ca^{2+} increases, mitochondria act as Ca^{2+} buffers due its ability to uptake and accumulate Ca^{2+} (Gunter, Yule et al. 2004). It has been suggested that intramitochondrial Ca^{2+} concentration, whose flow is directed in accordance with the protomotriz gradient, has a controlling function in metabolic rate of oxidative energy production through the activation of Ca^{2+}-sensitive dehydrogenases, F0F1ATPase and ANT (Glancy, Willis et al. 2013). Our results showed that DOX decreased maximal Δψ, repolarization and increased the lag phase. The lag phase represents the time elapsed to phosphorylate ADP. In the present study, exercise led to increased maximal Δψ, repolarization and decreased time to restore membrane potential after addition and consequent ADP phosphorylation in the DOX+FW and DOX+TM groups. 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 exercised groups (Ascensao, Lumini-Oliveira et al. 2011). It is however important to note that the Δψ values above ~200 mV in all experimental groups do not seem to compromise ATP synthase flow or the transport of ions and metabolites. In fact, the range of the Δψ 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 Δψ, reaching saturation at approximately ~ 100 mV (Kaim and Dimroth 1999).

6.2. Mitochondrial osmotic swelling during MPTP induction

In addition to their role in energy supply, mitochondria are also considered determinant players in the establishment of cytosolic Ca^{2+} homeostasis,
uptaking and accumulation of Ca\(^{2+}\) in the matrix, in a process that is favored by the electrochemical gradient formed across the inner mitochondrial membrane (Gustafsson and Gottlieb 2008). However, mitochondria have a finite capability to accumulate Ca\(^{2+}\) before undergoing Ca\(^{2+}\)-dependent MPTP opening, and thereafter to the release of pro-apoptotic proteins, which in turn results in apoptosis (Ascensao, Lumini-Oliveira et al. 2011). In this regard, the study of exercise in the context of MPTP modulation may assume an important clinical relevance. Also, it has been described that, among others, a characteristic of MPTP after \(\Delta \psi\) loss, is the increased osmotic swelling amplitude induced by Ca\(^{2+}\) \textit{in vitro} (Gunter, Yule et al. 2004). Furthermore, endogenous Ca\(^{2+}\) levels in matrix are greatly higher in oxidative tissue, limiting heart ability to uptake Ca\(^{2+}\) before MPTP induction (Picard, Csukly et al. 2008).

In the present study, only TM exercise \textit{per se} was able to increase the mitochondrial capability to accumulate Ca\(^{2+}\) after MPTP induction. However, both protocols afforded protection against DOX impairments. Briefly, our results showed that DOX \textit{per se} decreased the mitochondrial Ca\(^{2+}\) tolerance (SED+SAL vs. SED+DOX) and both exercise protocols counteract DOX-induced impairments, possibly activating some defense mechanisms that might contribute to prevent the increased DOX-induced MPTP opening susceptibility (Marcil, Bourduas et al. 2006).

As MPTP is known to be formed/regulated by several proteins including ANT, hexokinase VDAC, phosphate carrier or Cyp D (Ascensao, Lumini-Oliveira et al. 2011, Crompton 1999, Halestrap and Brenner 2003), it is possible that exercise may positively modulate the expression and activity of those proteins. Also, given the refereed close relationship between increased mitochondrial oxidative stress and the susceptibility to MPTP induction (Kowaltowski, Castilho et al. 2001), it is possible that the up-regulation and modulation of some mechanisms involving stress chaperones, as HSPs antioxidants or other defense systems (Ascensao, Ferreira et al. 2007), as well as the decreased heart mitochondrial free radical production found in rats undergoing regular exercise (Judge, Jang et al. 2005) may contribute to these protective effects. Moreover, the possible
increased functionality of the phosphorylative system in general, and the ETC in particular, induced by both exercise protocols may have some implications in Ca\(^{2+}\) uptake capacity. However, to better understand this phenomenon, further studies need to be addressed.

### 6.3 Oxidative stress markers

Prevailing hypotheses suggest that myocardial oxidative stress is a primary event in DOX-induced cardiotoxicity and it is believed to initiate several of the deleterious cellular events reported following DOX treatment (Zucchi and Danesi 2003). In fact, at present the principal mechanism of DOX-induced cardiotoxicity is believed to be increased mitochondrial oxidant production leading to protease activation and induction of apoptosis (Ascensão, Magalhães et al. 2006, Ascensao, Magalhaes et al. 2005, Chicco, Hydock et al. 2006, Chicco, Schneider et al. 2005). Accordingly, oxidative injury of fatty acids at subcellular level measured by increased levels of lipid peroxidation products has been frequently reported following DOX exposure (for refs see Chicco, Schneider et al. 2005).

