University of Porto

Faculty of Sport

Research Center of Physical Activity and Leisure

PROTECTIVE EFFECT OF REGULAR PHYSICAL ACTIVITY ON DELAYED SIGNS OF DOXORUBICIN-INDUCED CARDIOTOXICITY IN WISTAR

Dissertação académica apresentada às provas de Doutoramento no programada doutoral em Actividade Física e Saúde, nos termos do decreto-lei nº 74/2006 de 24 de Março. Esta dissertação foi concluída no Centro de Investigação de Actividade Física e Lazer (CIAFEL) sob supervisão do Professor Doutor José Alberto Ramos Duarte, Professor Catedrático da Faculdade de Desporto da Universidade do Porto.

Supervisor. Professor Doutor José Alberto Ramos Duarte

Ana Maria Seabra Moreira Ferreira de Carvalho

Porto, 2015

Carvalho, AM (2015) *Protective effect of Regular Physical Activity on delayed signs of Doxorubicin-induced cardiotoxicity in Wistar rat.* Tese de Doutoramento em Atividade Física e Saúde. Centro de Investigação em Atividade Física, Saúde e Lazer. Faculdade de Desporto. Universidade do Porto.

KEY WORDS: CARDIAC MUSCLE; DOXORUBICIN; MITOCHONDRIA; RUNNING WHEEL

Ao meus pais, Idalina e Francisco

À minha irmã, Mariana

À minha sobrinha, Luana

Agradecimentos

Chegar ao fim desta jornada foi possível apenas com a ajuda e apoio de várias pessoas. Mais do que as aprendizagens descritas nesta dissertação, as aprendizagens pessoais foram aquelas que mais se destacaram neste processo. E é neste ultimo momento quando despendo as últimas energias que devo não esquecer e devo referir todos aqueles que fazem parte deste trabalho.

Em primeiro lugar gostaria de agradecer ao meu orientador, o Professor Doutor José Alberto Duarte, sem o qual este trabalho não teria sido realmente possível. Obrigada por me ter acolhido no seu laboratório e me ter proporcionado um local onde pudesse desenvolver todo o meu trabalho. Nesta fase final, agradeço realmente a exigência que teve comigo, foi extremamente importante para a etapa final deste percurso.

Ao Hélder devo muito deste trabalho. Numa altura em que o teu percurso como aluno de Doutoramento estava a terminar nunca recusaste qualquer ajuda, ajuda essa que foi extremamente importante. Obrigada por teres partilhado comigo o laboratório e as actividades laboratoriais, obrigada por teres partilhado ideias e dado sempre importantes sugestões, obrigada especialmente pela amizade. Mais importante que tudo, obrigada por nunca teres desistido de mim! Mesmo sabendo que o meu caminho era difícil sempre acreditaste e fizeste-me a mim acreditar que era possível chegar ao fim. E parece que tinhas razão! Se aqui cheguei, realmente te devo muito a ti.

Ao Professor Jorge Mota quaisquer palavras que possa escrever serão poucas para aquilo que lhe devo. Desde o minuto que entrei nesta faculdade foi sempre o maior apoio e esteve sempre presente, mesmo não estando sempre lá. A sua preocupação com todos os alunos que passam por este centro de

investigação é realmente extraordinária. O facto de conhecer individualmente cada um de nós é algo que nos preenche e que nos faz sentir mais em casa, mais acompanhados. Obrigada por me ter dado força para continuar, por se ter preocupado constantemente com o meu bem estar, tanto a nível profissional como a nível pessoal. Foi realmente muito importante para que conseguisse chegar ao fim deste percurso. Obrigada!

A todos os meus colegas de laboratório, Tony, Cris, Daniel, Amparo e todos os por aqui passaram neste últimos cinco anos, um agradecimento por partilharem o laboratório e os momentos de lazer que foram também extremamente importantes. Especial agradecimento à Maria Teresa Baltazar, por todo o apoio e amizade durante este processo.

À Dona Celeste só posso agradecer todo o apoio durante estes últimos cinco anos. Sem a sua ajuda técnica não teria com certeza chegado ao final deste processo. Agradeço especialmente a sua ajuda no manuseamento dos animais e na preparação do material de microscopia.

Ao meu espelho, Rose Autran, obrigada por teres aparecido na minha vida. Vieste fazer-me ver que não estou sozinha no caminho tortuoso que é a elaboração de uma tese de doutoramento. Ainda iremos fazer o nosso jogral. Eduardo Teixeira fizeste este percurso desde o inicio comigo. Foste um grande companheiro nesta grande aventura. Saber que estavas sempre aí exactamente ao meu lado foi muito importante. Obrigada por teres estado sempre aí pronto a apoiar-me e a dar-me aquele abraço forte quando tudo parecia estar a cair. A vocês os dois o meu agradecimento não é sequer suficiente.

Ao meu Gang: vocês foram sem qualquer dúvida o que me manteve a sanidade mental, especialmente neste último ano. Speedy, Meias, Pingu, Xabala,

Beijokas, Sarinha, Pipa, Torres, foi muito importante para mim partilhar com vocês todas as minhas frustrações e tristezas, obrigada por me terem sempre posto a sorrir com os vossos/nossos devaneios. Iremos continuar a partilhar todos os nossos momentos, onde quer que estejamos, o Gang estará sempre presente.

A ti, obrigada por teres estado aqui. Obrigada pelo apoio e por me teres aturado quando tudo o resto estava a cair.

À minha famíia:

Nada do que eu possa escrever irá conter a eterna gratidão e o amor que tenho por vocês Mariana, Pai e Mãe. Não há palavras que expressem aquilo que sinto neste momento.

Maninha, vamos terminar juntas esta etapa! Durante estes cinco, seis anos estivemos pela primeira vez separadas durante tanto tempo, mas mesmo com todos os altos e baixos que possam ter existido, não há ninguém mais importante que tu. Só te posso agradecer teres sempre sido exigente comigo, não esperava de ti outra coisa.

Mãe e Pai, dizer obrigada não chega. Se termino esta etapa, 95% é vosso! Por todo o apoio e por terem estado sempre presentes para me ampararem quando eu estava a cair, porque sempre acreditaram e sempre se orgulharam de mim. Espero estar sempre à altura de toda a confiança que depositam em mim, espero continuar a merecer todo esse orgulho que vocês sentem. Vocês são o meu porto de abrigo, hoje e sempre. Obrigada!

Table of Contents

Introduction ----------1

Theoretical Background ----------11

I- Cancer and Cancer Treatment ----------13

II- Câncer and the use of Anthracyclines ----------15

III- Cardiomyopathy: Doxorubicin and Aging effects ----------18

i- Apoptosis and Autophagy ----------23

IV- Cardiomyopathy and Exercise ----------26

Methodology ----------31

I- Study Design ----------33

II- Animal Sacrifice and Organs Collection ----------34

III- Histology ----------36

i- Cardiac Muscle Damage Assessment ----------36

ii- Assessment of Fibrous Tissue in Cardiac Muscle ----------37

iii- Cardiac Muscle Ultrastructure Analysis ----------38

IV- Biochemistry ----------38

i- Spectrophotometry ----------38

a. Total Protein Assessment ----------38

b. Determination of Total Glutathione (TGSH), Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG) activity ----------38

c. Assessment of Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX) and Glutathione Reductase (GR) activity ----------39

d. Determination of Citrate Sintase (CS) activity ----------39

ii- Immunoblotting analysis ----------40

V- Measurement of Mitochondrial Respiratory activity ----------41

VI- Statistical Analysis ----------42

Results ----------43

I- Sample Characterization and Voluntary Physical Activity ----------45

II- Biochemical Analysis ----------46

i- Enzyme Activity ----------46

ii- Amount of Reduced Glutathione (GSH), Oxidized Glutathione (GSSG) and ratio GSH/GSSG ----------48

iii- Protein Expression ----------50

III- Mitochondrial Respiratory Activity ----------59

IV- Morphometric Analysis ----------64

Discussion ----------75

I- Methodology Discussion ----------77

II- Effects of Age and Sedentariness ----------81

III- Acute and Late Effects of Doxorubicin ----------85

IV- Sedentary behavior perpetuates Doxorubicin-induced Cardiotoxicity-89

Conclusions and Future Perspectives ----------95

References ----------99

LIST OF FIGURES

Figure 1. Schematic representation of the experimental design.----------33

Figure 2. Effect of Dox and exercise on the expression of tissue homogenate COXIV (15kDa). Immediately below the histogram, the panel shows a representative western blot of COXIV for each group. Values (mean and St. D.) are expressed as percentage of control (BSL). ----------51

Figure 3. Effect of Dox and exercise on the expression of tissue homogenate SOD (25kDa). Immediately below the histogram, the panel shows a

representative western blot of SOD for each group. Values (mean and St. D.) are expressed as percentage of control (BSL). ----------52

Figure 4. Effect of Dox and exercise on the expression of tissue homogenate HSP60 (60kDa). Immediately below the histogram, the panel shows a representative western blot of HSP60 for each group. Values (mean and St. D.) are expressed as percentage of control (BSL). ----------53

Figure 5. Effect of Dox and exercise on the expression of tissue homogenate HSP70 (70kDa). Immediately below the histogram, the panel shows a representative western blot of HSP70 for each group. Values (mean and St. D.) are expressed as percentage of control (BSL). ----------54

Figure 6. Effect of Dox and exercise on the expression of tissue homogenate Bax (48kDa). Immediately below the histogram, the panel shows a representative western blot of Bax for each group. Values (mean and St. D.) are expressed as percentage of control (BSL). ----------55

Figure 7. Effect of Dox and exercise on the expression of tissue homogenate Apg12 (16kDa). Immediately below the histogram, the panel shows a representative western blot of Apg12 for each group. Values (mean and St. D.) are expressed as percentage of control (BSL). ----------56

Figure 8. Effect of Dox and exercise on the expression of tissue homogenate GPX (22kDa). Immediately below the histogram, the panel shows a representative western blot of GPX for each group. Values (mean and St. D.) are expressed as percentage of control (BSL). ----------57

Figure 9. Effect of Dox and exercise on the expression of tissue homogenate GR (58kDa). Immediately below the histogram, the panel shows a representative western blot of GR for each group. Values (mean and St. D.) are

expressed as percentage of control (BSL). ----------58

Figure 10. Representative Oxygraph of Complex I-linked substrates (Malate/Glutamate) from all groups. ----------61

Figure 11. Light micrographs representative from animals of groups baseline (BSL), vehicle (V), vehicle + sedentary (VSED) and vehicle + exercise (VEXE). Sections were stained with hematoxylin-eosin. All micrographs depict a normal tissue structure without signals of cellular degeneration, necrosis or cell infiltration. The interstitial space seemed preserved without evidences of tissue disorganization. ----------64

Figure 12. Light micrographs representative from animals of group doxorubicin (DOX). Sections were stained with hematoxylin-eosin. In all micrographs, beyond the great heterogeneity of cardiomyocytes dimensions, it is depicted several enlarged cells with cytoplasmic vacuolization, intracellular edema and some of them the morphologic signs suggest to be in necrosis. It is also notorious an enlarged interstitial space fulfilled by collagen fibers and fibrin-like material, more evident in B and C. ----------65

Figure 13. Light micrographs representative from animals of group doxorubicin + sedentary (DSED). Sections were stained with hematoxylin-eosin. The heterogeneity of cardiomyocytes dimensions is evident in all micrographs with an enhanced interstitial space fulfilled with connective tissue. Some degenerated cells with cytoplasmic edema flanked by apparently normal cardiomyocytes. In C it is observed some infiltrative mononuclear cells. --------65

Figure 14. Light micrographs representative from animals of group doxorubicin + exercise (DEXE). Sections were stained with hematoxylin-eosin. Some punctual damaged cells with cytoplasmic edema or soft vacuolization can be identified in all micrographs, surrounded by a clean interstitial space. ----------66

Figure 15. Light micrographs representative from animals of groups baseline (BSL), vehicle (V), vehicle + sedentary (VSED) and vehicle + exercise (VEXE). Sections were stained with picrosirius red. In all groups the connective tissue (red stained) show a normal density. ----------68

Figure 16. Light micrographs representative from animals of groups doxorubicin (DOX), doxorubicin + sedentary (DSED), and doxorubicin + exercise (DEXE). Sections were stained with picrosirius red. It is evident an enhanced density of connective tissue (red stained) in DOX and DSED, more pronounced in DSED, with a lower density in DEXE group. ----------68

Figure 17. Effect of Dox and exercise on the fibrotic area. Results are shown as the percentage of Collagen deposition per total area obtained from Sirius-Red staining for all groups. Values (mean and St. D.) are expressed as percentage of control (BSL). ----------69

Figure 18. Electron micrographs representative from animals of groups baseline (BSL), vehicle (V), vehicle + sedentary (VSED) and vehicle + exercise (VEXE). All micrographs depict a normal ultrastructure with mitochondria showing a normal shape and well-defined lamellar cristae. ----------71

Figure 19. Electron micrographs representative from animals of group doxorubicin (DOX). In **A** it is observed a cardiomyocyte with cytoplasmic edema, traduced by an enlarged interspace between myofibrils, flanking a cardiomyocyte with mitochondria with less defined cristae and punctual mitochondrial swelling; **B** depict some mitochondrial swelling and heterogeneity of mitochondria dimensions with loss of well-defined cristae; in **C** it is possible to observe the different mitochondria dimensions and the presence of secondary lysosomes nearby. ----------72

Figure 20. Electron micrographs representative from animals of group doxorubicin + sedentary (DSED). In **A** and **C** it is observed a cardiomyocyte with secondary lysosomes surrounded by mitochondria, with a great heterogeneity in their dimensions, and with undefined cristae. **B** depicts a degenerated cardiomyocyte with signals of cytoplasmic edema. ----------72

Figure 21. Electron micrographs representative from animals of group doxorubicin + exercise (DEXE). **A**, **B**, and **C** depict cardiomyocytes with well-defined cristae and apparently with an enhanced mitochondrial density; these organelles are also more homogeneous in their dimensions. In **B** it is observed secondary lysosomes interspacing apparently normal mitochondria. ----------73

LIST OF TABLES

Table 1: Body Weights, Dissected Heart Weights and Physical Activity from all groups. ----------46

Table 2: Activity levels of the enzymes Glutathione Peroxidase (GPX), Glutathione Reductase (GR), Superoxide Dismutase (SOD), Superoxide Dismutase Mitochondrial (SODMit), Citrate Synthase (CS), and Citrate Synthase Mitochondrial (CSMit) measured for all groups. The amount of Reduced Glutathione (GSH), Oxidized Glutathione (GSSG) and ratio GSH/GSSG are also shown. ----------49

Table 3: Functional data obtained from cardiac muscle mitochondria from all groups with complex I-linked substrates (Malate/Glutamate). ----------60

Table 4: Functional data obtained from cardiac muscle mitochondria from all groups with complex II-linked substrates (Succinate/Rotenone). ----------63

Table 5: Data from cardiac muscle damage. ----------67

RESUMO

O coração é um órgão muito susceptível ao stress pois necessita grandes de energia. Embora os mecanismos ainda não sejam claros, pensa-se que o dano mitocondrial tem um papel fundamental no aumento desta toxicidade. A doxorubicina (Dox), uma das mais comuns e mais eficazes drogas usadas no tratamento do cancro, tem efeitos secundários agudos e crónicos nos sobreviventes de cancro, especialmente em órgãos que necessitam de grandes quantidades de energia como o coração. Efeitos similares acontecem no processo de envelhecimento onde se pensa que o stress oxidativo e o aumento do dano mitocondrial tem papel fundamental na degeneração dos tecidos. Na tentativa de contrariar os efeitos nefastos causados pela Dox e pelo envelhecimento nos vários tecidos, várias terapias foram propostas. A actividade física regular foi proposta como uma terapia não farmacológica para melhorar os efeitos causados pela Dox e pelo envelhecimento. O principal objetivo deste trabalho foi analisar o efeito da atividade física regular sobre a morfologia e bioquímica do coração e das mitocôndrias cardíacas de ratos que receberam doxorrubicina. Para atingir este objetivo foi utilizado um modelo animal, administramos doxorubicina a ratinhos durante sete semanas, e depois alojamo-los individualmente em gaiolas com acesso de roda livre durante dois meses. Analisou-se a lesão muscular e deposição de colagénio no músculo cardíaco usando coloração de H&E e Sirius Red. Para estudar a morfologia das mitocôndrias realizamos TEM. Medimos também marcadores bioquímicos de stress oxidativo (SOD, GPX, GR, GSH e GSSG), marcadores de quantidade de mitocondrias (CS e COXIV), a função respiratória mitocondrial (estado 3, 4 Estado e RCR), marcador de autofagia (ATG12) e marcador de apoptose (Bax) na fracção citoplasmática e mitocondrial. Os principais resultados do trabalho experimental apoiam a ideia de que a doxorrubicina causa toxicidade e aumenta o dano oxidativo no músculo cardíaco, e que esta toxicidade está associada ao aumento do dano mitocondrial. Os nossos resultados mostram que o sedentarismo perpetua o dano celular e mitocondrial, enquanto a atividade física parece acelerar a regeneração.

PALAVRAS-CHAVE: MÚSCULO CARDÍACO;DOXORUBICINA;MITOCONDRIA; RODA LIVRE

ABSTRACT

The heart is an organ very susceptible to stress due to its high-energy demand. Although mechanisms remain unclear, it is thought that mitochondrial damage has a major role in this toxicity. Doxoxrubicin (Dox), one of the most widely used and effective anticancer drugs causes acute and chronic damage to cancer patients, especially in organs with high-energy demands as heart. Similar effects are seen in aging process, where it is thought that oxidative stress and increase in mitochondrial damage has a major role in the degeneration of tissues. In an attempt to counteract the side effects provoked by doxorubicin and the effects of aging in several tissues, many therapies have been studied. Regular physical activity has been proposed as a non-pharmacological therapy either for healing Dox-induced toxicity or for delaying the aging-process, decreasing oxidative stress and mitochondrial dysfunction. Concerning this, the main goal of this work was to analyze the effect of regular physical activity on heart and heart mitochondria morphology and biochemistry of mice submitted to doxorubicin treatment. To achieve this goal we used a mouse model, were we administrated doxorubicin during seven weeks and after the rats were housed individually in cages with access of free wheel during two months. We analyzed the cardiac muscle damage and collagen deposition of the rat heart using H&E and Sirius Red staining. For accessing the morphology of mitochondria we performed TEM. Biochemical markers of oxidative stress damage (SOD, GPX, GR, GSH and GSSG), biochemical analysis and marker of healthy mitochondrial quantity (CS and COXIV), mitochondrial respiratory function (State 3, State 4 and RCR), autophagy marker (ATG12) and apoptosis marker (Bax) were measured in cytoplasmic and mitochondrial fractions. The main findings of the experimental work support the idea that doxorubicin causes toxicity and increased oxidative damage in cardiac muscle, and that this toxicity is associated to the increase in mitochondrial damage. Further our data show that exercise reverted this situation, altering the mitochondrial pool to a healthy mitochondrial pool and improving morphology of cardiac muscle. The overall data suggests that sedentariness exacerbates the cardiac toxicity and mitochondrial damage.

KEY WORDS: CARDIAC MUSCLE; DOXORUBICIN; MITOCHONDRIA; RUNNING WHEEL

LIST OF ABREVIATIONS

ADP, Adenosine Diphosphate;

ATG, Autophagy related-genes;

ATP, Adenosine Triphosphate;

BSA, Bovine Serum Albumin

CSCs, Cardiac Stem Cells

Dox, Doxorubicin;

ETC, Electron Transport Chain;

GPx, Glutathione Peroxidase;

GR, Glutathione Reductase

GSH, Reduced Glutathione;

GSSH, Oxidized Glutathione;

HSPs, Heat Shock Proteins;

IMF, Intermyofibillar Mitochondria;

MHC, Myosin Heavy Chain;

mtDNA, Mitochondrial DNA;

MTX, Mitoxantrone;

NADPH, Nicotinamide Adenine Dinucleotide Phospate;

OXPHOS, Oxidative Phosphorylation;

RCR, Respiratory Control Ratio;

ROS, Reactive Oxygen Species;

SOD, Superoxide Dismutase;

SSM, Subsarcollemmal Mitochondria

TEM, Transmission Electron Microscopy;

TFAM, Mitochondrial Transcription Factor A;

TGSH, Total Glutathione;



Introduction

Cancer is a malignancy that affects over than 12,7 million people in the world from which more than 175000 are children (WHO, 2008). The chances of surviving a childhood cancer in USA increased from 30% in 1960 to 70% in 1990 (Robison et al., 2002) and in Europe increased from 65% in children diagnosed between 1983 and 1985 to 75% in children diagnosed with cancer between 1992 and 1994 (Gatta et al., 2005). The results in survival rates are very similar in Europe and USA (Gatta et al., 2002). The survival rate is as high as 80% (for developed countries), but the side effects can be present through all life of the infants (WHO, 2008; "World Child Cancer", 2013b). They are at high risk of developing a second cancer, suffering from chronic or severe illness or have severe damages from the radiations and treatments (PAC2, 2013). At the age of 45, they have 95% chance of developing diseases such as hearing problems, pulmonary issues (Marcus, 2013) and specially cardiac problems due to anthracycline treatment (De Caro et al., 2011). Radiation therapy and chemotherapy, individually or both together, represent a risk factor for developing later diseases, specially if the treatment occurs at a very young age (Armstrong et al., 2010). In average, in United States for instance, costs with health are twice higher for a cancer survivor than for normal health care (de Moor et al., 2013). This acquires major concern regarding the high number of cancer survivors in the world population and their increased longevity. Medicines tend to be very expensive and there is awareness in finding treatments that can be cost effective, high effective and with minimal damage to the patients. Having a healthy life stile, changing some habits, some behaviors and implementing exercise may be the answer to this problem.

Several follow-up studies were conducted with children cancer survivors, either in Europe or in USA, to understand their quality of life after the cessation of the disease and how to improve their health regarding the side effects left by the cancer and the medicines to treat this illness.

In USA since 1994 was raised the Childhood Cancer Survivor Study (CCSS), which includes more than 20000 cancer survivors, diagnosed between 1970 and 1986, aged less than 21 when diagnosed, plus 4000 siblings of those

survivors to serve as control group (Robison et al., 2009; Robison et al., 2002). Due to the high importance to understand better how to deal with cancer survivors and because cancer treatments have changed quickly in the past decades, the study has been expanded to the patients diagnosed between 1987 and 1999. From the data of CCSS many reports and studies have came out, detailing the most frequent diseases that this survivors have to deal with (Armstrong et al., 2010; Demark-Wahnefried et al., 2008; Diller et al., 2009; Hudson et al., 2009; Lu et al., 2011) and describing possible ways to help this patients after the end of the cancer (Armenian & Robison, 2013; Nathan et al., 2009).

In Europe the compilation of data from childhood cancer survivors is more recent and is still under construction. There is an European registry for cancer survivors, and children are a population in focus (Gatta et al., 2005). Some European countries have their own CCSS, such as Swiss (Michel et al., 2007) and United Kingdom (Reulen et al., 2007). At European level, since February of 2011, it exists the PanCareSurFup (PanCare Childhood and Adolescent Cancer Survivor Care and Follow-up Study) with the aim to analyze the data available about long-term children cancer survivors in Europe. This group network was created to provide insights about cardiac diseases, second malignant neoplasm and late mortality due to first cancer episode. Other organizations collecting information about childhood cancer survivors are the SIOPE (European Society of Pediatric Oncology), the ENCCA (European network for Cancer Research in Children and Adolescents) and the ICCCPOC (International Confederation of Childhood Cancer Parent Organizations).

Childhood cancer survivors will face many problems in their growth. As they are under radiation treatments at a very young age, it will affect the good development of all the systems of the body. For instance, treatments for Hodgkin´s Disease affect largely the thyroid system, having the survivors a relative risk of 17 of developing hypothyroidism (Rodriquez et al., 2014; Sklar et al., 2000). Hyperthyroidism, thyroid nodules and cancer are also frequent and the risk is higher for patients treated with higher dose of radiation and with

longer radiations treatment (Rodriquez et al., 2014; Sklar et al., 2000). In a British cohort study of childhood cancer survivors the risk of developing thyroid cancer was 18 (Standard deviation 13,4-23,8) (Taylor et al., 2009). The overall growth is mostly commanded by the endocrine system under the supervision of the nervous system. If the endocrine system is affected by radiation, it might also affect the normal development of the child and these cancer survivors have higher probability of being below the percentile when adults (Gurney et al., 2003). Childhood cancer survivors are also at high risk of being overweight or underweight, with alterations in pubertal time and maturation and issues in fertility (Hudson et al., 2009). Female are even more affected as they have more chances to develop a secondary disease and also because cancer treatments as radiation therapy may provoke ovarian failure and premature menopause (Hudson et al., 2009). As the lungs are very radiosensitive organs, they are also highly affected by the radiotherapy and chemotherapy received during the treatment for cancer at young age. Radiotherapy was associated with lung fibrosis, recurrent pneumonia, as also with smaller lungs and decreased chest volume (Armstrong et al., 2010).

Childhood cancer survivors do also have increased risk of reporting pain throughout their lives, such as headaches, especially migraines, and those who were under brain radiation treatment have higher risk of reporting this kind of pain (Lu et al., 2011). Latter in life those patients are more likely to report a poor health status or some physical restriction when compared to the general population (Kenney et al., 2010).

Cancer survivors have 3 times more chance of having a chronic health condition, when compared with their siblings, and the incidence increases with time (Hudson et al., 2003; Oeffinger et al., 2006). They have higher chance of suffering from a second malignant neoplasm (SMN) than the general population (Taghian et al., 1991; Tester et al., 1984; Travis et al., 1997; Tucker et al., 1988), and specially children (Neglia et al., 2001). Among children, female patients, cancer survivors that were diagnosed at very young age (Neglia et al., 2001; Oeffinger et al., 2006), those who suffered from Hodgkin's or those who

were treated with anthracyclines and epipodophyllotoxins were at even higher risk of developing SMN (Neglia et al., 2001). In a Nordic cohort study the results showed that the risk of SMN was 2,5-7 times higher for cancer survivors diagnosed before the age of 20 than for normal population, and that the risk was highest for the first ten years after the cessation of the disease, although risk is high throughout life (Olsen et al., 1994). Radiotherapy is one of the main causes of SMN (Nygaard et al., 1991) but when combined with chemotherapy the risk is even higher (de Vathaire et al., 1989; Garwicz et al., 2000).

Heart problems are a major concern for childhood cancer survivors (Scott et al., 2013). Cardiac disease is the first noncancer disease causing death among childhood cancer survivors (Armstrong et al., 2009; Mertens et al., 2008; Reulen et al., 2010). Anthracyclines are widely used as antitumor drugs because they target for rapidly multiplying cells, but their use is associated with latter cardiac disease (Lipshultz et al., 2012) and premature death (Armstrong et al., 2009), as heart is also constituted with rapidly dividing cells (Lipshultz et al., 2013). This toxic effect can also be present in other organs, but their repair capacities tend to attenuate the late consequences. This does not happen in heart because of the limited regenerative capacity of its cells (Lipshultz et al., 2013). Congestive heart failure is 15 times more probable in a cancer survivor than in general population and this probability gets dramatically high with the increase in the cumulative dose of anthracyclines (Lipshultz et al., 2008). In adult cancer survivors cardiovascular issues induced by chemotherapy include arrhythmias, myocardial ischemia and infarction, hypertension and left ventricular contractile dysfunction (Lipshultz et al., 2013). Cancer survivors have increased risk factors for arthrosclerosis, high body mass index, high insulin levels and high fasting serum non-high-density lipoprotein levels (Lipshultz et al., 2012), all risk factors for cardiovascular disease.

As we have been discussing, childhood cancer survivors face a bunch of secondary health issues along their lives, and with the advances in the treatments more and more cancer patients will survive this illness. It is of high importance to develop ways to improve the quality of life of these survivors. This

patients will face psychological and physical issues, anxiety and sexual disturbance (Nie & Gao, 2014). As prevention, childhood cancer survivors should chose healthy habits such avoiding smoking or secondhand smoke, avoid alcohol in excess, be active and connect with friends and family and prevent the weight gain with exercise and healthy diet (Wolin et al., 2013).

Regular exercise may be a difficult task to cancer survivors in the beginning as they are more fragile, but the benefits go largely further this first barrier. Exercise when prescribed by experts and adapted to this particular group of people is very beneficial (Wolin et al., 2012). Sedentary behaviors as sitting time for long periods is related with high risks of mortality from all causes and especially cardiovascular diseases (Katzmarzyk et al., 2009). For general population having a physically active life is known to be an important detail for promoting a better quality of life, and this is even more important for cancer survivors, as they are at a higher risk of falling into a poor health status. Childhood cancer survivors are in risk of developing later in life chronic diseases as obesity, diabetes and cardiovascular diseases, and exercise can be a good therapy, combined with medical guidance, to heal this illnesses. Several meta-analysis and reviews in past few years found a positive relation between exercise and better quality of life for cancer survivors not only in preventing the appearance of other diseases but also in helping cancer survivors in all other domains as sociability, fatigue, self esteem and well being, anxiety, sleep disturbance sexual life and pain (Mishra, Scherer, Geigle, et al., 2012; Mishra et al., 2014; Mishra, Scherer, Snyder, et al., 2012). For children survivors conclusions of the firsts studies are in concordance with those found for adults (Braam et al., 2013), but more evidence is needed. Nonetheless exercise should be implemented as a nonpharmacological therapeutic for patients that survived cancer. Although quality of life can be improved, and several clinical trials confirm this association between physical activity and improvements in general health and lower risk factors for several diseases, such those for cardiovascular disease, as is reviewed by Baumann in his paper (Baumann et al., 2013), the underlying biological mechanisms that are affected

are not easy to assess in pediatric population due to ethical and technical reasons. There is a need to use animal models to understand the morphological and biochemical changes in heart morphology and cells, because in human is impossible to study directly the cardiac tissue or its cells being only possible to see the external outcomes from the internal changes.

With this work we aim to unlight some of the mechanisms behind the visible positive effects of exercise in cancer survivors, more precisely in heart tissue. It is well established that physical activity has a cardioprotective effect against ischemia-reperfusion (IR), a main underlying cause of coronary artery disease, because exercise training specially enhances the structure and functionality cardiomyocytes' mitochondria (Powers et al., 2014), but it is not totally understood the impact of exercise on mitochondria turnover in cardiac myocytes. It would be interesting to understand what is the extent of the effect of exercise over mitochondrial quality and how it influences the balance between mitophagy and mitochondrial biogenesis, fission and fusion processes, and thus how it promotes the formation of new healthy mitochondria and removal of damaged ones that produce high levels of reactive species of oxygen (ROS) (Yan et al., 2012). In cancer survivors, this would be highly important, as cardiac mitochondria are the organelle mostly affected by oncologic drugs, mainly anthracyclines, with increases in ROS production and failure in mitochondrial bioenergetics, what will affect the cell machinery and may lead to apoptosis. Exercise can have a protective role over cardiac cells long-term, preparing them better to the latte side effects from cancer treatment.

Therefore, our main objective is to understand if voluntary physical activity after doxorubicin, the most used anthracycline, treatment will attenuate the cardiotoxicity caused by cancer treatment and improve the cardiac condition of mice that received this drug. To do this we submitted mice to doxorubicin or to saline solution treatment during seven weeks and after that period animals were divided into sedentary and physical active, where this last group was under voluntary free wheel exercise during two months. At the end of the experimental protocol, we analyzed the morphological, biochemical and functional alterations

on the cardiac muscle and on the isolated cardiac mitochondria, comparing the effects of exercise on treated and non-treated animals. Our ultimate goal is to see the effect of voluntary physical activity on mitochondria phenotype in animals submitted to doxorubicin.

This thesis is divided in five main sections. The first two sections introduce the problematic and review the literature on the post-cancer adverse effects of doxorubicin for cancer survivors and the positive effect of exercise on cancer patients. The third section describes the experimental design and the methods used to achieve the results that are presented in the fourth section of this work. The interpretation of the results based on the literature is on the fifth section, as are the main conclusions of the work.



Theoretical Background

I. Cancer and Cancer treatment

The term cancer is used to define a group of diseases characterized by abnormal cell growth without any control, with the ability of invading other tissues. Cancer can spread to other parts of the organism through the blood or the lymphatic system. According to the tissue from which the abnormal cells start to develop, it can have different types of cancer. For instance, if the cancer starts in bone marrow and forms abnormal blood cells it is called leukemia. If it affects the immune system cell formation we have a lymphoma or myeloma. A sarcoma is a cancer that affects the formation of cells of the connective and support systems, such as bone, cartilage, fat, muscle and blood vessels. If the abnormal cell growth starts in the skin or tissues that cover other organs we are in the presence of a carcinoma ("Defining Cancer"). As we can see only for the variety of illnesses that comprehend cancer, there is a huge variability in the traits of this disease. Treatment can be very difficult, not only because of this variability, but especially because if the treatment is not soon enough, cancer can spread from the first organ to the rest of the body, and beyond treating the first problem, one has to treat all the subsequent malignancies ("World Child Cancer", 2013a).

How cancer spread along the body so quickly and how cancer cells can influence neighbor healthy cells to turn into malignant is not yet fully understood but in the last decades cancer is one of the main research subjects of the scientific community. It is known that normal undifferentiated cells develop and divide constantly during all our life and sometimes errors happen ("American Cancer Society"). Our body quickly detects these abnormal cells or detects old cells that lost their functionality, and induces their death and removal, through apoptosis or necrosis. When this repair system is ineffective or cell division of damaged cells is extremely fast and occurs when the organism does not need it, there is a growing mass of damaged cells that is called tumor ("American Cancer Society"; "Defining Cancer"). Tumors can be classified as benign if the

cells in this mass do not spread to neighbor tissues, and having this localized character normally they can be easily removed. A tumor is categorized as malignant if its cells invade nearby tissues, spread to other parts of the body (metastasis), influencing the healthy cells to turn into tumor cells, developing a cancer ("Defining Cancer"). Indeed, late evidences suggest that the waste of cancer cells affects healthy cells. Melo and her team (Melo et al., 2014) found that exossomes expelled from cancer cells would affect the neighbor healthy cells, turning these ones into malignant cells by means of changing the gene expression.

Treating this illness has always been challenging due to the wide variety of cancers. Researchers soon tried to understand ways to stop cell division of malignant cells and the rapid growth of the tumor, without damaging the healthy cells of the body.

Surgery is a technique vastly used to remove mass of abnormal cells, either to remove tissues that may become a cancer, or to check how cancer is spread in the body and also can be to remove solid cancer, if it is not spread to other parts of the body. It is still the most used technique to treat cancer ("Defining Cancer").

Radiotherapy is another common and old technique used by oncologists, because radiation affects the cell cycle and thus the cell division. It targets specially those cells that are actively dividing. This is why this technique is still in use today, besides its bad side effects ("Defining Cancer"). Radiation does not seek for malignant cells only, it also affects normal cells, stopping them to divide, and thus the use of radiotherapy is always dependent from the balance between the expectance of the cure and the damage to the healthy cells. This technique affects body tissue at different rates, because tissues as skin and bone marrow, that have fast growth, are affect immediately. In contrast, nerve system, breast, brain or bones are affected latter and the effects may arise late, after the cessation of treatment ("American Cancer Society").

Chemotherapy appeared in the late 60s as a coadjutant to the previous therapies, either to shrink mass of malignant cells before surgery or to kill

remaining damaged cells after surgery and radiation. Chemotherapy is normally used with cancers that have already spread to the rest of the body, but it has also negative side effects. It is an upgrade when compared to surgery or radiotherapy because it is a systemic treatment, while the two other are considered as local treatments (Arcamone et al., 1969; "Defining Cancer"; C Tan et al., 1967).

In the late decades new techniques appeared, such as targeted therapy, which is a technique that uses drugs that have the ability to target and attack more precisely cancer cells, or immunotherapy, a technique that induce the immune system of the patient to respond and defend the body more effectively ("American Cancer Society"; "Defining Cancer").

ii. Cancer and the use of Anthracyclines

Chemotherapy is used in cancer treatment trying to eliminate the malignant cells. Drugs administrated to the treatment of cancer mainly affect DNA and RNA, either directly damaging these molecules or by blocking and damaging other molecules involved in the replication of the nucleic acids. The main goal is to stop cancer cell division. This DNA and RNA damage causes stress inside the cell that will trigger apoptosis (Bagchi et al., 1995; Jain, 2000).

In the 60s, a compound isolated from *Streptomyces peucetius*, daunomycin, was found to have anticancer activity, but years later it was also discovered that it had fatal cardiotoxicity associated (C. Tan et al., 1967). Since the 70s several more compounds were isolated from other variants of *Streptomyces peucetius* (such as *Streptomyces peucetius caesius*) more effective and less toxicity, creating the family of Anthracyclines (Bonadonna et al.). The anthracyclines act over a wide variety of cancers, from solid tumors to leukemias (Kaur et al.), and doxorubicin (first knew as Adriamycin) is the derived compound with higher spectrum. Doxorubicin is a tetracycline aglycone with a quinone ring, which contributes to the highly redox instability, when it transforms in a semiquinone.

In the beginning it was thought that doxorubicin had its antitumor action by producing free radicals, and creating a cycle of damage due to the reactive species. Soon was found that in fact doxorubicin could react directly with the DNA, intercalating between the base pairs, and could inhibit DNA topoisomerase II, stopping the replication with both paths (Singal et al.). This last effect seems to be main target of doxorubicin (Simunek et al., 2009).

Even though the studies of the scientific community on this subject, it has not been yet possible to detect how to end the side effects of the use of this drugs. It is well documented its negative effects over some tissues, especially those with higher energetic demands, such as Heart (Ettinghausen et al.; Friedman et al.; Minow et al.), Liver (Nicolay & Kruijff, 1987) or Kidney (Yasuda et al., 2010).

It is known that the total dose of doxorubicin given to a patient is the main factor for its toxicity, and it has been a matter of research in order to find the better cumulative dose with the better treatment results (Minow et al.) (Ettinghausen et al.). It was found that the range of values is 200mg/m², for low probability of developing cardiomyopathy, and 550mg/m^2, for high probability of developing cardiomyopathy (Friedman et al.) This dose effect can be even higher when it is used with other drugs, due to synergetic effects (Jones et al.). If heart is more susceptible due to any previous insult, due to genetic predisposition, arterial hypertension or previous radiation therapy (Suter & Ewer, 2013), the effect of the drug will be even greater and it will have repercussion at lower doses. This dose effect is higher in children and in over-65 patients (Suter & Ewer, 2013). Friedman *et al*. (Friedman et al.), using myocardium biopsy analysis, also found that the ultrastructural changes in myocardium where doxorubicin specific. There have been some efforts in finding some strategies to diminish this toxicity, as lowering the level of doxorubicin in plasma (Shapira et al.), or the achievement of new compounds born from this drug (Singal et al.). Since the 70s and until today has not yet been found a substitute for doxorubicin with the same effectiveness and antitumor activity.

Despite this relation with dose, this cardiotoxicity has been even more difficult to characterize because it has a late onset for clinical manifestations, varying from

few years till 30 years late (Steinherz et al.). The effects of this drug are both acute and chronic, being the last one of those that raise more concerns, as they seem to be irreversible which contrast with the acute symptoms that are somewhat reversible (Singal et al.). Acute effects may be expressed as tachycardia, hypotension or electrocardiogram changes, and appear early after the Dox administration or even during the process (Singal et al.). Late repercussions of anthracyclines are manifested as chronic heart failure or reduced left ventricular ejection fraction. This is particularly important in pediatric population, where exists a higher probability of developing cardiac problems (Fulbright et al., 2010), and if treatment occurs before 4 years of age, the probability is even higher (Giantris et al., 1998). Follow ups of this particular patients gain high importance because they will live all their lives with the consequences of an earlier cancer event. Regarding this increased risk of cardiotoxicity, some researchers tried to find ways of minimizing these effects in children with this disease. Previous treatment with cardioprotective agents (such as dexrazoxane) and the use of similar but less toxic compounds (like liposomal doxorubicin) are the most effective new ways in fighting the doxorubicin-induced cardiotoxic-delayed effects (Fulbright et al., 2010). Many research is made with humans in cohort studies were patients are followed for several years to understand how previous cancer treatment affects daily life and future life. To understand the real mechanisms behind the late effects felt by cancer survivors many experimental studies using animal model were conducted, specially because it is would be really hard to study myocardial cells from living humans.

Similar drug to DOX, Mitoxantrone (MTX) is an antitumor and an imunossupressor that also intercalates in the base pairs of DNA and interacts with topoisomerase II to break the DNA double stranded chain (Rossato, Costa, Dallegrave, Arbo, Dinis-Oliveira, et al., 2014). Sadly it shares with Dox the ability of damaging heart, and this cardiotoxicity is also dose dependent. Rossato and her group (Rossato, Costa, Dallegrave, Arbo, Dinis-Oliveira, et al., 2014; Rossato, Costa, Dallegrave, Arbo, Silva, et al., 2014) designed an

experiment were animals would receive a cumulative dose of 7,5 mg/kg of MTX three times during 20 days (at 1st, at 10th and at 20th) and would be sacrificed 2 or 28 days after the last MTX dose. Cardiac (Rossato, Costa, Dallegrave, Arbo, Silva, et al., 2014) and hepatic (Rossato, Costa, Dallegrave, Arbo, Dinis-Oliveira, et al., 2014) damage were assessed and results showed that MTX cardiotoxicity was mediated by MTX mitochondrinopathy and ATP levels were decreased both in cardiomyocytes and hepatocytes from the latter group, possibly because of the impairment on the respiratory chain, evidence supported by the cardiac-decreased activity of complex V in the 28 days' after group (Rossato, Costa, Dallegrave, Arbo, Silva, et al., 2014).

III. Cardiomyopathy: Doxorubicin and Aging effects

Aging is the progressive loss of body functionality, with loss in repair mechanism, being an inevitable and irreversible phenomenon (Tiana et al., 1998). Interestingly, many of the biochemical processes that occur during aging are similar to those that happen due to chemotherapy (Arola et al., 2000; Raha & Robinson, 2000).

Mitochondria seem to have a central role in the process of aging (Lemieux et al., 2010), as they are the primary source of cellular ROS production. Although there is not certainty how aging is initiated at biochemical level, it is accepted that oxidative stress has a major role in the process (Wei & Lee, 2002)

Heart is more susceptible to Dox because cardiomyocytes tend to retain anthracyclines longer than other cells, they have higher amounts of mitochondria (being simultaneously a source and a target of oxidative stress), cardiac tissue lacks catalase (leading to weaker antioxidant activity) and also anthracycline binds to a cardiac specific phospholipid in the membrane, cardiolipin, and this connection inhibits the respiratory chain, leading to accumulation of free oxygen (reviewed on (Simunek et al., 2009)).

Doxorubicin is a quinone that quickly changes to a semiquinone, by gaining an electron from NADPH, cytochrome P-450 reductase or other flavin – containing enzyme (Kaur et al., 2012). This semiquinone, which is highly unstable in the presence of oxygen, reducing this molecule to the instable superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), the first two molecules of the redox cycle, turning again into the first quinone structure (Shi et al., 2011; Wallace, 2003). The hydrogen peroxide in the presence of iron and other heavy metals will be converted in very toxic hydroxyl radicals. Researchers think that this quinone-semiquinone cycle is the key element for doxorubicin anti-cancer effect (Kaur et al., 2012). Moreover, this redox-cycle is highly damaging to the cell homeostasis and only a small amount of doxorubicin is needed for a huge harm effect (Simunek et al., 2009; Sterba et al., 2013). Ichikawa *et al.* (Ichikawa et al., 2014) found that the oxidative stress is amplified because Dox may also increase levels of iron inside the mitochondria. Doxorubicin alters the function of aconitase/iron regulatory protein-1 (IRP-1), an important protein for maintaining the iron pool inside the cell in a metabolic level, because this metal is of high importance for some cell activities, but over a threshold it is harmful for the cell (Simunek et al., 2009; Sterba et al., 2013). Minotti and colleagues (Minotti et al., 1999; Minotti et al., 2001) found that DOX transforms this aconitase/IRP-1 complex into a null protein, because it inactivates IRP-1 and cells can't detect the levels of iron inside the cell, thus cells have a dysfunctional level for this metal. Besides its importance, iron also reacts with the reactive oxygen species (ROS) formed in the course of the quinone-semiquinone cycle. Through the Haber-Weiss reaction, $O_2^{\cdot -}$ and H_2O_2 react and give rise to hydroxyl radical ($HO^{\cdot}$), O_2 and OH^-. Hydroxyl radical is very toxic and unstable, with a very quick half-life, can't be eliminated and tend to oxidize any macromolecule, such as lipids (peroxidation), nucleic acids (mutations) and proteins (Minotti et al., 1999). Iron has the power of accelerating that reaction, normally slow, and in a two step reaction, the Fenton reaction, first Fe^{3+} is reduced by $O_2^{\cdot -}$, being formed Fe^{2+} and O_2, and secondly this Fe^{2+} is oxidized by H_2O_2, with the formation of $HO^{\cdot}$, OH^- and Fe^{3+} (Simunek et al., 2009).

Oxidative damage will occur over proteins, lipids and nucleic acids, and thus affecting all the machinery of the mitochondria and subsequently the cell. Once the proteins are involved in every physiological process of the cell, the oxidative stress may cause damage in activation site of many proteins, leading to their loss of functionality with severe repercussions to cell function (Figueiredo, Mota, et al., 2008). Doxorubicin seems to changes the protein pool of the cell (Kumar et al., 2011), but evidences are not very clear. Nonetheless, there is common over expression of ß-enolase, troponin and alpha B-crystalin, proteins present in high quantity normally in stress conditions. Oxygen reactive species will also react with the cellular membrane because membrane is rich in unsaturated lipids, which are very sensitive to peroxidation. Cardiolipin is especially affected by oxidative stress because it is rich in unsaturated fatty acids and is in close proximity to the formation source of ROS. Cardiolipin-damaged molecule will cause diminished activity of ETC-complex, which in turn will cause more electron leakage that will reduce oxygen into superoxide radical, with consequent amplification of the redox signaling (Petrosillo et al., 2009).

Due to doxorubicin treatment there is also a decrease in some antioxidant defenses, like ascorbat (Ghibu et al., 2012), being these considered late effects (Ghibu et al., 2012; Richard et al., 2008).

DNA is highly affected by ROS as this unstable oxidative species break the nucleotide chain and change some of those nucleotides. Due to its proximity to the main cellular source of ROS, mtDNA is more vulnerable to suffer form oxidative damage, such as mutation, oxidized base pair and deletions (Chomyn & Attardi, 2003). Aging is also intimately linked to mtDNA damage and to the fact that this damage molecules can be perpetuated trough mitochondrial division (Larsson, 2010; Lemieux et al., 2010; Li et al., 2010). These mutations generate heteroplasmy, which means that there is a co-existence between mutated and wild-type molecule, being the ratio between this two types of mtDNA what determines if the respiratory chain will be efficient or deficient for the whole cell functioning (Larsson, 2010).

Doxorubicin also affects DNA in several ways. First, due to oxidative stress, which will attack and damage DNA. Secondly, doxorubicin can also interact directly with the DNA molecule, by intercalating inside the base pair causing large deletions (Adachi et al., 1993) or by binding and inhibiting topoisomerase-II, an important protein for DNA replication process (Tewey et al., 1984). This effect is higher in mtDNA than in nuclear DNA (Ashley & Poulton, 2009) and the effect stays longer in life (Yakes & Houten, 1997), due its particular characteristics. mtDNA is located closer to the font of ROS production because mitochondria is the primary source of oxidative stress due to electron chain transporter and respiration and thus is more affected by oxidative stress (Chomyn & Attardi, 2003). Additionally, mtDNA it is not involved and protected by histones as the nuclear DNA and it lacks a repair system, which clear contrasts with its high mutation rate and high copy number of molecules (either because inside a mitochondria can co-exist 2-10 mtDNA molecules, or because each cell can arbor up to a hundred mitochondrias) (D Lebrecht et al., 2005).