The present results show that TM, but not FW *per se* was able to decrease MDA level and increase -SH groups. In accordance to previous reports, DOX induced a significant decrease in -SH, indicating increased disulfide linkages from both proteins and GSH. As polyunsaturated fatty acids are considered highly susceptible to ROS attack, the increased oxidative stress caused by DOX led to peroxidative modification of lipid membranes affecting membrane integrity and permeability, which leads to decoupled mitochondria, altering normal mitochondrial respiratory function.

Myocardial antioxidant enzymes defend the heart against the damaging effects of ROS and have been hypothesized to play an important role in exercise-induced resistance to oxidative stress (Powers, Lennon et al. 2002) and in the attenuation of DOX cardiotoxicity (Singal, Iliskovic et al. 1997). In particular,
some studies suggested that the presence of myocardial SOD might be important for the prevention of DOX cardiotoxicity (Ascensao, Lumini-Oliveira et al. 2011, Sarvazyan, Askari et al. 1995, Yen, Oberley et al. 1996). This is reasonable, as SOD dismutates superoxide into H$_2$O$_2$, thereby providing the first line of defense against DOX-induced oxidative stress. Furthermore, increasing evidence suggest that myocardial HSP72 induction plays a pivotal role in exercise-induced cardioprotection against oxidative stress (Powers, Lennon et al. 2002, Powers, Locke et al. 2001, Taylor and Starnes 2003).

### 6.4 Meaning for exercise-induced cardioprotection in aging

Considering the present results in the context of exercise-induced cardioprotection in advanced age, they can be interpreted as preliminary. Indeed, it can be carefully speculated that the observed protective phenotype caused by both chronic models of exercise against DOX can also be observed in aged rats. In fact, Quindry et al. (2005) reported that aged rats submitted to exercise training ameliorate cardiac hemodynamic response with significant improvements in the apoptotic levels and signaling caused by IR injury. Furthermore, the authors observed that trained old rats increased MnSOD activity, which can be interpreted as a sign of cardiac mitochondrial adaptations induced by chronic exercise in old rats compared to their young counterparts. Similar results were found by Starnes et al. (2003), which suggest that, although observing cardioprotective protein phenotype alterations with age, exercise can enhance cardioprotection regardless of elderly. Furthermore, physical exercise has the ability to positively modulate some gene expression associated with improved heart function in aged rats. In fact, heart is known for its ability to produce energy from fatty acids because of its important β-oxidation equipment, which capacity is reduced with age (Starnes, Beyer et al. 1983). Confirming the potential beneficial effects of physical exercise on cardiac metabolism in elderly, Iemitsu et al. (2002) reported that exercise training improved the aging-induced decreased expression of peroxisome proliferator-activated receptor, which regulates genes related to fatty acid metabolism in the heart. Giving those
alterations reported in aged hearts, it is possible to speculate that the results of the present work could also be observed in aged rats; affording protection and mitigating the deleterious consequences associated with sub-chronic DOX treatment schedules.
7. Conclusion

In summary, the data from the present work provide additional support about the effect of two types of physical exercise (treadmill endurance training and free-wheel voluntary physical activity) against heart mitochondrial dysfunction induced by sub-chronic treatment of Doxorubicin (DOX). Our results showed us that:

- Regarding mitochondrial respiratory function both types of exercise reverted the effects induced by DOX on state 3, RCR and ADP/O. Interestingly, free wheel voluntary physical activity was more efficient at counteracting DOX-induced defects on RCR;
- Both types of exercise were able to counteract DOX-induced impairments in mitochondrial transmembrane endpoints. Importantly, free wheel voluntary physical activity was also more efficient at normalizing DOX-induced increases in lag phase;
- Regarding mitochondrial osmotic swelling during MPTP induction, both exercise protocols reverted DOX-induced impairments. In fact, both types of exercise mitigated DOX-induced increases in swelling amplitude and average swelling rate. However, once again free wheel voluntary physical activity was more efficient at counteracting Ca$^{2+}$-induced MPTP induction;
- Exercise protocols were able to revert DOX-induced increases in MDA content and decrease in sulphhydryl groups.

The mechanisms by which treadmill endurance training and free-wheel voluntary physical activity seems to confer additional protection against DOX remain elusive and further studies need to be addressed in order to comprehend the role of the different systems, such as those related to mitochondria, in this process.
8. References