Lebrecht and his group studied the influence of doxorubicin in the mtDNA, either in cardiac tissue (D. Lebrecht et al., 2005; D. Lebrecht et al., 2003) and in kidneys (D. Lebrecht, 2004). In the study performed in a mouse model (D. Lebrecht et al., 2003), they found that there was specificity in the alterations on mtDNA and this caused a toxicity that prevailed longer after the cessation doxorubicin administration. They also found the same mutation standard that exists in the aging process, a 4777bp deletion, commonly named mtDNA deletion (Yang et al., 1994). This mutation is heteroplasmic (is not present in all the mtDNA molecules, only in a few), having a co-existence with wild-type mtDNA. Due to this characteristic, the effects may vary from situation to situation: in some cases it may affect only the non-coding region of mtDNA, it may affect the coding region but not actually the expressed proteins, it may affect some proteins of the electron transport chain turning them to not functional proteins, and in a severe case, the mutation may occur into a tRNA gene, which will affect all the protein machinery, affecting the synthesis of all mitochondrial proteins (Ballinger, 2005). In cardiomyocytes under the effect of

doxorubicin, the common deleted mitochondrial DNA is seen more often whereas the wild-type is in lower number (D. Lebrecht & U. A. Walker, 2007). Dox affects differently the respiratory chain, once it affects the mtDNA and not the nuclear DNA. This way, subunits of the respirator chain encoded by mtDNA will be damage, while the other subunits will not be affected (D Lebrecht et al., 2005). This will lead to a dysfunctional ETC and in this way, more and more oxidative stress will affect more and more mtDNA, in a vicious cycle of oxidative damage caused by doxorubicin leading to mitochondrial dysfunction (D Lebrecht & U A Walker, 2007). This mitochondrial dysfunction is of high importance when dealing with an energy-demanding organ such heart. 90% of the energy used by the cardiomyocytes is produced by the mitochondrial respiration (Ventura-Clapier et al., 2004). Impairment of ETC will lead to lower energy supply. Tokarska-Schlattner (Tokarska-Schlattner et al., 2006) reviewed the findings from several works on this issue, and found that doxorubicin affects mitochondrial respiration through the inhibition of state 3 or stimulation of state 4. ADP is not totally transformed in ATP and there is less energy available for the cardiomyocytes. Another topic reviewed by this author was the fact that doxorubicin may interact with cardiolipin from the inner membrane, which is very close to the ETC complexes. Studies demonstrate that this impairment in complex I of ETC will lower the rate of state 3 respiration, demonstrating that diminished activity of complex I is a key factor for changes in the mitochondrial respiratory status in aged rat hearts (Petrosillo et al., 2009). These problems on the ETC and in the mitochondrial respiration are of major importance in cardiac dysfunction in general, due to the well-known high-energy demands of this organ. In animal model, Figueredo *et al.* (Figueiredo, Ferreira, et al., 2008) studied the effect of aging in isolated mitochondria from skeletal muscle. He and his group compared adult and young mice and found that old mice had lower rate of state 3 respiration for both complexes, but more accentuated for complex I, meaning that complex I mediated respiration was more affected than complex II, an expected result as complex II is Succinate dehydrogenase, an

nuclear encoded complex, while complex I is NADH dehydrogenase, being partially encoded by mtDNA.

Cell death is the ultimate effect of this cardiotoxicity induced by doxorubicin (Zhang et al., 2009) and aging. Apoptosis, necrosis and autophagy can all be triggered directly by Dox, by activating or inhibiting some proteins and effectors, or indirectly with all the events that were described earlier as consequences of exposure to this drug (Shi et al., 2011; Zhang et al., 2009). Dox induces stress creating an unstable environment inside the cell, increasing the levels of Beclin-1 and decreasing the levels of Bcl-2, thus promoting the formation of autophagosome in autophagy process (Smuder et al., 2013). If in the early stages of the tumor increase in autophagy is a tumor suppressor, in the late stages autophagy can be used by the cell to fight against the stress provoked by the cancer itself and by chemotherapy (Chen & Debnath, 2010), trying to maintain homeostasis. Autophagy is decreased in several types of cancer because the Atg gene beclin-1 is deleted, and thus formation of the autophagosome is impossible. With age, the autophagy is also decreased, leading to an accumulation of damaged organelles and proteins (Mizushima et al., 2008).

i. Apoptosis and Autophagy

Apoptosis is a normal and programmed cell process that exists to control the number of cells and to eliminate cells that are old or undergo a wrong path turning into damaged cell. This programmed cell death is beneficial because it helps to eliminate cells that are no longer needed to the organism. It is important to distinct apoptosis from necrosis. Necrosis, unlike apoptosis, is a detrimental process that leads to cell death influencing neighbor cells, starting an inflammatory response. Normally it is a consequence of an acute injury and is characterized by swelling and burst, with the loss of membrane integrity and the release of the cellular content to the exterior, especially cell death signals,

that will affect neighbors cells. In contrast, apoptosis is a process that happens independently only in localized cells, not affecting the tissue surrounding (Ouyang et al., 2012).

Apoptosis is a complex process characterized by a cascade of reaction that has its starting point in the activation of a procaspase into caspase. These proteins are a big family of cysteine proteases that exist in each cell as an inactive precursor called procaspase (Elmore, 2007). The activation of caspases may occur via exogenous or endogenous pathways: the cytoplasmic membrane receptor and the mitochondrial pathways, respectively. In the receptor path a death receptor, for instance Fas ligand produced by killer lymphocytes, bond to the plasma membrane receptor creating a protein complex that will now call for substances inside the cell and activate procaspase-8. In the mitochondrial path the signaling comes from inside the cell and happens when a cell is damaged or under stress. There is the release of substances that act as proapoptotic factors such as cytochrome C, endonuclease G, caspase- 2 and 9, Smac/Diablo and Omi/HtrA2. These factors are released and bind to an adaptor, Apaf-1, that will in turn activate the procaspase-9, forming a complex called apoptosome. This mitochondrial pathway is controlled by proteins of the Bcl-2 family that either inhibit apoptosis such as Bcl-2 or Bcl-Xl and or promote apoptosis, via inactivation of cell death-inhibitors (Bad) and promotion of the release of cytochrome C from the mitochondria (Bax and Bak) (Debatin & Krammer, 2004). Caspase-8 and -9 are categorized as initiators, as they are in the beginning of the chain and activate all other caspases and substances amplifying the cell death signaling. The cell can use this mitochondrial pathway either to start the apoptotic process or to amplify the death response initiated by the receptor path. After the first activation, there is a cascade of cleavages of the subsequent procaspases that amplifies the death signaling. Both pathways will activate procaspase-3 into caspase-3, an effector caspase and it initiates the cascade of cell death (Debatin & Krammer, 2004; Elmore, 2007; Ouyang et al., 2012). It is thought that once this process is initiated it is impossible to stop it, and cell death is irreversible, but this subject is still in discussion inside the

scientific community.

Autophagy is a process of degradation of cellular organelles inside the lysosome system. Autopahgy can be activated as a physiological process, during development or to eliminate organelles and proteins aggregates that no longer have their functionality, promoting the balance inside the cell, or can be triggered by a pathological situation, such as neurodegenerative disease, general stress (starvation, low energy, radiation and chemotherapy, oxidative stress, hypoxia), infection, and cancer (Levine & Kroemer, 2008). Mitochondria can be eliminated through this autophagic process. This organelle is initially involved in a double layer membrane from endoplasmic reticulum, forming an autophagosome that will fuse with a lysosome, forming an autolysosome. During this fusion, the inner membrane is destroyed along with the captured material. This autophagic process is regulated by a kinase, rapamycin TOR (mTOR), since its activation suppresses autophagy, and its inactivation promotes it. Akt and MAPK activate this kinase, responding to insulin-like and some other growth factors (Glick et al., 2010; Yang & Klionsky, 2010). Bcl-2 and Bcl-X_L, two antiapoptotic proteins, also inhibit autophagy by linking to Beclin 1, which disrupt the complex Beclin1/Vps34, an important complex in the formation of the autophagosome. During cellular stress situations several other molecules act over mTOR, inactivating it, so autophagy can occur. mTOR can be activated by AMPK, eukaryotic initiation factor 2α (eIF2α) and tumor suppressor protein (p53) (Levine & Kroemer, 2008; Yang & Klionsky, 2010). Autophagy-related genes (ATG) encodes for the majority of these proteins with major tasks during this process. After the formation of the complex Beclin/Vps34, two other complex are formed to signalize the formation of the autaphagosome. ATG5 and ATG12 bind together and this complex will also recruit APG16L, forming a multimeric complex that will act on the autophagosome formation helping the membrane to curve and to involve the targeted organelle or complex to eliminate (Glick et al., 2010; Levine & Kroemer, 2008). This curvature is formed by asymmetric recruitment of other complex, LC3B-I, and once the membrane is closed, this autophagosome will

finally fuse with the lysosome and the lysosome will degrade what is inside (Glick et al., 2010; Homer et al., 2010; Klettner et al., 2013; Levine & Kroemer, 2008; Yang & Klionsky, 2010).

IV. Cardiomyopathy and Exercise

It is well established that regular exercise or physical activity improves cardiovascular capacity and cellular oxidative respiration (Jacobs & Lundby, 2013). This has been proven to be truth not only for healthy subjects (Werner et al., 2008), but also for aged individuals (I. R. Lanza et al., 2008; Short et al., 2003) and for individuals with some health issues (Libonati, 2013; Siciliano et al., 2012). This effect last long the time of regular exercise or physical activity, but seams to disappear after its end (Lennon et al., 2004). For many years patients with heart disease were advised to stay still and rest as much as possible but in the past years results came out to show the beneficial effect of exercise either as therapeutic or protective measure (T Taivassalo et al., 2006).

Jeppesen and colleagues (Jeppesen et al., 2006) have worked with patients harboring mitochondrial myopathy and found out that exercise improves the oxidative capacity after a 12 weeks program, and that after the end of program the values of Citrate Sintase activity and VO_{2max} were the same as those before exercise conditioning. This last effect of detraining was also seen somewhere else as also the snowball effect “use it or lose it” (T. Taivassalo et al., 2006).

Cardiac myocytes are renewed throughout life by stem cells (CSCs), being recruited to become myocytes and maintain the functionality of the organ. This process is normally enough for healing small lesions, but for bigger insults to the heart the repair system will not be so effective and heart will present some damaged myocytes. It is suggested in literature that CSCs activation and differentiation happens in response of mechanical overload of the heart. As exercise causes a stress situation to heart and is associated with great overload

to heart, it is expected that exercise may activate stem cells and thus helps in regenerating cardiac tissue (Ellison et al., 2012; Figueiredo et al., 2014).

Exercise affects all the biochemistry of the survived cell. It alters the antioxidant system in acute stimuli or in endurance training (Gul et al., 2006). Ellison (Ellison et al., 2012), on her paper about the cardiac adaptations in the molecular and cellular mechanisms due to the effect of endurance exercise, revised several experimental works on the same subject. It is well documented that cardiomyocytes increase in shape due to exercise stimuli and left ventricular wall thickness and dilatation also increase. In endurance athletes there is an eccentric hypertrophy characterized by increased left ventricular thickness and moderate dilatation of the left ventricle while in strength athletes there is concentric hypertrophy characterized by mild thickening of the left ventricular wall as also mild dilatation of the left ventricular. Athletes that combine both trainings are the ones with better adaptations, as they are the ones with higher levels of wall thickness and dilatation in the left ventricle. Although exercise can be deleterious to the organism if it is done in an exaggerated way, regular moderate endurance exercise has several health benefits, giving among others an enhanced cellular tolerance to other harmful stimuli of different nature that reduces the probability of cell death (Ascensao et al., 2007). Several experimental evidences support this concept, such as the Bax/Bcl2 ratio in the left ventricular and the age-related apoptosis that were attenuated in a 12 week 5 days a week treadmill program (Kwak et al., 2006). Similar results for Bcl2 and Bax levels were found in an 8 week 5 days a week moderate intensity treadmill program, with increased production of HSP70 (Siu et al., 2004), an inhibitor of apoptosis.

Many researches have been studying the effect of exercise in aging process, with great evidence pointing the improvement of some senescence events due to endurance exercise (Johnston et al., 2008; I R Lanza et al., 2008). Lanza and his group (I. R. Lanza et al., 2008) stated that oxidative capacity was normalized in aged trained individuals and that insulin sensitivity was improved in exercised young and old individuals. Short and colleagues (Short et al., 2004)

searched the changes in whole body and muscle proteins due to aging and exercise in men and women, finding that there is a generalized lower synthesis of protein, probably because there is a lower recovery activity in elderly, resulting from the aging process. When prescribing 4 months of aerobic exercise protein synthesis in muscle was improved, but not whole body protein synthesis (Short et al., 2004).

Another important fact is the effect of sedentary behaviors instead of lack of regular physical activity. A study of our group showed that sedentary behavior trough out life will speed up the aging process in skeletal muscle and enhance the oxidative damage to mitochondria in mice (Figueiredo, Powers, Ferreira, Amado, et al., 2009). When comparing old sedentary animals to old active animals, it was found that oxidative damage to the mitochondria due to aging was present in both groups, but the levels were higher in the sedentary group, meaning that aging happens anyway but sedentary behavior enhances its consequences.

Following these evidences, a lot of work has been done about the effect of exercise on subjects under doxorubicin treatment. Since early the 90s there were not many studies, but after this date and after the first knowledge about how physical activity could help cancer survivors, many more came out. One of the first was the research performed by Kanter in 1985 (Kanter et al., 1985) with evidences for the positive effect of exercise on the antioxidant defenses. Since than and until today most of the researches support the idea of positive effect of exercise over patients under doxorubicin treatment (Hydock et al., 2009; Matsuura et al., 2010; Wonders et al., 2009).

The majority of works on this area are in animal model and we can divide this works in those that used the exercise before the doxorubicin treatment or those that used exercise during the drug delivery.

Endurance exercise before single dose of doxorubicin (15 to 20 mg/Kg) appears to ameliorate myocardial tolerance and cardiomyocytes to the stress (Kavazis et al., 2010), it improves the glutathione system response and increases HSP (important proteins in the repair and degradation system of damaged proteins)

(A. Ascensao, J. Magalhaes, J. Soares, et al., 2005; A. J. Chicco et al., 2006), it prevents doxorubicin-induced mitochondrial respiration dysfunction and apoptosis (increasing Bax, Bax/Bcl-2 ratio and caspase-3 activity) (Ascensao, Ferreira, et al., 2006; A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005). Additionally, the exercise preconditioning also seems to protect dysfunction of the heart because it maintains the myosin heavy chain (MHC) isoform pool (ß isoform upregulation is related to cardiotoxicity) (D. S. Hydock et al., 2008). Hydock *et al.* (D. S. Hydock et al., 2011) in another work found similar results with endurance exercise in treadmill or in free wheel with cumulative dose of doxorubicin (1mg/kg for 10 consecutive days). Others found that exercise had protective effect against specific autophagy proteins, normally increased due to doxorubicin treatment (Smuder et al., 2013). Acute exercise before a single bout of doxorubicin has also positive effect in preventing doxorubicin cardiotoxicity (A. Ascensao et al., 2011), because it protects against reduced cardiac mitochondrial function, reduced functioning of cardiac mitochondrial chain complexes I and V and increased activity of pro-apoptotic proteins caspase 3 e 9. Martins and colleagues (R. A. Martins et al., 2012) also found evidences that endurance exercise prior to single bout of doxorubicin had protective effect on doxorubicin-induced genotoxicity.

Few researchers studied the effect of exercise during a cumulative dose of the drug and in a work performed with two modalities of doxorubicin administration (1mg/Kg for 15 and 2,5mg/kg weekly for 6 weeks) and free wheel exercise during the drug protocol lasting for more 10 weeks, evidences were found of cardioprotective effect on exercised animals when compared to the sedentary receiving the drug, more specifically in decreased expression of ß-MHC (D. S. Hydock et al., 2012). In another research using treadmill exercise during two weeks concomitantly with doxorubicin treatment, 3 days a week 2,5mg/Kg each time, the same cardioprotective effect of exercise was seen with protection in the doxorubicin-induced increased activity of caspase 3 (A. J. Chicco et al., 2005).

In what concerns to pediatric population, there are not many research papers regarding the cardioprotective effect of exercise. Huang (Huang & Ness, 2011) in his review compiled all the articles on the topic of exercise interventions in children with cancer until 2011, but they were only focused on physical fitness and none elicited any biochemical analysis of any kind. In 2012 Hayward and his group (R. Hayward et al., 2012) performed a research using a juvenile animal model (25 days old rats), administrated doxorubicin for 7 days (2mg/kg each day) and voluntary wheel beginning the first day of drug injection and lasting for more ten weeks. They found that exercise didn't prevent the delayed on growth caused by the drug, but helped to protect late cardiotoxicity in the adult life. Baumann *et al.* (Baumann et al., 2013) saw this lack of information in exercise intervention for pediatric cancer survivors and conducted in 2013 a systematic review on the subject. Their conclusions are very clear: exercise when performed with supervision is only bit beneficial because it improves fatigue, physical fitness, quality of life and strength in cancer survivors.

Studies are lacking on the effect of exercise over mitochondria morphology and functionality, the main target of doxorubicin toxicity. In concrete there is a void in the literature about the possible therapeutic effect of exercise after doxorubicin treatment. It would be very important to understand if exercise could revert some biological deleterious effects left by doxorubicin treatment, mainly in pediatric population.



Methodology

I. Study Design

In order to reach the main goals of this work, we built a model where we used a cardiotoxic drug (doxorubicin) to induce toxicity on the heart and used stainless-steel running wheel cages to mimic voluntary exercise practice. Our experimental approach is represented in figure 1 and is thoroughly explained bellow.

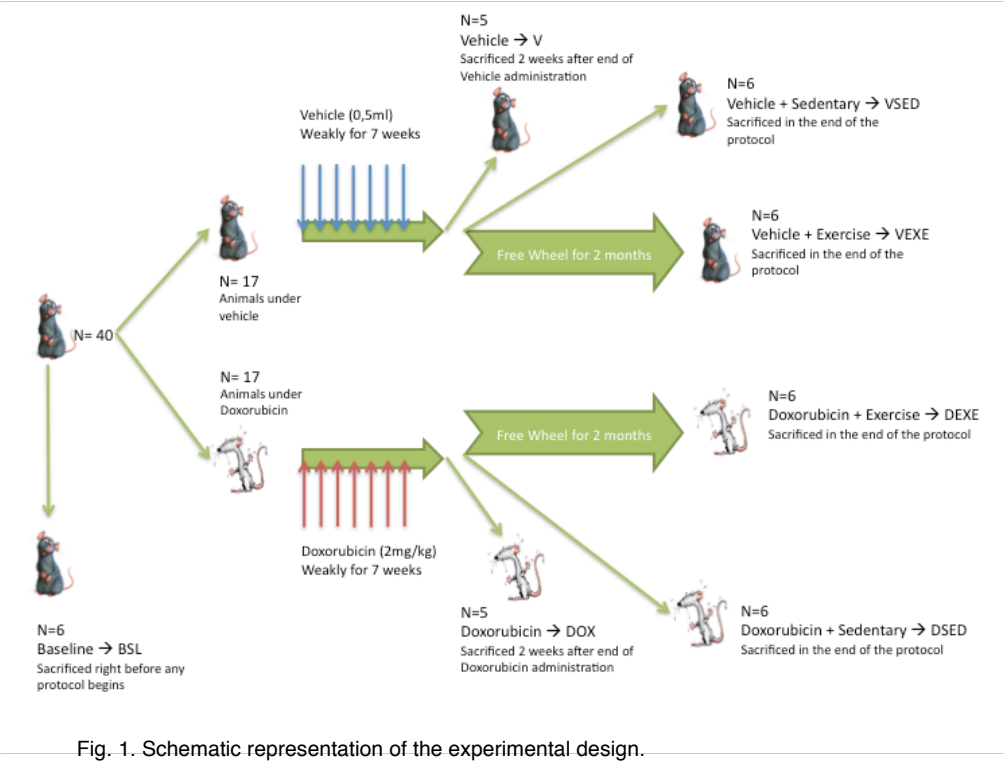


Fig. 1. Schematic representation of the experimental design.

Forty male Wistar rats were obtained from Charles River Laboratories, aged 8 weeks, weighing about 250g. The animals were maintained in a room with normal atmosphere (21 – 22 ºC; 50 – 60% humidity), in a 12 h light/dark cycles, receiving food for rodents and water ad libitum. Males were used over females due to the protective effect of estrogen on cardiac tissue (A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005). All animal interventions were in accordance with the *Guidelines for Care and Use of Laboratory Animals* in research.

After 1 week of acclimation, the animals were randomly divided into Doxorubicin-treated animals (DOX, n=17), Saline/Vehicle-treated animals (V, n=17) and baseline controls (BSL, n=6). Baseline animals were not under any protocol and were sacrificed right before the beginning of the experimental design.

Animals under Doxorubicin treatment received a weekly intra peritoneal (I.P.) injection of Doxorubicin (2mg/kg diluted in 0,5 ml of sterile saline solution; D1515 Sigma Aldrich Co. LLC) during seven weeks (cumulative dose of 14 mg/kg). The animals under Saline/vehicle received the same volume of a sterile saline solution (0,9% NaCl) by I.P. injection. The objective was to mimetize a cancer treatment and this protocol was already used before (Ascensao, Ferreira, et al., 2006; A Ascensao et al., 2005; D Lebrecht et al., 2003).

After the seven weeks of treatment, animals had a two weeks' period of rest and 5 animals in each group were sacrificed. The remaining 12 DOX and 12 V animals were once again randomly divided into sub-groups housed in different conditions. They were either housed individually in a cage with a stainless-steel running wheel, with free access to the wheel for two months (DEXE, n=6; VEXE, n=6), or housed in standard cages with no access to the free wheel (DSED, n=6; VSED, n=6). An attached counter recorded the number of wheel rotations through out the period.

After these two months, animals from these four remaining subgroups were sacrificed. Body weights of the animals were monitored along the experiments.

II. Animal sacrifice and organs collection

Animals were euthanized with an i.p. injection of Xylazine (20 mg/Kg) and Ketamine (80 mg/Kg).

Each animal was placed in supine position, the chest cavity was opened and the heart was exposed to be quickly excised, rinsed, carefully dried and weighted. The atria and the great vessels were removed, and two pieces from the apical part were prepared for optical and electron microscopy.

The rest of the heart was cut quickly into small pieces in ice-cold isolation medium containing 250mM Sacarose, 10mM Hepes and 0,5mM EGTA, pH 7,4. The small heart pieces were washed in the same medium and the blood-free tissue was suspended in 10ml of isolation medium containing a protease (0,5mg/g of initial tissue of Subtilisin). The suspension was homogenized with a tightly fitted glass Potter-Elvehjen homogenizer and a Teflon pestle, in a 0º-4º temperature and at low speed. After homogenization were added three volumes (30 ml) of Subtilisin-free isolation medium and the suspension was centrifuged at 14000g for 10 minutes to remove all the protease. The supernatant was discarded and the pellet was ressuspended in 40 ml of isolation medium containing 0,2% BSA. A low-velocity centrifugation of 800g for 10min was performed in order to pellet the debris, mainly the nucleus, and to isolate better our organelle of interest. This pellet was discarded and the supernatant kept for continuing mitochondrial isolation. 5ml of this supernatant were collected for further biochemical analysis on the all status of the cell (cardiac muscle homogenate). It was added 0,1% of Triton X 10% and 50 μl of protease inhibitor cocktail, and it was vigorously shacked for membrane rupture. 4ml of this solution was divided in aliquots of 500μl and stored at -80ºC for latter experiments. The remaining 1 ml was prepared for latter assessment of TGSH, GSH and GSSG (total glutathione, reduced glutathione and oxidized glutathione). It was added equal volume of percloric acid 5% and centrifuged at 10000g for 10min. The pellet was discarded and the supernatant was stored at -80ºC.

The remaining supernatant was centrifuged at 12000g for 10 minutes and the resulting pellet was suspended in isolation medium free of BSA, and re-centrifuged one last time at 12000g for 10 minutes. This last pellet containing mitochondria was ressuspended in 1ml of a ressuspension medium containing 250mM Sacarose, 10mM Hepes and 0,2mM EGTA, pH 7,4. One aliquot was used for measuring mitochondrial oxygen consumption and the rest of this suspension was stored at -80ºC for posterior biochemical analysis.

All centrifugation steps were executed at 4ºC and mitochondria suspensions were kept at a temperature around 4ºC throughout the experiments.

III. Histology

For Light microscopy, one small piece was removed from the apical part of the heart of each animal was fixed in 4% paraformaldehyde (in 0,1M PBS, pH 7,4; with 2,5% w/v Sucrose and 0,1% Gluteraldehyde). Then the samples were washed in PBS 0,1M, pH 7,4, dehydrated in graded ethanol solutions, cleared in xylene and embedded in paraffin. Paraffin blocks were then cut in pieces of 5 μm in a Leica 2125 rotary microtome (Leica Microsystems Inc.), stained with H&E for qualitative and quantitative morphology assessment of cardiac muscle damage or stained with Sirius Red for assessment of collagen deposition.
For transmission electron microscopy (TEM) analysis, the pieces were fixed 2,5% Gluteraldehyde (in 0,2M Sodium Caccodylate, pH 7,4), pos-fixed in 2% osmiumtetroxide, dehydrated in graded ethanol and embedded in Epon (TAAB) at 60ºC until polymerization. Ultrathin (100mm) sections were cut in an ultramicrotome (Reicher-Jung Ultracut) with a diamonf Knife (ultra 45º Diatome). These slices were mounted on copper grids and contrasted with uranyl acetate and lead citrate (Sigma) and analyzed qualitatively with a transmission electron microscope (Zeiss EM10A; Carl Zeiss, Oberkochen, Germany), at an accelerating voltage of 60kV. Micrographs were recorded with couple photographic camera.

i. Cardiac muscle damage assessment

Cardiac Muscle damage was assessed according to Dinis-Oliveira et al. (Dinis-Oliveira et al., 2007), where the severity and incidence were calculated in each slide. Briefly, for each group, at least more than 1000 cells per slide were analyzed in a blind fashion in order to semi quantify the severity of the following

parameters: i) cellular degeneration, ii) interstitial inflammatory cell infiltration, iii) necrotic zones, and iv) loss of tissue organization. The severity of cellular degeneration was scored according to the number of cells showing any alterations (dilatation, vacuolization, pyknotic nuclei, and cellular density) in the LM visual field: grade 0 = no change from normal; grade 1 = a limited number of isolated cells (until 5% of the total cell number); grade 2 = groups of cells (5–30% of cell total number); and grade 3 = diffuse cell damage (30% of total cell number). The Inflammatory activity was graded semi-quantitatively into: grade 0 = no cellular infiltration; grade 1 = mild leukocyte infiltration (1 to 3 cells by visual field); grade 2 = moderate infiltration (4 to 6 leukocytes by visual field); and grade 3 = heavy infiltration by neutrophils. The severity of tissue disorganization was scored according to the percentage of the affected tissue: score 0 = normal structure; score 1 = less than one third of tissue; score 2 = greater than one third and less than two thirds; score 3 = grater of two thirds of tissue. For each visual field the highest possible score was 12 and the lowest was 0.

ii. Assessment of fibrous tissue in cardiac muscle

Cardiac muscle sections were stained with Picrosirius Red (PSR) according to Sweat et al (Sweat et al., 1964). Briefly, the sections were incubated on 0,1% Sirius red in saturated picric acid for 1h and then rinsed in 0,5% acetic acid, dehydrated in ethanol and cleared in xylene. This technique stains collagen bright red and muscle fibers yellow. Images were analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) quantifying the percentage area covered by collagen (red) and muscle tissue (yellow). The images were analyzed in the ImageJ software (NIH, Bethesda, MD).

iii. Cardiac muscle ultrastructure analysis

The morphometric analysis of the cardiac muscle ultrastructure was performed on the digitalized TEM micrographs. Intermyofibrillar (IMF) and subsarcolemmal (SSM) mitochondria size (area) as well as totall mitochondria density (n/μm^2) were determined using image J software (NIH, Bethesda, MD) considering each image magnification. Qualitative morphological analysis of sub cellular structures was also made.

IV. Biochemistry

i. Spectrophotometry

All the following procedures were measured in the spectrophotometer iEMS reader (Labsystems, Washington, USA).

a. ***Total protein assessment:*** Total protein concentration in heart homogenate and in mitochondrial suspension was spectrophotometrically estimated, according to Gornall an colleagues (Gornall et al., 1949), using bovine serum albumin (BSA) as a standard.

b. ***Determination of Reduced Glutathione (GSH), Total Glutathione (TGSH) and Oxidized Glutathione (GSSG) activity:*** GSH, TSGH and GSSG activities were measured in heart homogenate spectrophotometrically at 414 nm, according to Anderson (Anderson, 1985). Briefly, for total GSH analysis, after neutralization with potassium hydrogen carbonate (0,76M), samples were centrifuged for 10 min at 4ºC at 13000 rpm. Then the resulting supernatant was incubated for 15 minutes at 30ºC in a microtiter plate with a reagent solution (0,7mM 5,5-dithiobis (2-nitrobenzoate) (DTNB) and 0,24mM nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)) and it was added

Glutathione reductase (10U/ml). A kinetic analysis was performed at 414nm during 3 minutes, in intervals of 10 seconds. For GSSG assessment, a 2-vinylpiridine addition (final concentration 5% (v/v)) was made prior the neutralization to inactivate the sulfhydryl groups. The following procedures were similar to those of the TGSH analysis. TGSH and GSSG concentrations (nmol/mg protein) were established according to calibration curves performed with standard samples and dilutions were taken into account for final result.

c. ***Assessment of Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX) and Glutathione Reductase (GR) activity:*** Total SOD activity in cardiac muscle homogenate and mitochondrial suspension were measured spectrophotometrically at 550nm using commercial RANSOD kit (catalogue no. SD 125, Crumlin, United Kingdom) and GPx activity heart homogenate was assessed spectrophotometrically at 340 nm using a commercial RANSEL kit (catalogue no. RS 504, Crumlin, United Kingdom). Methodological recommendations from the manufacturer were followed, with minor adaptations. GR activity was assessed in cardiac muscle homogenate according to Calberg (Carlberg & Mannervik, 1985) by spectrophotometric assay at 340nm. Briefly, samples were diluted in a phosphate buffer solution, sonicated for 5 minutes and centrifuged at 15000g for 5 minutes. Into a microtiter plate were added the phosphate buffer at 30ºC, the needed substrates, GSSG and NADPH, and the sample. A kinetic analysis was performed at 340nm in intervals of 10 seconds for 15 min at 30ºC.

d. ***Determination of Citrate Synthase (CS) activity:*** CS activity was assessed spectrophotometrically at 414nm in heart homogenate and in total mitochondrial suspension after treatment with 0,1% Trinton X-100, according to Coore (Coore et al., 1971). This assay measures the quantity of DTNB that reacts with acetyl-coenzyme A (CoA) after its release from the reaction between acetyl-CoA and oxaloacetate. The absorbance was measured for 7min with intervals of 15 seconds at 37ºC. The activity was calculated as the velocity of

enzyme consumed expressed per mg of protein content.

ii. Imunoblotting analysis

To determine the level of different proteins a volume containing 30 μg of protein was resolved by SDS-PAGE (12,5% acrylamide gels of 1mm thickness) at 130 V for 2h as described elsewhere (Laemmli, 1970) and transferred to a PVDF membrane (Immobilon®-P Transfer Membrane; Cat. No. IPVH00010, Milipore) with Trans-Blot® Turbo Transfer Starter system (Cat. No. 170-4155, BioRad), according to manufacturer instructions.

To control for protein loading and transfer differences, membranes were stained with Ponceau S, analyzed with the ChemiDoc™ XRS+ system and quantified with the Image Lab software (Cat. No. 170-8265, BioRad). Non-specific sites were blocked for 2 hours at room temperature in phosphate-buffered saline (PBS) solution containing 0,1% Tween and 5% non-fat milk. Membranes were incubated overnight at 4ºC with primary antibodies directed against the proteins of interest: HSP60 (1/5000; ab46798 rabbit polyclonal), HSP70 (1/1000; ab47455 mouse monoclonal), Bax (0,4μg/ml; ab7977 rabbit polyclonal), SOD2 (1/1000; ab13533 rabbit polyclonal), COXIV (0,5μl/ml; ab16056 rabbit polyclonal), GPX 1 (1μg/ml; ab22604 rabbit polyclonal), GR (1/1000; ab16801 rabbit polyclonal) or Apg12 (1/500; ab64223 rabbit polyclonal)(all Abcam, Cambridge).

After incubation with primary antibodies, membranes were vigorously washed with PBS-Tween and then incubated with secondary horseradish peroxidase-conjugated anti-mouse (ab98808; Abcam, Cambridge) or anti-rabbit (ab97069; Abcam, Cambridge) IgG antibodies (1:1000) for 2 hours at room temperature. After washing the membranes for totally removable of secondary antibodies, the membranes were treated for 1 minute with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech, Buckinghamshine UK) to detect the labeled proteins and visualized using the ChemiDoc™ XRS+ system with ImageLab software analysis (Cat. No. 170-8265, BioRad). Optical density results were

expressed as percentage according to baseline values.

V. Measurement of mitochondrial respiratory activity

Mitochondrial respiratory function was measured polarographically, at 25ºC using an Oxytherm system and the Clark-type S1/MINI oxygen electrode (Hansatech Instruments). Reactions were conducted in a 0,75ml closed thermostated and magnetically stirred chamber. The tests were performed using the isolated mitochondria in a reaction buffer of 130 mM Sucrose, 50 mM KCl, 2,5 mM KH_2PO_4, 0,5 mM Hepes, pH 7,4. It was added 1mg/ml of mitochondrial protein for testing complex I activity and 0,5 mg/ml of mitochondrial protein for testing complex II. After 1 minute of equilibration period, respiration was initiated by adding malate (5mM) and glutamate (10mM) or Succinate (10mM) plus Rotenone (4μM), for complex I or complex II assessment, respectively. State 3 respiration was determined after addition of ADP (500μM) and sate 4 respiration was measured as the rate of oxygen consumption in the absence of ADP phosphorylation. In order to mimic the i*n vivo* stress under which is mitochondria during exercise, was performed a consecutive ADP-stimuli test. To the chamber containing 0,5 mg/ml of mitochondria protein was added malate (5mM) and glutamate (10mM) to initiate respiration. After 30s of stabilization, 4 pulses of ADP (500μM each time) were added, with 1 minute and 30 seconds of interval between each pulse.

The respiratory control ration (RCR), meaning the ratio between state 3 and state 4 respiration was calculated according to Estabrook (Estabrook, 1976), using 235 nm O_2/ml as the value of solubility of oxygen at 25ºC.

VI. Statistical analysis

The Shapiro-Wilk test was used to investigate within-group normality for each variable. This test was chosen instead of Kolmogarov-Smirnov test for normality because of its power to detect differences in smaller samples. Levene´s test was used to check homogeneity of variance.

Once many of our variables were nor not normally distributed and showed high standard deviation from the mean, we chose to perform non-parametric test. We tested for differences among groups using Kruskal-Wallis and if differences were significant, we performed the Mean-Whitney to assess which groups differed.

The IBM SPSS Statistics 20 was used for all analysis and significance was established a priori as $p<0.05$.



Results

I. Sample characterization and Voluntary Physical Activity

Animal body weights, heart weights and physical activity are shown in table 1.
Animal body weight had an increase associated with age, as observed for the groups V and VSED compared to the BSL ($p<0.05$). Heart weight maintained in a first moment (BSL vs. V, $p>0.05$) and increased until the end of the protocol (BSL vs. VSED, $p<0,05$). Ratio between heart and body weights was increased in V animals when compared with BSL animals ($p<0.05$), and did not changed for VSED (V vs. VSED, $p>0.05$). Once the exercise did not affect body weight in vehicle animals but increased slightly heart weight (VSED vs. VEXE), the ratio between heart and body weights was similar between these two groups.
Doxorubicin affected the normal growth of the animals, delaying it slightly during the treatment, as it can be seen when comparing V and DOX animals ($p<0.05$), but heart weight and ratio between heart and body weights were similar. The body and heart weights almost did not changed two months after the cessation of doxorubicin treatment (DSED vs. DOX, $p>0.05$), and the values for DSED groups were not different from those of VSED groups ($p>0.05$). Heart to body weights ratio was not different between DOX and DSED animals, but DSED heart to body weight ratio was lower than for VSED animals ($p<0.05$). Exercise did not affect body or heart weights in animals under doxorubicin treatment (DSED vs. DEXE, $p>0.05$). Comparing exercised vehicle with exercised doxorubicin animals, it can be seen that animals under doxorubicin treatment have lower body weight ($p<0.05$) and heart to body weight ratio ($p<0.05$).
The voluntary distance run on the free wheel was not different between doxorubicin and vehicle animals ($p>0.05$).

Table 1: Body Weights, Dissected Heart Weights and Physical Activity from all groups.

		BSL	Saline Solution			Doxorunicin		
			V	VSED	VEXE	DOX	DSED	DEXE
Body Weight (g)	Mean (SD)	268,33 (±15,06)	386,40 (±29,70) [a]	434,67 (±57,46)[a]	422,67 (±29,70)[a]	337,75 (±19,60)[a,b,e]	393,80 (±74,62)[a]	363,17 (±46,61)[a,d,e]
	Median (IQR)	272,5 (250;281,5)	283 (361;413,5)[a]	440,5 (408,5;481,25)[a]	431 (403,25;442,5)[a,]	345 (317,25;351)[a,b,e]	433 (321,5;446,5) [a]	374 (325,25;401,5)[a,d,e]
Heart Weight (g)	Mean (SD)	0,52 (±0,05)	0,69 (±0,13)	0,79 (±0,02)[a]	0,81 (±0,04)[a]	0,74 (±0,15)[a]	0,88 (±0,22)[a]	0,84 (±0,11)[a,b]
	Median (IQR)	0,585 (0,523;0,613)	0,74 (0,565;0,81)	0,79 (0,77;0,8)[a]	0,815 (0,758;0,845)[a]	0,7 (0,62;0,885)[a]	0,97 (0,655;1,065) [a]	0,86 (0,77;0,93)[a,b]
Ratio Heart/Body Weight	Mean (SD)	0,00194 (±0,00018)	0,00180 (±0,00030)[a]	0,00189 (±0,00023)	0,00191 (±0,00011)[a]	0,00242 (±0,00058)	0,00222 (±0,00020)[b,d, e]	0,00232 (±0,00010)[b,d, e]
	Median (IQR)	0,0021 (0,00198;0,0022 7)	0,00185 (0,00156;0,002) [a]	0,00181 (0,00174;0,002)	0,00192 (0,00180;0,002) [a]	0,0025 (0,189;0,0029 2)	0,00215 (0,0020;0,002 4)[b,d,e]	0,00236 (0,0022;0,002 4)[b,d,e]
Distance (Km)	Mean (SD)	-	-	-	23,88 (±6,99)	-	-	30,52 (±19,84)
	Median (IQR)	-	-	-	26,16 (16,34;29,81)	-	-	23,99 (13,00;51,31)

Notes: Data is expressed as Mean±Standard Deviation (SD) and Median & Interquiartil Range (IQR). BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

[a] p≤ 0,05 vs BSL [d] p≤0,05 vs VSED

[b] p≤0,05 vs V [e] p≤0,05 vs VEXE

[c] p≤0,05 vs DOX

II. Biochemical Analysis

i. Enzyme Activity

Results from enzyme activity are shown in table 2.

Comparing animals from BSL and V it can be seen that GPX activity did not change, however it increased in the final time (BSL vs. VSED, $p<0.05$). Exercise itself did not affect GPX activity (VSED vs. VEXE, $p> 0.05$). Doxorubicin didn´t affect the activity of this enzyme (V vs. DOX, $p>0.05$). Exercise did not seem to have any impact on this enzyme activity in doxorubicin treated animals (DSED vs. DEXE, $p>0.05$).

GR activity increased from BSL to V animals ($p<0.05$), but it did not change until the end of protocol (V vs. VSED, $p>0.05$). Voluntary physical exercise did not affect GR activity (VSED vs. VEXE, $p>0.05$). The effects of doxorubicin were different with time. Right after doxorubicin administration (DOX group), GR

activity was lower when compared to V animals ($p<0.05$), but in DSED animals the activity was dramatically increased, and differences between DOX and DSED were statistically significant ($p<0.05$). Exercise in doxorubicin animals had the same effect as in normal animals, because it decreased significantly GR activity (DSED vs. DEXE, $p<0.05$), but GR activity was still higher for exercised vehicle animals (VEXE vs. DEXE, $p<0.05$).

Regarding SOD, it can be seen that the activity of this enzyme was not different between BSL and V animals, however with age it tended to increase (V vs. VSED, $p<0.05$). Once the results of VEXE were very close to those from VSED ($p>0.05$), exercise did not seem to alter the activity of SOD. Because results from DOX were close to V group, doxorubicin did not seem to have an acute effect on this enzyme activity; however, this drug had latter effects decreasing this enzyme activity in DSED animals (DOX vs. DSED, $p<0.05$). In doxorubicin treated animals, the voluntary exercise did not affect the activity of this enzyme (DSED vs. DEXE, $p>0.05$).

SOD inside the mitochondria was very active in young animals but its activity decreased highly during growth (BSL vs. V, $p<0.05$), increasing again in older animals (V vs. VSED, $p<0.05$), being the values of BSL and VSED very similar. Exercise did not affect the activity of this enzyme on vehicle animals (VSED vs. VEXE, $p>0.05$). Doxorubicin did not seem to have any effect on the activity of SODMit, once V and DOX animals presented similar results ($p>0.05$), being the same observed for VSED and DSED animals (VSED vs. DSED, $p>0.05$). Voluntary exercise didn't affect the activity of this enzyme in doxorubicin treated animals (DSED vs. DEXE, $p>0.05$).

CS activity increased slightly with age in a first time (BSL vs. V, $p<0.05$), increasing dramatically latter in time (V vs. VSED, $p<0.05$). CS Mit decreased in the first moment (BSL vs. V, $p<0.05$) but increased latter in time (V vs. VSED, $p<0.05$), to values similar to those of BSL animals (BSL vs. VSED, $p>0.05$). Voluntary physical exercise diminished CS activity, as it can be seen when comparing VEXE & VSED groups ($p<0.05$). CS Mit activity was not changed with exercise in vehicle animals. The activity of CS and CS Mit was not affected

by doxorubicin once the values from DOX are similar to those from V ($p>0.05$) as well as the values from DSED are similar to VSED ($p>0.05$). Exercise in doxorubicin treated animals did not have any effect either in CS or in CS Mit activities (DSED vs. DEXE, $p>0.05$), and when compared to exercised vehicle animals it can be seen that CS and CS Mit activities were higher in DEXE animals ($p<0.05$).

ii. Amount of Reduced Glutathione (GSH), Oxidized Glutathione (GSSG) and ratio GSH/GSSG

Comparing BSL and V with VSED animals, it was clear that the amount of GSH did not change in a first moment (BSL vs. V, $p>0.05$), increasing latter in time (BSL vs. VSED and V vs. VSED, $p<0.05$). Exercise diminished the amount of GSH (VSED vs. VEXE, $p<0.05$). Doxorubicin did not have any effect on the amount of GSH, as the results of DOX animals were similar to V animals and, moreover, DSED values were close to VSED values as well as VEXE results were also similar to DEXE results.

In vehicle animals the amount of GSSG did not alter with time, but it increased dramatically with exercise (VSED vs. VEXE, $p<0.05$). Values of DOX were similar to V animals ($p<0.05$). The amount of GSSG in doxorubicin treated animals increased latter in time (DOX vs. DSED, $p<0.05$), and maintained in exercised doxorubicin animals. Considering the influence of voluntary exercise, values for vehicle and doxorubicin animals were similar (VEXE vs. DEXE, $p>0.05$).

Ratio between GSH and GSSG amounts didn't change in vehicle animals with time, but it decreased in VEXE when compared with VSED ($p<0.05$). The GSH/GSSG was not affected by doxorubicin, as values for DOX, DSED and DEXE were similar.

Table 2: Activity levels of the enzymes Glutathione Peroxidase (GPX), Glutathione Reductase (GR), Superoxide Dismutase (SOD), Superoxide Dismutase Mitochondrial (SODMit), Citrate Synthase (CS), and Citrate Synthase Mitochondrial (CSMit) measured for all groups. The amount of Reduced Glutathione (GSH), Oxidized Glutathione (GSSG) and ratio GSH/GSSG are also shown.

		BSL	Vehicle			Doxorunicin		
			V	VSED	VEXE	DOX	DSED	DEXE
GPX (U/g)	Mean (SD)	9,86 (±2,20)	14,74 (±4,23)	18,34 (±3,03)[a]	17,38 (±4,99)[a]	21,86 (±7,75)[a]	17,38 (±4,99)	17,42 (±7,77)[a]
	Median (IQR)	10,58 (7,69;11,69)	16,22 (10,27;17,74)	17,88 (16,12;20,57)[a]	18,09 (15,07;20,03)[a]	21,78 (17,96;25,72)[a]	14,30 (7,81;21,02)	17,70 (13,58;20,17)[a]
GR (U/g)	Mean (SD)	0,905 (±0,432)	1,608 (±0,228)[a]	1,075 (±0,443)	0,873 (±0,190)[b]	0,740 (±0,121)[b]	2,291 (±0,385)[a,b,c,d]	1,419 (±0,117)[c,e,f]
	Median (IQR)	0,92 (0,54;1,05)	1,63 (1,42;1,80)[a]	1,06 (0,71;1,44)	0,78 (0,76;0,94)[b]	0,73 (0,68;0,80)[b]	2,45 (2,15;2,51)[a,b,c,d]	1,045 (1,35;1,49)[c,e,f]
SOD (U/g)	Mean (SD)	0,272 (±0,172)	0,169 (±0,096)	1,212 (±0,233)[a,b]	1,052 (±0,140)[a,b]	0,145 (±0,084)[d,e]	0,738 (±0,085)[a,b,c,d,e]	0,711 (±0,127)[a,b,c,d,e]
	Median (IQR)	0,21 (0,16;0,39)	0,15 (0,12;0,21)	1,15 (1,04;1,39)[a,b]	1,05 (0,94;1,17)[a,b]	0,15 (0,08;0,16)[d,e]	0,76 (0,69;0,79)[a,b,c,d,e]	0,73 (0,65;0,77)[a,b,c,d,e]
SOD Mit (U/mg prtmit)	Mean (SD)	392,81 (±54,67)	169,93 (±73,40)[a]	367,26 (±79,70)[b]	292,70 (±88,15)	116,20 (±27,09)[a,d,e]	335,76 (±83,43)[b,c]	332,78 (±31,99)[b,c]
	Median (IQR)	391,11 (34792;437,69)	162,71 (107,41;232,46)[a]	338,56 (322,22;397,95)[b]	270,79 (244,18;330,27)	130,65 (107,80;131,82)[a,d,e]	337,27 (263,33;403,96)[b,c]	338,68 (322,93;346,23)[b,c]
CS (U/g)	Mean (SD)	146,09 (±15,45)	196,21 (±26,16)[a]	525,00 (±54,81)[a,b]	340,71 (±19,74)[a,b,d]	176,39 (±12,42)[a,d,e]	520,95 (±49,34)[a,b,c]	475,30 (±78,18)[a,b,c]
	Median (IQR)	150,26 (136,18;156,01)	201,37 (184,61;210,39)[a]	548,98 (495,92;554,09)[a,b]	348,79 (33,50;549,30)[a,b,d]	181,99 (1172,08;183,51)[a,d,e]	544,55 (504,39;549,30)[a,b,c]	493,88 (413,66;536,94)[a,b,c]
CS Mit (U/g)	Mean (SD)	3,588 (±0,538)	1,389 (±0,355)[a]	4,055 (±0,477)[b]	3,809 (±0,482)[b]	1,637 (±0,219)[a,d,e]	4,403 (±1,097)[b,c]	4,098 (±1,281)[b,c]
	Median (IQR)	3,69 (3,44;3,79)	1,23 (1,13;1,54)[a]	3,99 (3,76;4,34)[b]	3,73 (3,47;4,01)[b]	1,66 (1,46;1,81)[a,d,e]	4,69 (3,59;5,21)[b,c]	3,84 (3,06;5,14)[b,c]
GSH (U/g)	Mean (SD)	713,27 (±84,38)	871,25 (±236,57)	1468,99 (±152,13)[a,,b]	1074,93 (±100,91)[a,d]	978,17 (±54,99)[a,d]	1444,53 (±159,16)[a,b,c,e]	1029,29 (±374,66)
	Median (IQR)	719,21 (644,64;788,14)	759,64 (735,39;951,31)	1416,72 (1383,31;1528,55)[a,b]	1047,87 (1007,46;1142,41)[a,d]	999,84 (942,23;1014,11)[a,d]	1487,38 (1377,86;1532,62)[a,b,c,e]	984,34 (718,04;1340,55)
GSSG (U/g)	Mean (SD)	21,99 (±5,20)	27,89 (±16,94)	23,74 (±7,14)	58,65 (±19,43)[a,b,d]	29,59 (±3,78)[e]	50,19 (±12,05)[a,c,d]	53,06 (±9,81)[a,b,c,d]
	Median (IQR)	19,90 (19,03;24,97)	25,33 (14,20;41,58)	24,15 (17,59;28,97)	60,86 (39,94 (60,46)[a,b,d]	27,93 (27,57;29,33)[e]	50,93 (39,94;78,87)[a,c,d]	53,63 (53,60;54,49)[a,b,c,d]
GSH/ GSSG	Mean (SD)	33,59 (±6,33)	43,20 (±13,77)	41,01 (±5,24)[a]	26,85 (±3,58)[b,d]	33,00 (±4,33)[d]	29,22 (±10,24)	30,99 (±5,00)
	Median (IQR)	32,90 (30,30;34,77)	38,56 (33,27;53,12)	41,23 (36,77;45,25)[a]	26,72 (25,03;28,61)[b,d]	33,59 (29,92;36,08)[d]	27,06 (21,02;37,43)	29,51 (28,20;33,04)

Notes: Data is expressed as Mean±Standard Deviation (SD) and median & Interquartil Range (IQR). BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

[a] p≤ 0,05 vs BSL
[b] p≤0,05 vs V
[c] p≤0,05 vs DOX
[d] p≤0,05 vs VSED
[e] p≤0,05 vs VEXE
[f] p≤0,05 vs DSED

iii. Protein Expression

Immunoblots were performed to semiquantify target proteins.

Results from COXIV expression and a representative example of western blot analysis are shown in figure 2.

COXIV expression increased with age as it can be observed by the results of BSL, V and VSED groups ($p<0.05$). Exercised animals didn't differ from sedentary ones in vehicle treated groups (VSED vs. VEXE, $p>0.05$).

In doxorubicin animals it can be seen that the expression of this protein didn't increase after doxorubicin administration as registered in vehicle animals (V vs. DOX, $p<0.05$), but with time its expression levels increased (DOX vs. DSED, $p<0.05$) and tended to equalize vehicle and doxorubicin animals (VSED vs. DSED, $p>0.05$). Exercise did not affect COXIV expression in doxorubicin treated animals (DSED vs. DEXE, $p>0.05$), however the values of DEXE were significantly lower than in VEXE animals ($p<0.05$).

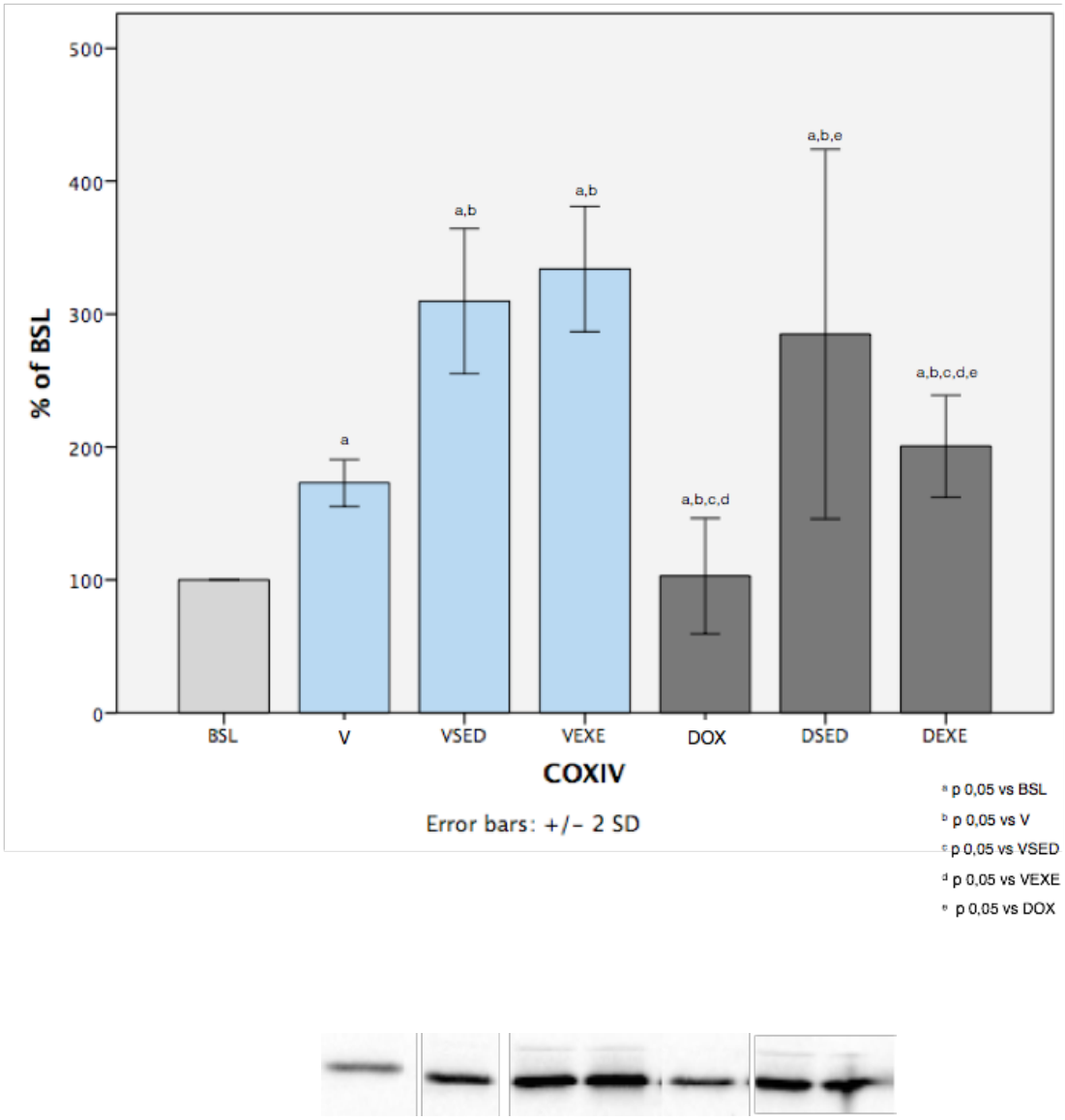


Figure 2. Effect of doxorubicin and voluntary exercise on the expression of tissue homogenate COXIV (15kDa). Immediately below the histogram, the panel shows a representative western blot of COXIV for each group. Values (mean and Standard Deviation) are expressed as percentage of control (BSL).

BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

Results from SOD expression and an example of western blot analysis are shown in figure 3. SOD expression increased dramatically in a short period (BSL vs. V, $p<0.05$) and stabilized with age (V vs. VSED, $p>0.05$). In vehicle groups, exercise decreased SOD expression (VSED vs. VEXE, $p<0.05$).

Doxorubicin had great effect on the expression of this protein. In the first moment, the expression of SOD was highly decreased (V vs. DOX, $p<0.05$), but with time this protein expression dramatically increased (DOX vs. DSED, $p<0.05$). Exercise did not affect the expression of this protein in doxorubicin animals (DSED vs. DEXE, $p>0.05$).

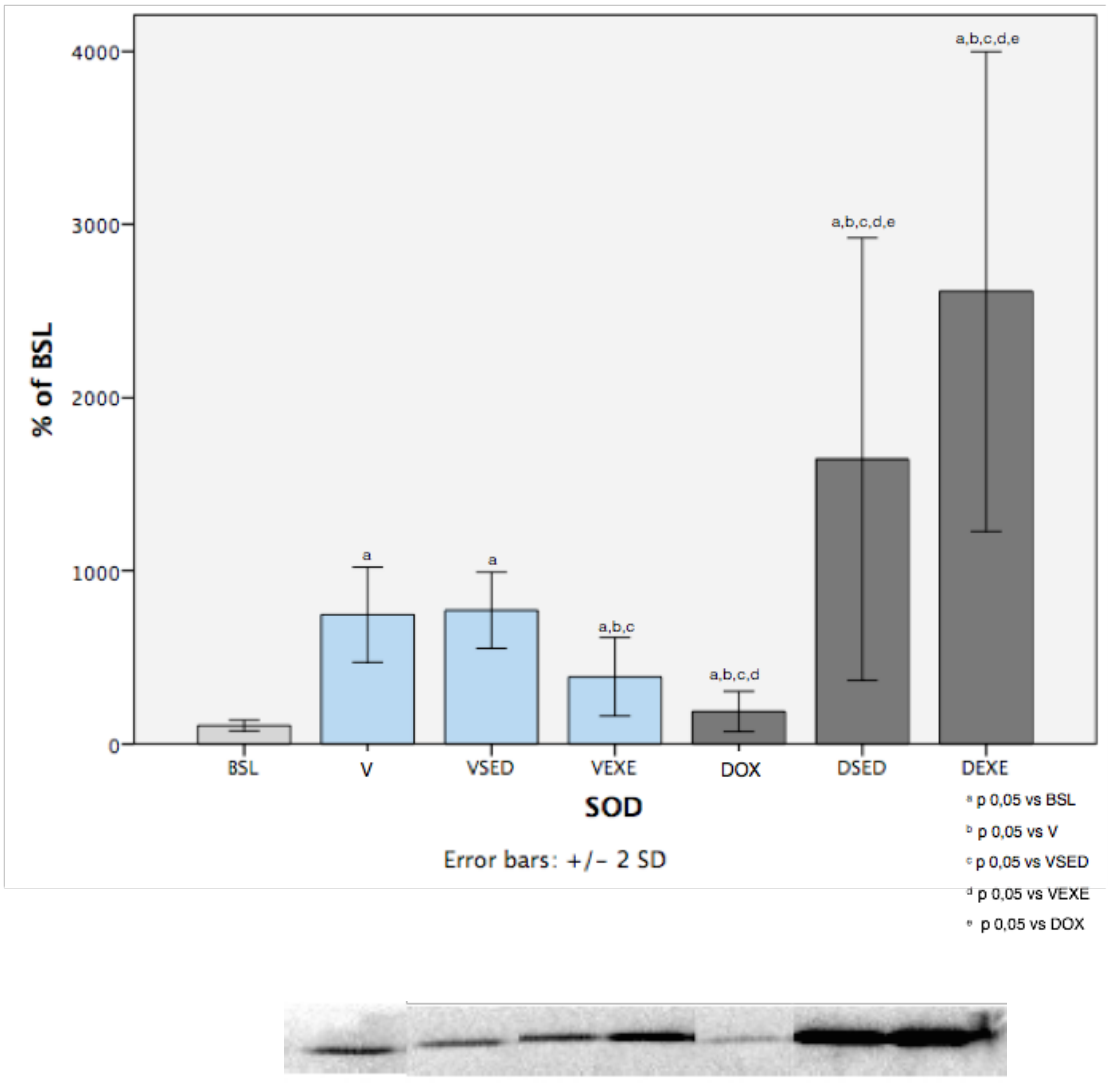
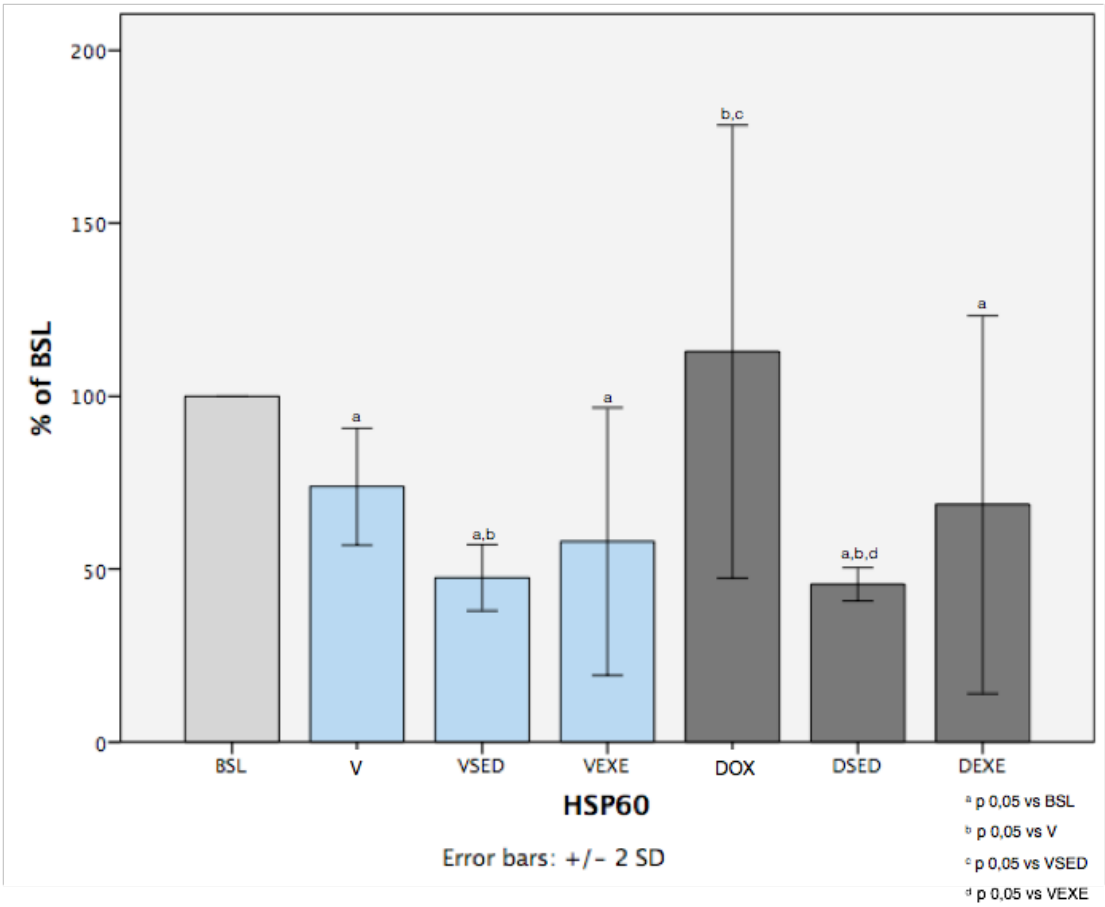


Figure 3. Effect of doxorubicin and voluntary exercise on the expression of tissue homogenate SOD (25kDa). Immediately below the histogram, the panel shows a representative western blot of SOD for each group. Values (mean and Standard Deviation) are expressed as percentage of control (BSL). BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

Results from HSP60 expression and an example of western blot analysis are shown in figure 4. With age, HSP60 expression tended to decrease (BSL vs. V, $p<0.05$; V vs. VSED, $p<0.05$). The voluntary physical exercise did not influence HSP60 in vehicle animals (VSED vs. VEXE, $p>0.05$).

In DOX the HSP60 expression levels were highly increased, however the standard deviation among the values within this group is very high. HSP60 expression in DSED animals was similar to HSP60 expression in VSED animal ($p>0.05$), and it was not altered by exercise (VEXE vs. DEXE, $p>0.05$).



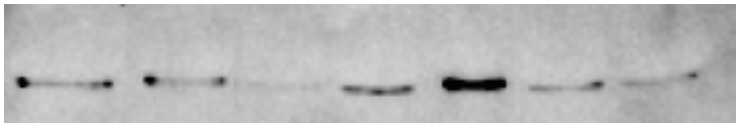
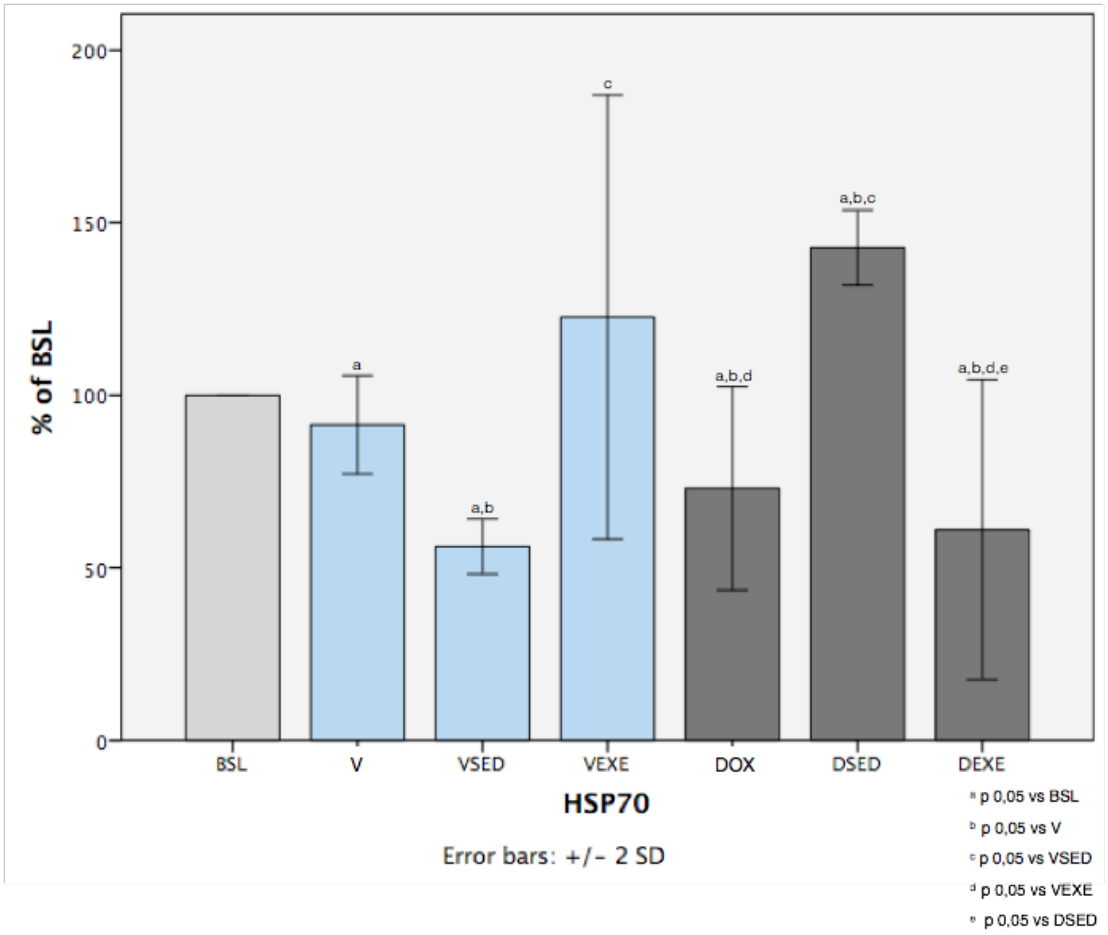


Figure 4. Effect of doxorubicin and voluntary exercise on the expression of tissue homogenate HSP60 (60kDa). Immediately below the histogram, the panel shows a representative western blot of HSP60 for each group. Values (mean and Standard Deviation) are expressed as percentage of control (BSL).

BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

Results from HSP70 expression and an example of western blot analysis are shown in figure 5. HSP70 expression was decreased with age in vehicle animals ($p<0.05$). In exercised animals, HSP70 expression was increased (VSED vs. VEXE, $p<0.05$) reaching similar values to those of BSL and V animals ($p>0.05$). Doxorubicin decreased slightly its expression (V vs. DOX, $p<0.05$), but with age it maintained similar (DOX vs. DSED, $p>0.05$). Exercise decreased the expression of this protein in doxorubicin treated animals (DSED vs. DEXE, $p<0.05$), in contrast to what happened in vehicle animals (VEXE v. DEXE, $p<0.05$).



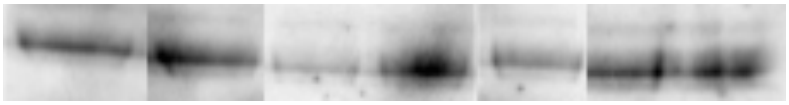


Figure 5. Effect of doxorubicin and voluntary exercise on the expression of tissue homogenate HSP70 (70kDa). Immediately below the histogram, the panel shows a representative western blot of HSP70 for each group. Values (mean and Standard Deviation) are expressed as percentage of control (BSL).

BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

Results from Bax expression and an example of western blot analysis are shown in figure 6. Bax expression increased with age in vehicle animals in a first moment (BSL vs. V, $p<0.05$), but maintained latter in time (V vs. VSED; $p>0.05$). Exercise leaded to a decrease in Bax expression (VSED vs. VEXE, $p<0.05$). V and VEXE had similar expression levels ($p>0.05$).

Doxorubicin didn't have any acute effect on Bax expression (V vs. DOX, $p>0.05$), but with age this drug decreased Bax expression (DOX vs. DSED, $p<0.05$). Doxorubicin exercised animals didn't differ from doxorubicin sedentary animals ($p>0.05$).

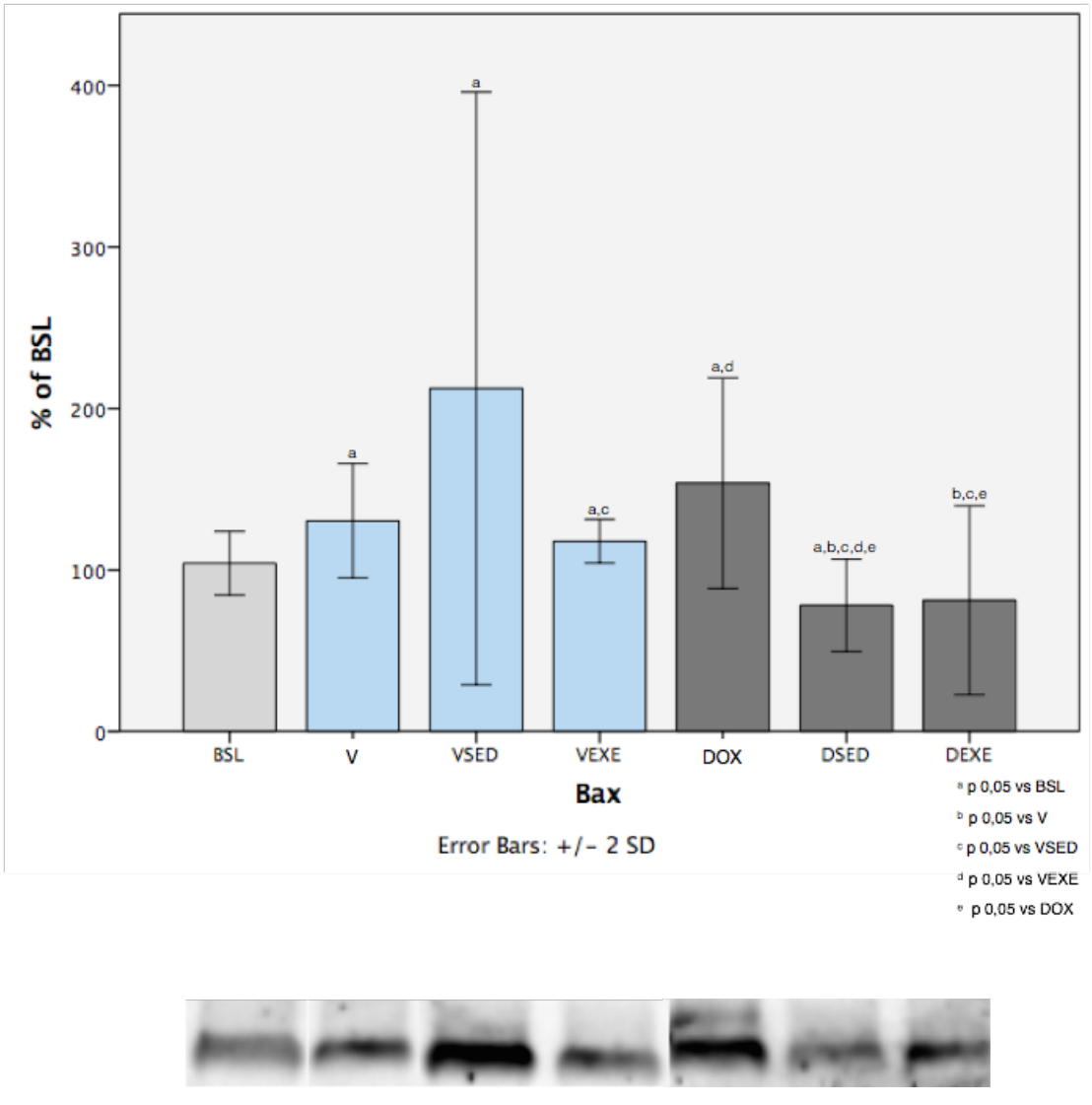


Figure 6. Effect of doxorubicin and voluntary exercise on the expression of tissue homogenate Bax (48kDa). Immediately below the histogram, the panel shows a representative western blot of Bax for each group. Values (mean and Standard Deviation) are expressed as percentage of control (BSL).

BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

Results from Apg12 expression and an example of western blot analysis are shown in figure 7. In vehicle animals, Apg12 expression tended to maintain equal in all groups and exercise did not have any effect on the expression of this protein.

Doxorubicin didn't affect Apg12 expression in a short period of time (V vs. DOX, $p>0.05$), but the effect of this drug was felt latter and very marked. DSED had incredible high levels of Apg12 when compared to vehicle animals ($p<0.05$), and also when compared to DOX group ($p<0.05$). Exercised doxorubicin animals didn't show any significant differences of Apg12 expression from sedentary doxorubicin ones.

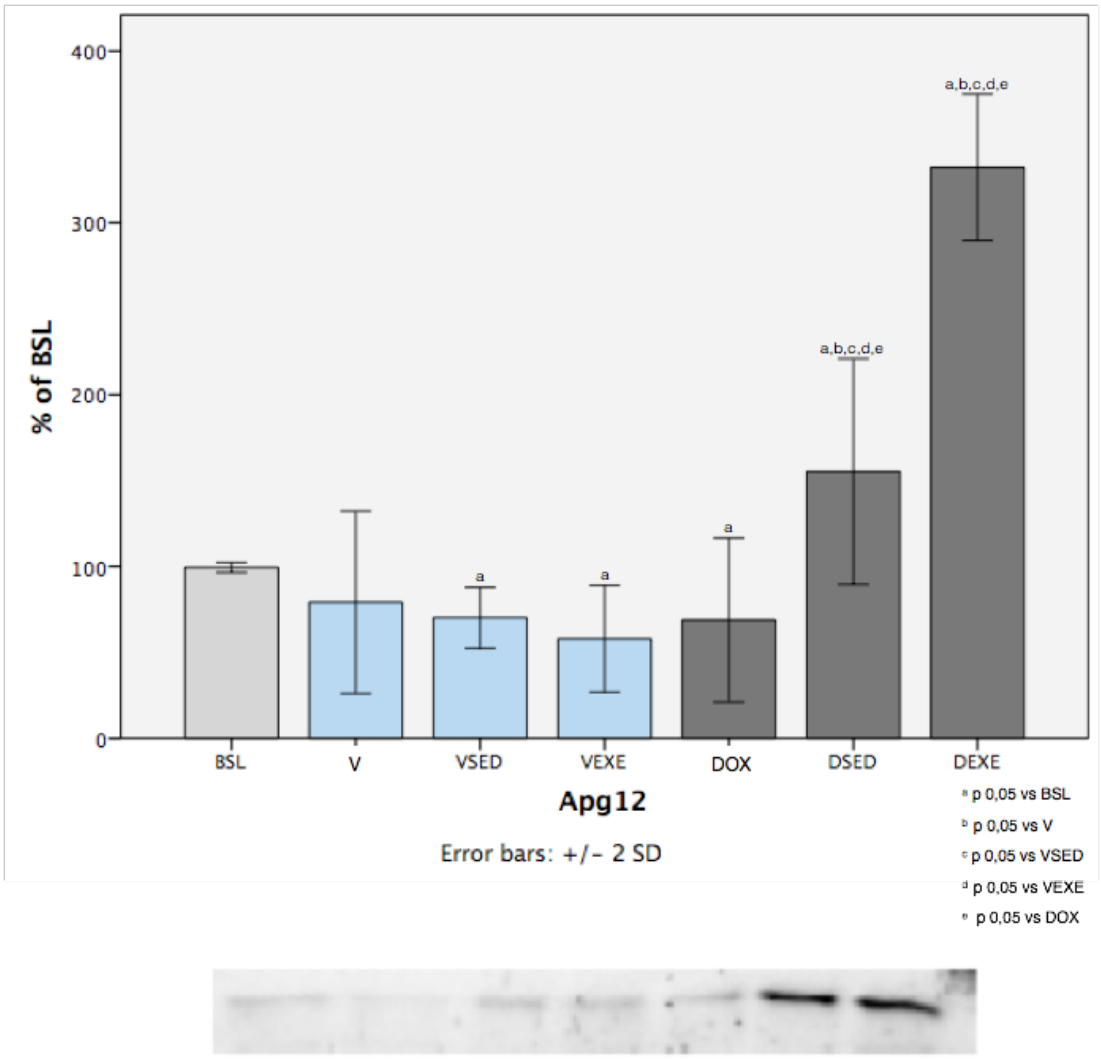


Figure 7. Effect of doxorubicin and voluntary exercise on the expression of tissue homogenate Apg12 (16kDa). Immediately below the histogram, the panel shows a representative western blot of Apg12 for each group. Values (mean and Standard Deviation) are expressed as percentage of control (BSL).

BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

Results from GPX expression and an example of western blot analysis are shown in figure 8. GPX expression in vehicle animals increased in a first moment (BSL vs. V, $p<0.05$) and maintained stable after (V vs. VSED, $p>0.05$). Exercise did not affected GPX expression in vehicle treated animals (V vs. VEXE, $p>0.05$).

Doxorubicin did not affect the expression of this protein.

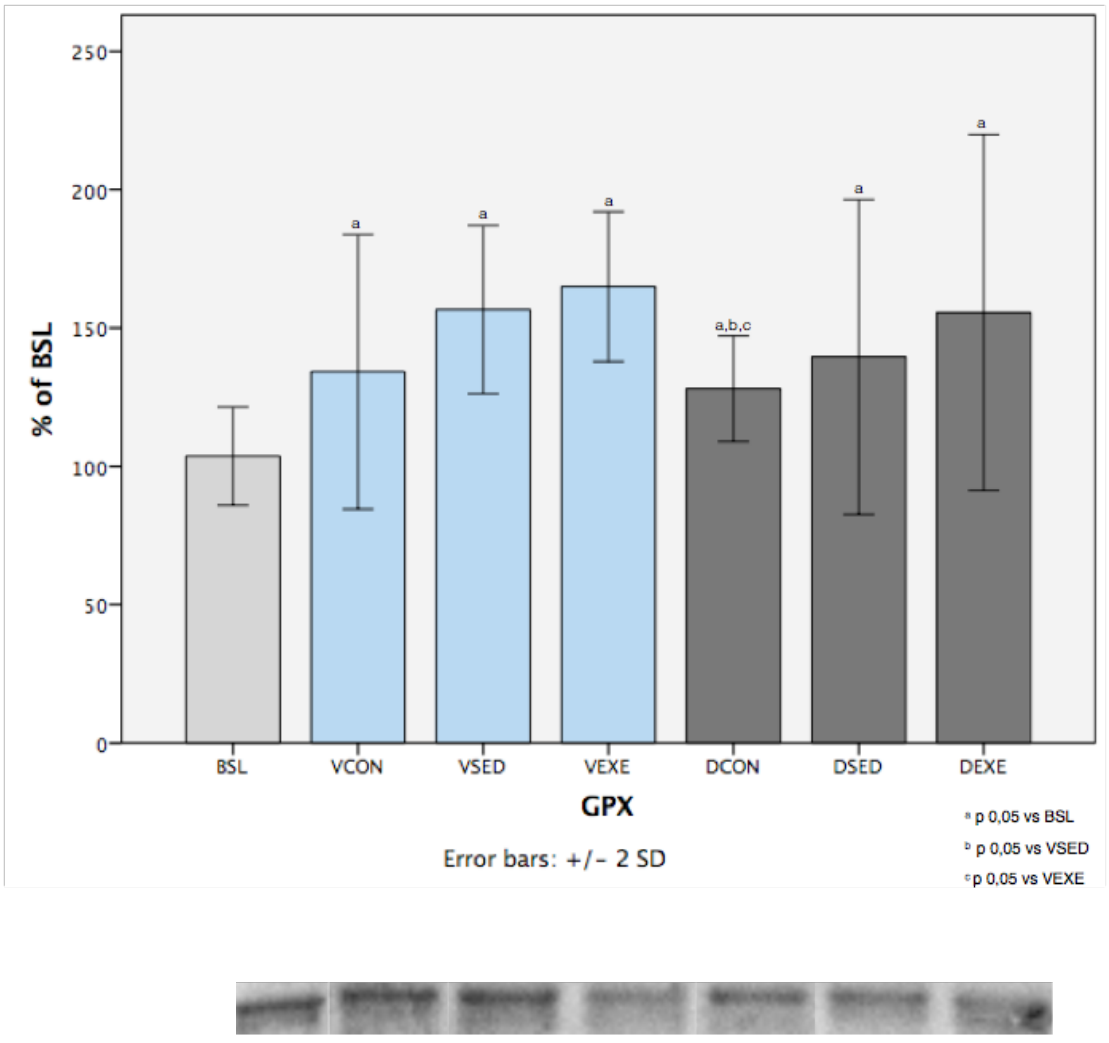


Figure 8. Effect of doxorubicin and voluntary exercise on the expression of tissue homogenate GPX (22kDa). Immediately below the histogram, the panel shows a representative western blot of GPX for each group. Values (mean and Standard Deviation) are expressed as percentage of control (BSL).

BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

Results from GR expression and an example of western blot analysis are shown in figure 9. GR expression in vehicle animals was not altered in a first moment (BSL vs. V, $p>0.05$) but it tended to decrease with age (V vs. VSED, $p<0.05$). Exercise did not have any effect on the expression of this protein in vehicle animals.

Doxorubicin did not affect GR expression. Exercise decreased GR expression in doxorubicin treated animals (DSED vs. DEXE, $p<0.05$).

Exercised animals (DEXE and VEXE) had similar results ($p>0.05$).

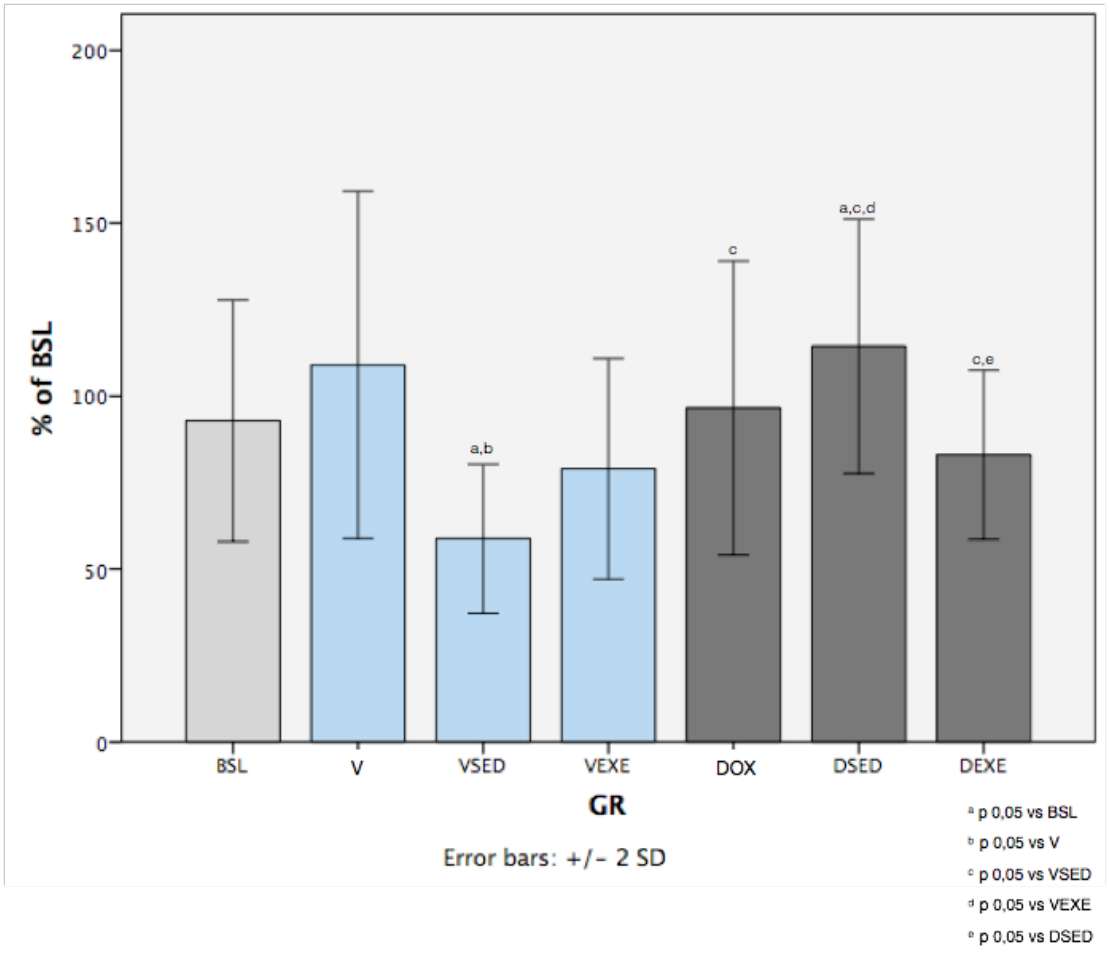




Figure 9. Effect of doxorubicin and voluntary exercise on the expression of tissue homogenate GR (58kDa). Immediately below the histogram, the panel shows a representative western blot of GR for each group. Values (mean and Standard Deviation) are expressed as percentage of control (BSL).

BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

III. Mitochondrial respiratory activity

Functional data obtained from cardiac muscle mitochondria of all groups with complex I-linked substrates (Glutamate/Malate) of the mitochondrial respiratory chain is shown in table 3. Examples of Oxygraph output from all groups, for complex I-linked substrates are shown in figure 10.

Comparing vehicle animals, it can be seen that State 3 increases with age (BSL vs. V, $p<0.05$; V vs. VSED, $p<0.05$). State 4 increased only in the final time (V vs. VSED, $p<0.05$). For State 3 and State 4 rated to CSMit it can be seen that there was also an increase with aging (BSL vs. V, $p<0.05$; V vs. VSED, $p<0.05$). The State3/State4 ratio increased in the first moment (BSL vs. V, $p<0.05$) but decreased highly with age (V vs. VSED, $p<0.05$). State 3, State 3 rated to CSMit and State 4 rated to CSMit increased with exercise (VSED vs. VEXE, $p<0.05$), while State 4 maintained equal. State3/State4 ratio was also not affected by exercise in vehicle animals.

Doxorubicin did not affected State 3, State 4 and State 4 rated to CSMit in the first moment (V vs. DOX, $p>0.05$), and there was only differences in State 3 rated to CSMit where DOX values were lower than those for V animals (V vs. DOX, $p<0.05$) and similar to BSL values (BSL vs. DOX, $p>0.05$). With age, the influence of doxorubicin was more prominent, as DSED animals had higher State 3, State 4 and State 3 and 4 rated to CSMit (DOX vs. DSED, $p<0.05$). State 3/State 4 ratio was similar between DOX and DSED groups ($p>0.05$). Exercise in doxorubicin treated animals had no effect, as values from DEXE animals were similar to those from DSED animals ($p>0.05$), for variables presented in table 3.

Table 3: Functional data obtained from cardiac muscle mitochondria from all groups with complex I-linked substrates (Malate/Glutamate).

		BSL	Vehicle			Doxorunicin		
			V	VSED	VEXE	DOX	DSED	DEXE
State 3 (nmol/min/mg)	Mean (SD)	35,11 (±2,56)	44,54 (±2,41)[a]	87,06 (±9,01)[a,b]	145,17 (±47,11)[a,b,d]	51,35 (±7,77)[a,d,e]	167,68 (±24,16)[a,b,c,d]	137,53 (±17,93)[a,b,c,d]
	Median (IQR)	35,36 (33,12;37,11)	44,88 (43,43;45,82)[a]	90,02 (83,49;92,12)[a,b]	126,6 (109,3;192,2)[a,b,d]	44,16 (26,43;50,29)[a,d,e]	165,8 (150,05;185,30)[a,b,c,d]	143,4 (130,4;147,6)[a,b,c,d]
State 4 (nmol/min/mg)	Mean (SD)	10,78 (±2,62)	12,18 (±3,36)	50,51 (±12,88)[a,c]	61,31 (±5,53)[a,c]	10,14 (±3,96)[d,e]	51,63 (±7,04)[a,b,c]	51,18 (±9,09)[a,b,c]
	Median (IQR)	10,69 (8,58;12,97)	14,03 (11,17;14,12)	53,66 (41,19;59,82)[a,c]	62,29 (57,59;65,03)[a,c]	8,96 (7,93;11,76)[d,e]	54,09 (47,44;56,47)[a,b,c]	54,92 (47,88;58,16)[a,b,c]
State 3 (nmol/CS)	Mean (SD)	10,11 (±2,24)	38,34 (±6,75)[a]	19,29 (±2,55)[a,b]	42,50 (±13,57)[a,d]	17,29 (±2,84)[a,b,e]	43,08 (±24,11)[a,c,d]	50,52 (±19,05)[a,c,d]
	Median (IQR)	10,00 (8,73;11,49)	38,66 (33,55;43,14)[a]	19,21 (17,66;20,92)[a,b]	40,83 (31,47;50,98)[a,d]	18,61 (16,32;18,92)[a,b,e]	33,85 (29,84;48,52)[a,c,d]	49,55 (34,62;66,42)[a,c,d]
State 4 (nmol/CS)	Mean (SD)	3,07 (±1,07)	4,91 (±1,86)[a]	9,95 (±2,81)[a,b]	18,13 (±4,05)[a,b,d]	3,54 (±2,37)[d,e]	10,98 (±1,66)[a,b,c,e]	10,77 (±2,66)[a,b,c,e]
	Median (IQR)	3,34 (2,23;3,96)	4,60 (3,46;6,35)[a]	10,26 (7,56;12,34)[a,b]	16,63 (16,48;18,19)[a,b,d]	3,73 (2,40;4,77)[d,e]	10,98 (10,15;11,81)[a,b,c,e]	11,88 (9,81;12,29)[a,b,c,e]
RCR	Mean (SD)	4,04 (±0,62)	6,55 (±2,45)[a]	2,80 (±0,85)[a,b]	2,25 (±0,57)[a,b]	4,29 (±3,78)	3,05 (±1,19)[b]	2,84 (±0,646)[a,b]
	Median (IQR)	4,28 (3,79;4,49)	5,77 (5,05;8,05)[a]	2,77 (2,35;2,96)[a,b]	2,35 (1,73;2,57)[a,b]	2,42 (2,12;5,53)	2,72 (2,49;3,63)[b]	2,76 (2,46;3,19)[a,b]

Notes: Data is expressed as Mean±Standard Deviation (SD) and median & Inaterquartil Range (IQR). BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise; RCR- Respiratory Control Ratio

[a] p≤ 0,05 vs BSL [d] p≤0,05 vs VSED

[b] p≤0,05 vs V [e] p≤0,05 vs VEXE

[c] p≤0,05 vs DOX

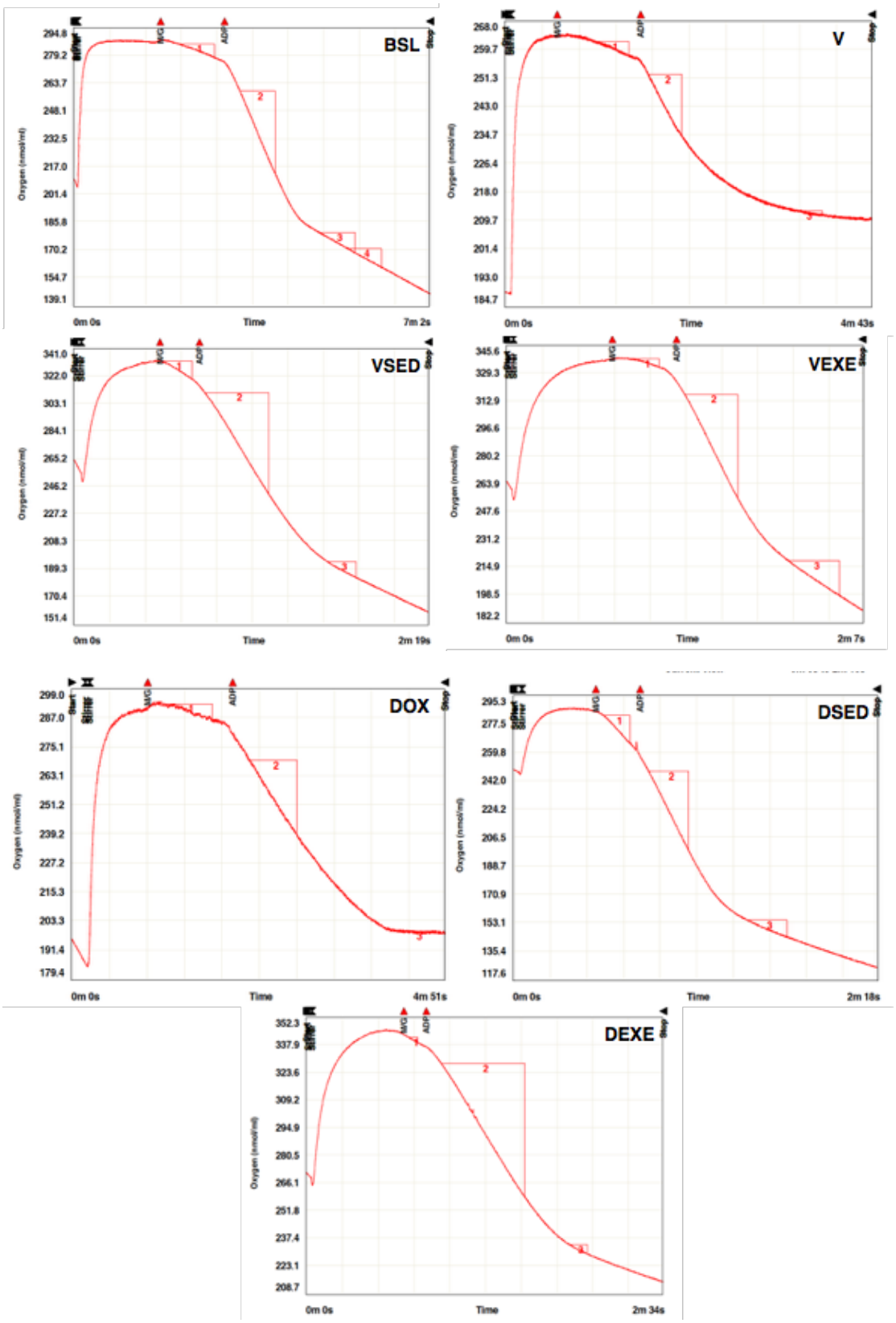


Figure 10. Representative Oxygraph of Complex I-linked substrates (Malate/Glutamate) from all groups.

BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

Functional data obtained from cardiac muscle mitochondria of all groups with complex II-linked substrates (Succinate/Rotenone) of the mitochondrial respiratory chain is shown in table 4.

For complex II-linked substrates, State 3 increased with age (BSL vs. V, $p<0.05$; V vs. VSED, $p<0.05$) but State 4 maintained equal. When State 3 and State 4 were rated to CSMit, it can be seen that they increased highly from BSL to V ($p<0.05$). After two months, State 3 increased, as it can be seen for VSED values when compared to V values ($p<0.05$). State 4 and State 3 rated to CSMit maintained unchanged and State 4 rated to CSMit decreased (V vs. VSED, $p<0.05$). The State3/State4 ratio increased with time ($p<0.05$). Exercise had no effect over the variables measured for complex II-linked substrates in vehicle animals.

Doxorubicin seems to have no effect in a short time (V vs. DOX, $p>0.05$), except for State 3 rated to CSMit, where values were significantly lower in DOX animals than in V animals ($p<0.05$). State 3 was affected by doxorubicin latter in time, as values of DSED animals are significantly higher than for DOX animals ($p<0.05$), but not as high as were for VSED animals ($p<0.05$). State 4 rated to CSMit was decreased in DSED when compared with DOX animals ($p<0.05$) and all other variables maintained similar values. Exercise had no effect on doxorubicin treated animals, increasing only State 4 rated to CSMit (DSED vs. DEXE, $p<0.05$).

Table 4: Functional data obtained from cardiac muscle mitochondria from all groups with complex II-linked substrates (Succinate/Rotenone).

		BSL	Vehicle			Doxorunicin		
			V	VSED	VEXE	DOX	DSED	DEXE
Stage 3 (nmol/min/mg)	Mean (SD)	58,79 (±21,30)	85,08 (±17,49)[a]	167,55 (±31,09)[a,b]	186,66 (±33,15)[a,b]	64,68 (±7,67)[c,d]	111,93 (±8,09)[a,b,c,d,e]	156,98 (±51,85)[a,b,e]
	Median (IQR)	52,14 (40,20;79,21)	91,65 (74,10;96,01)[a]	166,00 (148,50;187,05)[a,b]	182,80 (158,70;213,5)[a,b]	67,10 (59,52;69,84)[c,d]	115,30 (109,00;116,55)[a,b,c,d,e]	160,25 (112,85;201,10)[a,b,e]
Stage 4 (nmol/min/mg)	Mean (SD)	24,92 (±6,99)	32,88 (±9,36)	47,91 (±22,33)[a]	38,47 (±16,90)	30,06 (±18,03)	36,53 (±12,07)	38,24 (±10,76)
	Median (IQR)	26,08 (19,50;30,34)	32,35 (28,08;37,43)	40,38 (34,57;61,26)[a]	34,21 (27,27;49,68)	23,86 (19,91;37,12)	34,36 (30,03;41,95)	38,93 (29,00;47,48)
Stage 3 (nmol/CS)	Mean (SD)	17,29 (±6,29)	67,16 (±12,63)[a]	54,37 (±30,66)[a,b]	49,60 (±12,47)[a]	39,61 (±2,12)[a,b]	33,98 (±12,88)[a,b]	50,18 (±28,82)[a]
	Median (IQR)	19,69 (11,06;21,15)	61,91 (61,40;72,45)[a]	35,56 (32,76;81,67)[a,b]	45,70 (40,13;53,18)[a]	39,46 (38,14;41,09)[a,b]	33,41 (23,56;44,40)[a,b]	48,97 (26,47;73,90)[a]
Stage 4 (nmol/CS)	Mean (SD)	8,56 (±0,74)	23,11 (±2,88)[a]	6,04 (±2,85)[b]	3,68 (±1,86)[a,b]	18,68 (±9,07)[a,c,d]	7,03 (±1,93)[b,c,e]	14,01 (±2,61)[a,b,c,d,f]
	Median (IQR)	8,72 (8,24;8,96)	21,87 (21,47;24,14)[a]	5,67 (3,97;8,1)[b]	3,08 (2,64;4,42)[a,b]	17,37 (13,85;22,85)[a,c,d]	8,03 (6,42;8,14)[b,c,e]	15,46 (13,22;15,52)[a,b,c,d,f]
RCR	Mean (SD)	2,29 (±0,53)	4,46 (±0,77)[a]	5,30 (±1,07)[a]	7,74 (±4,25)[a]	2,58 (±1,38)[c]	5,59 (±2,08)[a]	4,75 (±2,84)[a]
	Median (IQR)	2,45 (2,26;2,64)	3,47 (2,79;4,13)[a]	5,23 (4,52;6,08)[a]	7,14 (4,35;11,12)[a]	2,25 (1,83;3,17)[c]	5,38 (4,09;7,10)[a]	4,17 (2,13:7,54)[a]

Notes: Data is expressed as Mean±Standard Deviation (SD) and median & Interquartil Range (IQR). BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise; RCR- Respiratory Control Ratio.

[a] p≤ 0,05 vs BSL [d] p≤0,05 vs VEXE

[b] p≤0,05 vs V [e] p≤0,05 vs DOX

[c] p≤0,05 vs VSED [f] p≤0,05 vs DSED

IV. Morphometric analysis

The optical microscopy analysis from the H&E staining revealed in the groups BSL, V, VSED and VEXE a cardiac muscular tissue with normal morphology, without any structural changes (figure 11).

In a qualitative analysis, the animal from DOX and DSED groups showed a marked vascular congestion, with increased interstitial space, in which it could be observed a deposition of fibrin-like material suggesting a great quantity of protein content (figure 12A&13A). In these groups it can be seen many necrotic cells as other cells with degenerative signs, like edema and cytoplasmic vacuolization (figure 12B&13B). The heterogeneity of the cardiomyocytes dimensions was seen in the DOX and DSED groups (figure 12C&13C). Similar changes were observed in DEXE group, but slightly tenuously, more dispersed and punctual (figure 14).

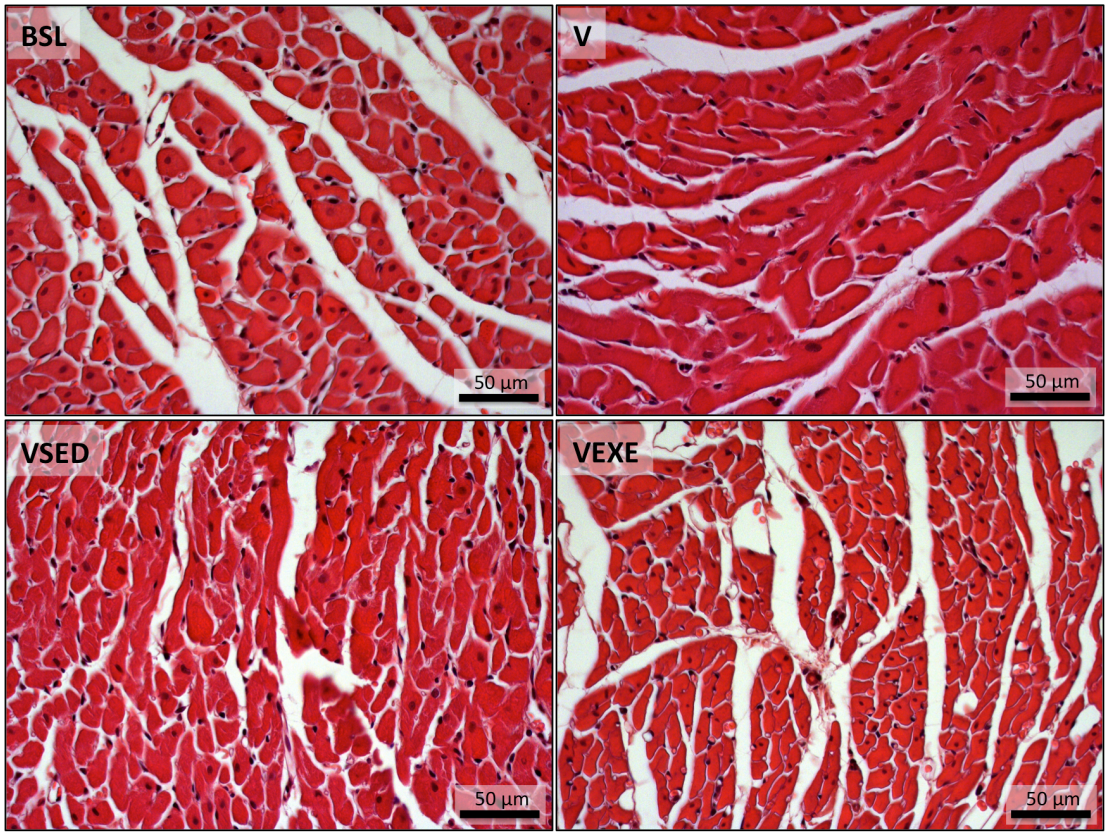


Figure 11. Light micrographs representative from animals of groups baseline (BSL), vehicle (V), vehicle + sedentary (VSED) and vehicle + exercise (VEXE). Sections were stained with hematoxylin-eosin. All micrographs depict a normal tissue structure without signals of cellular degeneration, necrosis or cell infiltration. The interstitial space is preserved without evidences of tissue disorganization.

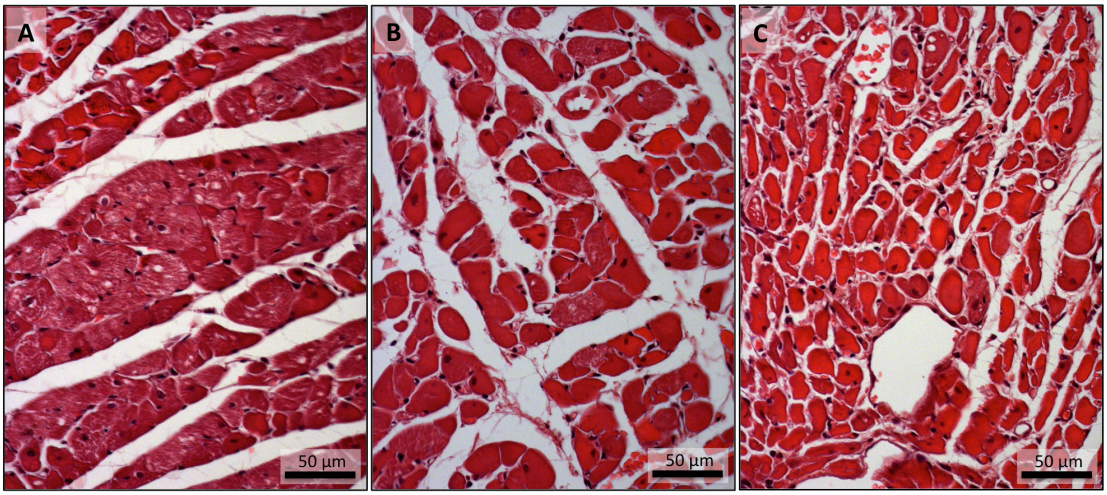


Figure 12. Light micrographs representative from animals of group doxorubicin (DOX). Sections were stained with hematoxylin-eosin. In all micrographs, beyond the great heterogeneity of cardiomyocytes dimensions, it is depicted several enlarged cells with cytoplasmic vacuolization, intracellular edema and in some of them the morphologic signs suggest to be in necrosis. It is also notorious an enlarged interstitial space fulfilled by collagen fibers and fibrin-like material, more evident in B and C.

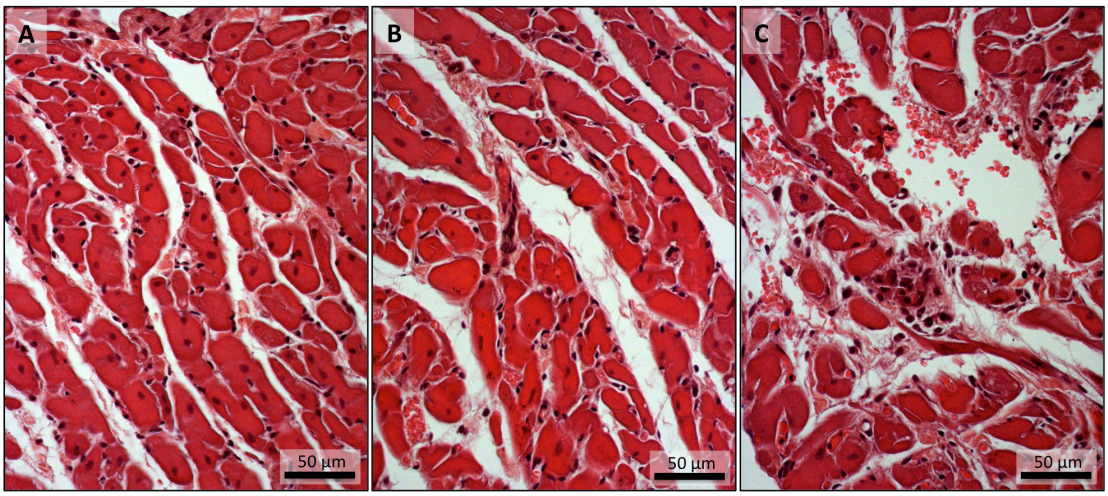


Figure 13. Light micrographs representative from animals of group doxorubicin + sedentary (DSED). Sections were stained with hematoxylin-eosin. The heterogeneity of cardiomyocytes dimensions is evident in all micrographs with an enhanced interstitial space fulfilled with connective tissue. Some degenerated cells with cytoplasmic edema are flanked by apparently normal cardiomyocytes. In C it is observed some infiltrative mononuclear cells.

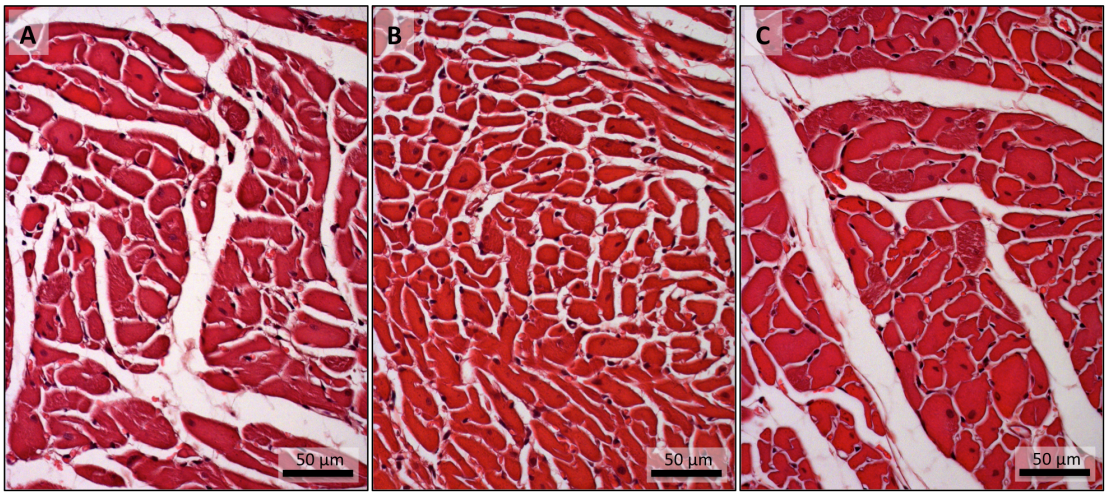


Figure 14. Light micrographs representative from animals of group doxorubicin + exercise (DEXE). Sections were stained with hematoxylin-eosin. Some punctual damaged cells with cytoplasmic edema or soft vacuolization can be identified in all micrographs, surrounded by a clean interstitial space.

Quantitative data from cardiac muscle damage obtained from slides stained with H&E is shown in table 5. In accordance with the heart normal structure observed in the qualitative analysis, there were no significant differences between vehicle animals.

Doxorubicin affected the cardiac muscle ultrastructure, increasing dramatically the damage in cardiomyocytes. DOX and DSED values for cardiac damage differed from every other group for all parameters of cardiac damage measured and results were statistically significant ($p<0.05$). Exercise restored the damage, as it can be seen by the results shown for DEXE, statistically different from DOX and DSED ($p<0.05$), and values of DEXE were close to those from vehicle groups.

Table 5: Data from cardiac muscle damage

		BSL	Vehicle			Doxorunicin		
			V	VSED	VEXE	DOX	DSED	DEXE
Cell Deg	Mean (SD)	0,044 (±0,206)	0,102 (±0,305)	0,167 (±0,377)	0,050 (±0,221)	2,136 (±0,765)[a,b,c,d,e]	1,613 (±0,715)[a,b,c,d,e]	0,097 (±0,300)[f]
	Median (IQR)	0 (0)	0 (0)	0 (0)	0 (0)	2 (±2;3)[a,b,c,d,e]	2 (±1;2)[a,b,c,d,e]	0 (0)[f]
Inflam	Mean (SD)	0 (0)	0 (0)	0 (0)	0 (0)	0,409 (±0,497)[a,b,c,d,e]	0,355 (±0,486)[a,b,c,d,e]	0 (0)[f]
	Median (IQR)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0;1)[a,b,c,d,e]	0 (0;1)[a,b,c,d,e]	0 (0)[f]
Necro	Mean (SD)	0 (0)	0,017 (±0,130)	0 (0)	0,025 (±0,158)	0,659 (±0,645)[a,b,c,d,e]	0,807 (±0,749)[a,b,c,d,e]	0,065 (±0,249)[f]
	Median (IQR)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0;1)[a,b,c,d,e]	1 (0;1)[a,b,c,d,e]	0 (0)[f]
Loss Org	Mean (SD)	0 (0)	0 (0)	0 (0)	0 (0)	0,364 (±0,487)[a,b,c,d,e]	0,452 (±0,506)[a,b,c,d,e]	0 (0)[f]
	Median (IQR)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0;1)[a,b,c,d,e]	0 (0;1)[a,b,c,d,e]	0 (0)[f]
Sum	Mean (SD)	0,044 (±0,206)	0,119 (±0,375	0,167 (±0,377)	0,075 (±0,267)	3,568 (±0,998)[a,b,c,d,e]	3,226 (±1,117)[a,b,c,d,e]	0,161 (±0,374)[f]
	Median (IQR)	0 (0)	0 (0)	0 (0)	0 (0)	4 (3;4)[a,b,c,d,e]	3 (3;4)[a,b,c,d,e]	0 (0)[f]

Notes: Data is expressed as Mean±Standard Deviation (SD) and median & Interquartil Range (IQR). BSL-Baseline; V-Vehicle; VSED-Vehicle+Sedentary; VEXE-Vehicle+Exercise; DOX-Doxorubicin; DSED-Doxorubicin+Sedentary; DEXE-Doxorubicin+Exercise.

[a] p≤ 0,05 vs BSL [d] p≤0,05 vs VSED

[b] p≤0,05 vs V [e] p≤0,05 vs VEXE

[c] p≤ 0,05 vs DOX [f] p≤0,05 vs DSED

In the qualitative analysis of Sirius-Red staining, the groups BSL, V, VSED and VEXE showed a normal collagen deposition in the interstitial space and similar among these groups (figure 16).

Doxorubicin increased dramatically the degree of collagen, more intensely in DSED. Exercise decreased the amount of collagen in DEXE animals (figure 17).

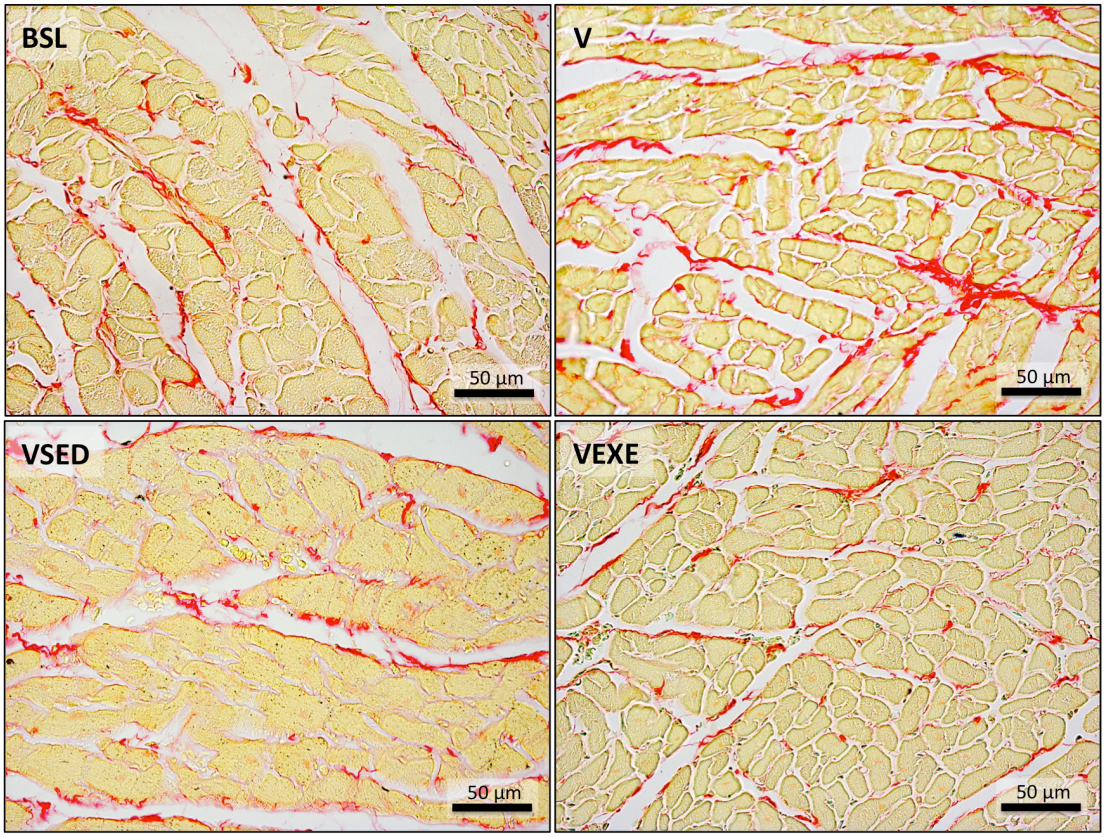


Figure 15. Light micrographs representative from animals of groups baseline (BSL), vehicle (V), vehicle + sedentary (VSED) and vehicle + exercise (VEXE). Sections were stained with picrosirius red. In all groups the connective tissue (red stained) show a normal density.

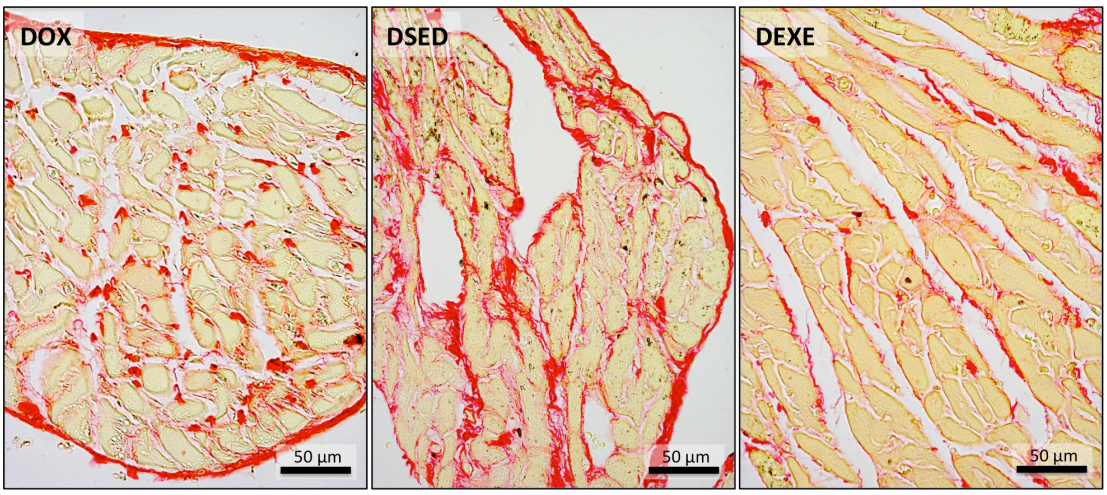


Figure 16. Light micrographs representative from animals of groups doxorubicin (DOX), doxorubicin + sedentary (DSED), and doxorubicin + exercise (DEXE). Sections were stained with picrosirius red. It is evident an enhanced density of connective tissue (red stained) in DOX and DSED, more pronounced in DSED, with a lower density in DEXE group.

The quantitative analysis of collagen deposition measured in slides with Sirius Red staining is shown in figure 11.

Analysis of the amount of collagen deposition in vehicle animals showed that it decreased after two weeks (BSL vs. V, $p<0.05$), and increased until the end of protocol (V vs. VSED, $p<0.05$). Exercise had no effect on the amount of collagen deposition in vehicle animals (VSED vs. VEXE, $p>0.05$).

Doxorubicin increased the amount of collagen deposition (V vs. DOX, $p<0.05$) that was maintained with time (DOX vs. DSED, $p>0.05$). Exercise decreased the amount of collagen deposition when compared to DOX animals (DOX vs. DEXE, $p<0.05$), but this exercise protocol was not sufficient to show differences between DSED and DEXE animals ($p>0.05$).

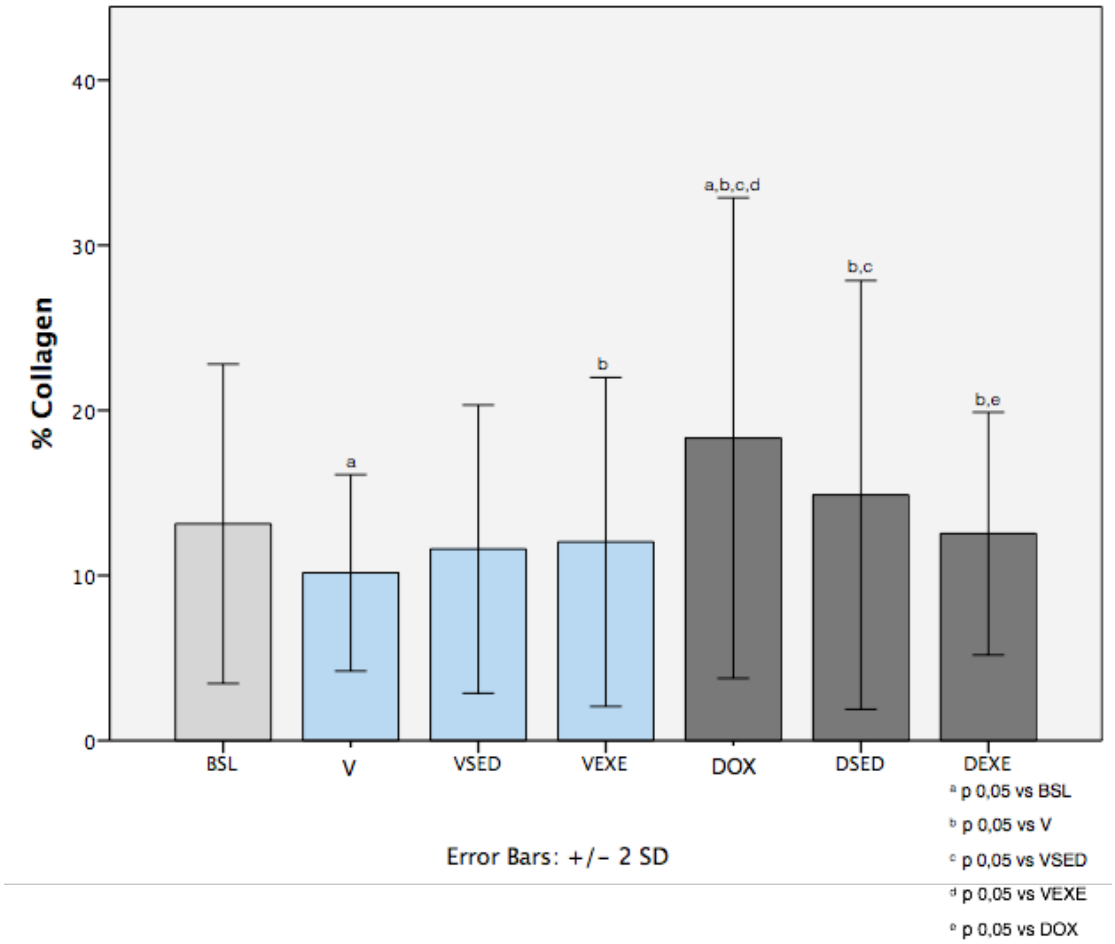


Figure 17. Effect of doxorubicin and voluntary exercise on the fibrotic area. Results are shown as the percentage of Collagen deposition per total area obtained from Sirius-Red staining for all groups. Data is expressed as Mean±Standard Deviation (SD).

BSL- Baseline; V- Vehicle; VSED- Vehicle+Sedentary; VEXE- Vehicle+Exercise; DOX- Doxorubicin; DSED- Doxorubicin+Sedentary; DEXE- Doxorubicin+Exercise.

In the qualitative analysis of TEM, the cardiomyocytes of BSL, V, VSED and VEXE animals show a normal ultra-structure, without any apparent morphological abnormality (figure 18). In these groups, mitochondria had normal shape and dimensions, with lamellar cristae well defined. The VEXE group seems to evidence an increased mitochondrial density, although this parameter was not quantified.

In the DOX and DSED groups were found regularly necrotic cells or cardiomyocytes with signs of intracellular edema, with enlarged space between the myofibrils and dilated perinuclear cisterns. In these two groups the mitochondria had repeatedly strange forms, with giant mitochondria being side-by-side with very small mitochondria, showing a great heterogeneity of these organelles on these groups (figure 19A&20B). In general these organelles had very badly defined cristae, apparently lamellar (figure 19B&20B). In the surroundings or even between the mitochondria, the presence secondary lysosomes was frequently found (figure 19C&20C). In these two groups the existence of fibroblast with signs of activation and large deposition of collagen fibers, more pronounced in DSED group, were also observed. Frequent macrophages and mononuclear infiltrative cells were repeatedly seen in interstitial space. All these changes were also observed on DEXE group but with a lower pronounced character (figure 21). In this group the mitochondria had a round shape with well-defined cristae, which were lamellar in their appearance, and the organelle heterogeneity was not as pronounced as observed in DOX and DSED analysis (figure 21).

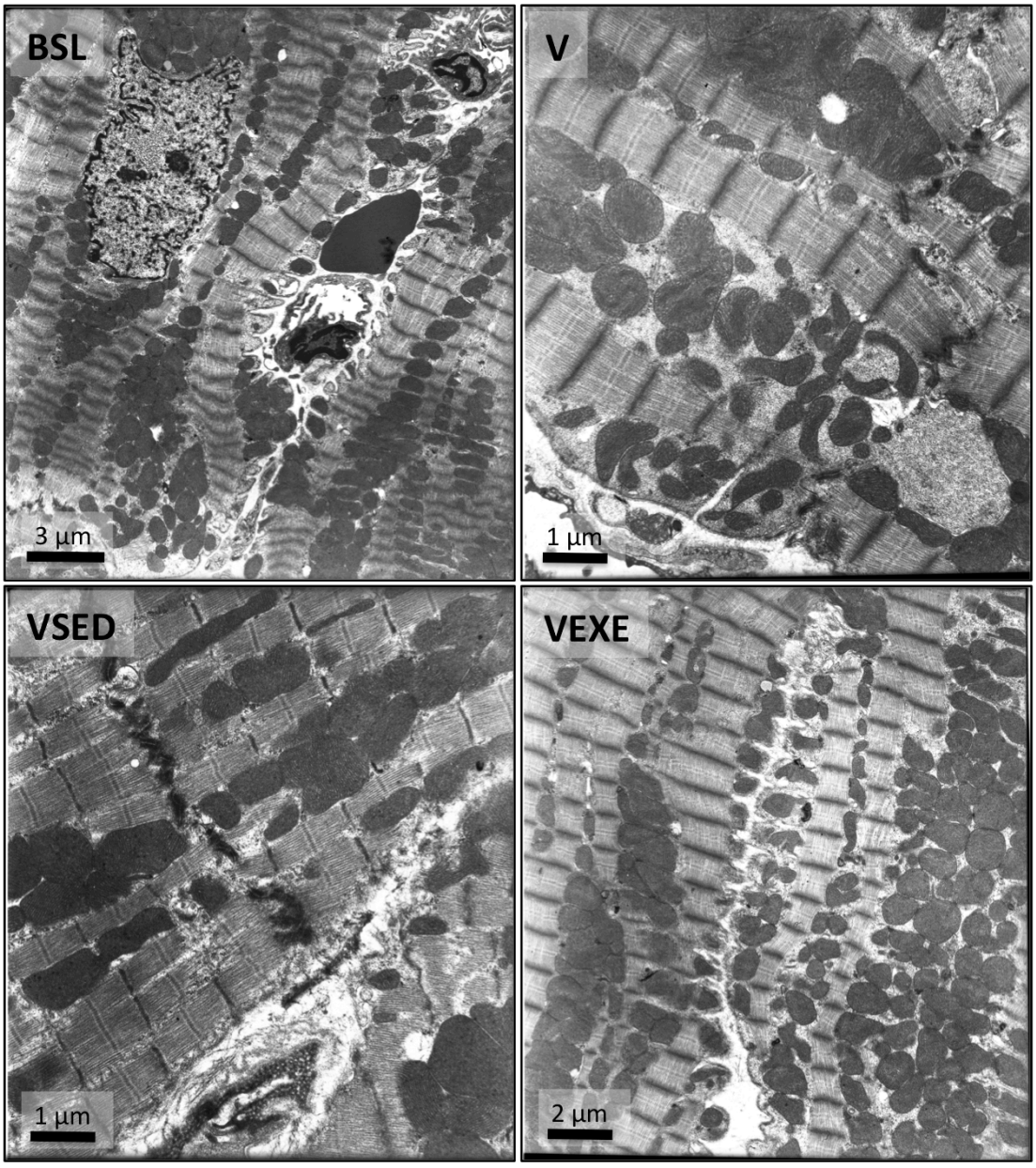


Figure 18. Electron micrographs representative from animals of groups baseline (BSL), vehicle (V), vehicle + sedentary (VSED) and vehicle + exercise (VEXE). All micrographs depict a normal ultrastructure with mitochondria showing a normal shape and well-defined lamellar cristae.

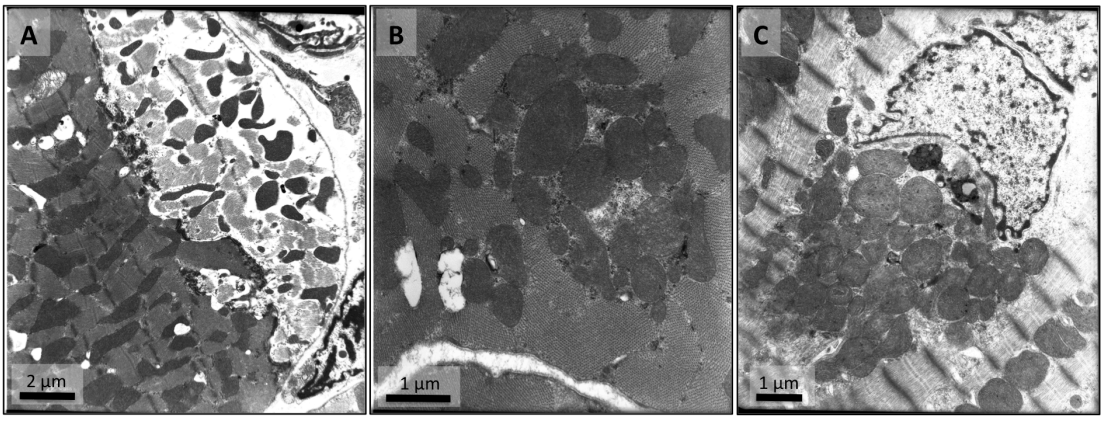


Figure 19. Electron micrographs representative from animals of group doxorubicin (DOX). In **A** it is observed a cardiomyocyte with cytoplasmic edema, traduced by an enlarged interspace between myofibrils, flanking a cardiomyocyte with mitochondria with less defined cristae and punctual mitochondrial swelling; **B** depict some mitochondrial swelling and heterogeneity of mitochondria dimensions with loss of well-defined cristae; in **C** it is possible to observe the different mitochondria dimensions and the presence of secondary lysosomes nearby.

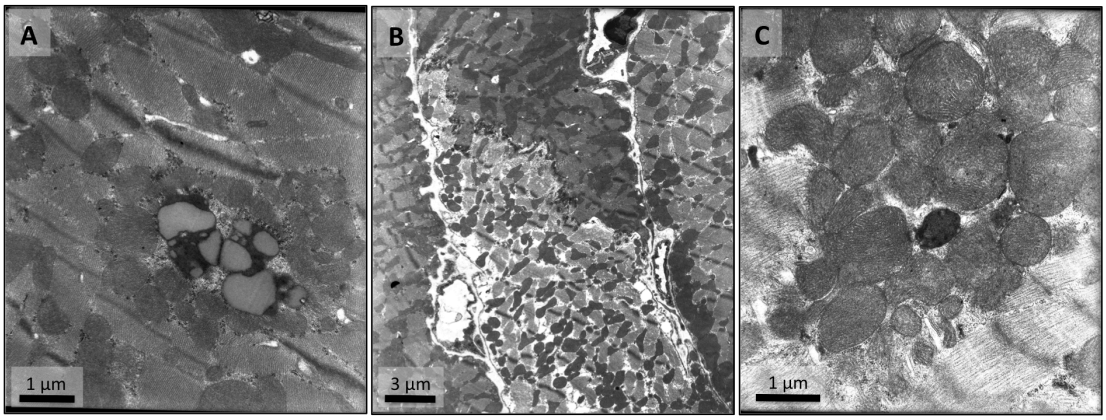


Figure 20. Electron micrographs representative from animals of group doxorubicin + sedentary (DSED). In **A** and **C** it is observed a cardiomyocyte with secondary lysosomes surrounded by mitochondria with a great heterogeneity in their dimensions, and with undefined cristae. **B** depicts a degenerated cardiomyocyte with signals of cytoplasmic edema.

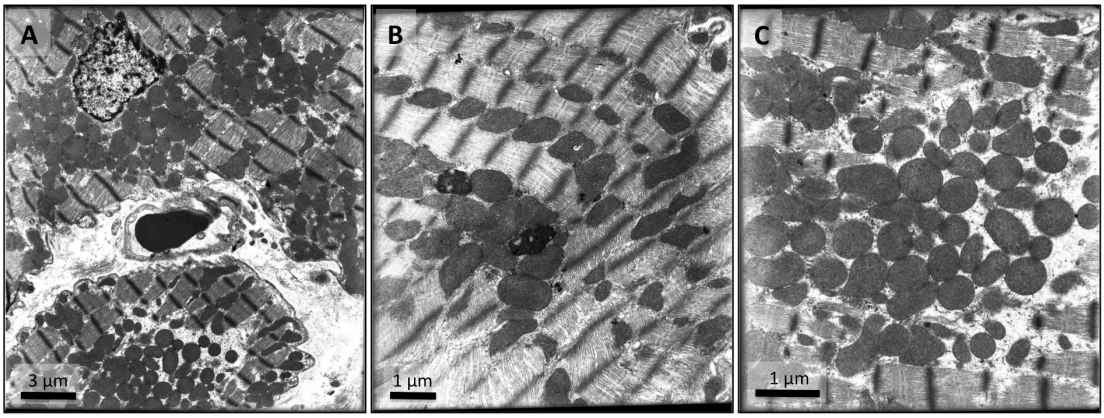


Figure 21. Electron micrographs representative from animals of group doxorubicin + exercise (DEXE). **A**, **B**, and **C** depict cardiomyocytes with well-defined cristae and apparently with an enhanced mitochondrial density; these organelles are also more homogeneous in their dimensions. In **B** it is observed secondary lysosomes interspacing apparently normal mitochondria.



Discussion

The main findings of the present document support the hypothesis that sedentary behavior after doxorubicin treatment is prejudicial to cardiac muscle metabolism and morphology. Additionally, cardiac muscle impairment is associated with damaged heart mitochondria, more notorious in doxorubicin sedentary animals. Moreover, there are biochemical and morphological signs that the mitochondria pool is renewed by exercise in animals under doxorubicin. A general discussion of these findings and of the experimental methodology is in the following chapters.

I. Methodology discussion

One of the crucial steps of this work was to induce a chronic and sub-lethal cardiotoxicity in the animals similarly to the observed in survived subjects with a history of cancer chemotherapy (Armstrong et al., 2010; Landier et al., 2004; Lipshultz et al., 2012). The chosen model was used by many other researchers in the past, always showing positive results to induce toxicity in the mice heart (Arola et al., 2000; A Ascensao et al., 2011; A Ascensao et al., 2005; Ascensao et al., 2012; A. J. Chicco et al., 2005; A J Chicco et al., 2005, 2006; D S Hydock et al., 2012; D S Hydock, C Y Lien, et al., 2011; D S Hydock et al., 2008; D S Hydock, T L Parry, et al., 2011; Hydock et al., 2009) and also in other organs such as brain (Mohamed et al., 2011), liver (R A Martins et al., 2012), kidneys (D Lebrecht, 2004), and lungs (Machado et al., 2010). Doxorubicin has also been used to test the cardioprotective effect of physical activity protocol before an acute dose of doxorubicin (Ascensao, Ferreira, et al., 2006; A Ascensao et al., 2011; A Ascensao et al., 2005; A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005; Ashrafi & Roshan, 2012; A J Chicco et al., 2005, 2006; D S Hydock et al., 2008; Kavazis et al., 2010; Smuder et al., 2013; Wonders et al., 2009) or during the doxorubicin administration (A. J. Chicco et al., 2005; R Hayward et al., 2012; D S Hydock, C Y Lien, et al., 2011). In the present work, however, the doxorubicin administration was used not to verify the *a priori* protective effect of exercise, but to understand the adverse effect of exacerbated sedentariness

after its administration. It was used a protocol to mimic an anti-neoplastic treatment, with chronic administration of low doses of doxorubicin. With the chosen experimental longitudinal design, it was also possible to differentiate doxorubicin acute from chronic effects on the heart. With this protocol, animals under doxorubicin treatment had signs of damage, being the doxorubicin-induced cardiotoxicity successfully achieved.

The morphometric technique to assess cardiac muscle damage was used already in the past as a valid technique to quantify the cardiac damage degree (Dinis-Oliveira et al., 2007; Yagmurca et al., 2003). Parameters measured using optical microscopy H&E staining such as cellular degeneration, inflammation, necrosis or loss of tissue organization, are the most frequent toxicological manifestations expected from doxorubicin cardiotoxicity (Saad et al., 2001; Santos et al., 2002). The analysis of collagen deposition using Sirius Red is also a valid technique and was already used for the same purpose by other authors (Richard et al., 2011).

The observed morphological changes were paralleled with some biochemical changes, also meaning that our experimental model has worked. Always bearing in mind the morphological data, we chose some specific biochemical analysis to confirm or to explain those morphological results. To assess the degree of oxidative stress we analyzed the activity and the expression of some enzymes that promote the equilibrium of reactive species inside the cell. We analyzed the activity and the expression of GR (Ascensao, Ferreira, et al., 2006; A Ascensao et al., 2005; A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005), that transforms the GSSG in its reduced form (GSH), and GPX (Ascensao, Ferreira, et al., 2006; A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005; A. J. Chicco et al., 2005; Childs et al., 2002) that reduces the GSH again to GSSG, transforming hydrogen peroxide in water. The importance of those two enzymes is to maintain the reduced environment inside the cell and to wipe out the increased hydrogen peroxide, a reactive oxygen specie that originates a cascade of many other reactive oxygen species. Thus, in addition we also have analyzed the level of GSH, GSSG and TGSH (A Ascensao et al.,

2005). Another important antioxidant enzyme expression and activity analyzed was superoxide dismutase (Ascensao, Ferreira, et al., 2006; A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005; A J Chicco et al., 2005). This enzyme catalyzes the dismutation of the superoxide anion into water and hydrogen peroxide. The superoxide anion is a high reactive oxygen specie that needs to be neutralized quickly before arming the cell (Fridovich, 1997). Some other proteins are normally high expressed during a stress situation, as the heat shock proteins and, consequently, the expression levels of HSP60 and HSP70 were assessed (Ascensao, Ferreira, et al., 2006; A Ascensao et al., 2005; A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005). To check the approximate quantity of undamaged mitochondria we measured the expression levels of Citochrome Oxidize subunit IV, because being coded by nuclear DNA it is not so affected by stress as the mitochondrial DNA (D Lebrecht, 2004; D Lebrecht et al., 2005). We also measured the levels of Citrate Sintase, also a marker of intact mitochondria, as it is a crucial protein in the Krebs cycle (Ascensao, Ferreira, et al., 2006; A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005; Figueiredo, Ferreira, et al., 2008; Figueiredo, Powers, Ferreira, Amado, et al., 2009; D Lebrecht et al., 2005). Another important process that should be analyzed in stress situations is apoptosis, which was indirectly checked by the expression levels of a pro-apoptotic protein, Bax (A Ascensao et al., 2011; A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005; Childs et al., 2002). It was also important to understand the mitochondrial turnover and thus it was analyzed the expression of Apg12, an important protein for autophagy process, forming the Apg12-Apg5 complex that initiates a cascade of events that culminate in the autophagosome formation (Mizushima et al., 2003).

These biochemical analyses were made in the cardiac homogenate to which contribute normal cells, damaged and necrotic cells, once it is known that cells are not equally susceptible to doxorubicin damage. So, results from biochemical analysis should be analyzed with caution and always together with the morphological data because the extent of changes and damage may be underestimated.

In terms of laboratory work, the process of mitochondrial isolation was particularly critical. During the process the sample undergo delicate steps that may corrupt the organelle such as differential centrifugation, where the mitochondria are under a huge mechanical stress. For this reason, although being a frequent technique used to assess the mitochondrial function (Ascensao, Ferreira, et al., 2006; A Ascensao et al., 2011; A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005; Ascensao, Magalhaes, et al., 2006; Figueiredo, Ferreira, et al., 2008; Figueiredo, Powers, Ferreira, Amado, et al., 2009; Figueiredo, Powers, Ferreira, Appell, et al., 2009; Judge, Jang, Smith, Hagen, et al., 2005; Judge, Jang, Smith, Selman, et al., 2005), one should not exclude the possibility that this might be a selective process, compromising the final results. In fact, this isolation process might select the solider and more functional mitochondria, which may bias the obtained results. We may infer that results from mitochondrial respiratory rates could be away from reality. Indeed, samples from animals under doxorubicin treatment might have more susceptible or even damaged mitochondria that may be totally destroyed during the isolation process and will be not present in the final pellet. Thus the final pellet of mitochondria might not be representative of the real pool of mitochondria in the *in vivo* cardiomyocytes, influencing the mitochondrial respiratory rates measured.

Concerning the exercise protocol, animals belonging to VEXE and DEXE had free access to free wheel after doxorubicin-administration during two months. This pattern of activity of exercised animals that were under voluntary exercise tried to mimic the motor pattern experienced by animals in the wildlife (Figueiredo, Powers, Ferreira, Amado, et al., 2009). In contrast, sedentary animals were confined to their cages representing an abnormal pattern of activity, trying to simulate a sedentary behavior (Figueiredo, Powers, Ferreira, Amado, et al., 2009). These considerations may change the traditional concept of experimental and control groups, as animals under exercise resemble the normal pattern of activity and sedentary animals are under exacerbated sedentariness. Bearing this in mind we considered that exercised animals were

the real control for the sedentary animals (groups VSED and DSED), considering that these animals show the effect of sedentariness, rather than considering that exercised animals showed the effect of physical training. This kind of exercise/sedentariness protocol was already used in the past in a study performed in our laboratory to study the aging process (Figueiredo, Powers, Ferreira, Amado, et al., 2009) and it does not seem to cause any extra stress to animals.

Another concern in our work was the small number of animals in each group. This detail raises the concern about the interindividual variability with a lower statistical power. Nevertheless, based on data from the literature (Ascensao, Ferreira, et al., 2006; A Ascensao et al., 2005; Figueiredo, Ferreira, et al., 2008; Figueiredo, Powers, Ferreira, Amado, et al., 2009), an *a priori* statistical power calculation considering a large effect size of doxorubicin (d=0.8) and a medium effect size of sedentariness (d=0.5), the minimum sample size required for a statistical power of 0.95 was 32 animals. Consequently, the sample size used in our study was adequate to safe extrapolate the obtained results. Still, one consequence of the small number of individuals in each group was the lack of normality in the distribution of some variables and the high standard deviation of the means, as it may be seen in the results presented. Bearing this in mind it was used non-parametric tests and results were presented using median and interquartil range for several variables. Protein expression and collagen deposition percentage were presented as mean and standard deviation of the mean. Although the abnormal distribution of data, mean and SD were also presented in parallel with the median and interquartil range for all other variables, so information would be more complete and easier to understand.

II. Effects of age and sedentariness

As expected body weight and heart weight increased with age.

The morphological analysis of optical microscopy or collagen deposition did not show differences between animals from BSL, V and VSED animals, either

optical microscopy or TEM analysis, meaning that there isn't an increase in damaged tissue or damaged mitochondria until the adult life in mice. The age difference was perhaps to small to be seen any change in the morphological analysis, unlike with previous work where the age difference was big enough to induce changes (Figueiredo, Ferreira, et al., 2008). Probably with older animals the age effect would be seen. However, some biochemical analyses showed a different result, as for some variables there were found changes with age. The antioxidant system seems to act differently with time because some enzymes are changed in V animals and others are only altered latter in time in VSED. GR activity was increased in animals V, and decreased in VSED to values similar to those of BSL animals. This pattern was also seen in the GR expression, once it was increased in V animals, and highly decreased in VSED, when compared to BSL animals. GPX activity, the other crucial enzyme in the reaction of oxidation-reduction of the GSSG-GSH complex, increased with time when comparing BSL animals to V animals, and maintains high with time in VSED. This increased activity is in concordance with the increased expression of this enzyme. Due to this unbalance between GPX and GR in VSED animals, there is an accumulation of GSH in VSED animals, suggesting that oxidative stress may be increasing with time. SOD and MitSOD showed different patterns of evolution with time. Total SOD activity increased latter in time (in group VSED), indicating an enhanced oxidative environment in older animals. Although activity of this enzyme is only increased in VSED animals, its expression is already highly increased in V animals and maintained stable with time, in VSED animals, possibly because cardiomyocytes are living over an enhanced oxidative environment. MitSOD activity decreased drastically when comparing BSL with V animals, and increased in VSED to values similar to those of BSL probably because inside mitochondria the oxidative environment has a higher degree in young (BSL) and older (VSED) animals, more than in juvenile (group V). The increased CS activity with age is in accordance with previous research (Figueiredo, Ferreira, et al., 2008), where they found the same pattern in the skeletal muscle that we found in cardiac muscle. MitCS decreases in V animals

and than increases again in VSED animals to values similar to BSL group. Because MitCS decreased in V animals we may infer that the number of normal mitochondria may be slightly lower or that Krebs cycle is not occurring properly. Nonetheless it is important to refer that the morphological data does not confirm this hypothesis of higher number of damaged mitochondria in V animals. COXIV expression levels confirm the morphological data, as it is a marker for normal mitochondria, with an increased in V animals and even more increased in VSED animals. Expression of heat shock proteins HSP60 and HSP70 were decreased with time, meaning that cellular stress might be higher in younger animals (BSL). All these results may be analyzed along with the analysis of the expression of Bax. This protein is increased in V animals, but it drastically increased in VSED animals, meaning that the occurrence of apoptosis is probably more prominent in VSED animals. Thus, if more cells undergo apoptosis, damage cells are eliminated and there exist higher percentage of normal cells. This may explain why some antioxidant enzymes and proteins have higher levels in V animals than in VSED animals, because the damaged cells are being selectively removed.

Considering the mitochondria functionality, it may be seen a decline in the State 3 either for complex I and complex II-linked substrates rated to CS content in VSED but not in V animals. This decline was more prominent in complex I-linked substrates. State 4 for complex I-linked substrates increased with age and for complex II-linked substrates state 4 increased in V animals, but decreased in VSED animals to values similar to those observed in BSL animals. Thus, RCR for complex I-linked substrates increased in V animals and highly decreased in VSED animals, while RCR for complex II-linked substrates increased with age. The alterations in RCR values seem to be mainly affected by changes in State 3 results than from alterations in State 4. The decline with age for complex I-linked substrates either in State 3 and RCR is supported by previous works (Figueiredo, Ferreira, et al., 2008), and supports the idea that the mitochondrial function and the maximal rate of oxygen consumption are compromised with age. Complex II-linked substrates does not appear to be as

affected as complex I-linked substrate perhaps because complex II is totally encoded by nuDNA whereas complex I as subunits encoded by mitDNA, which is more affected by the aging process than is nuDNA (Mandavilli et al., 2002). The mitochondrial turnover, indirectly accessed by the AP12 expression, may be slightly lower in VSED animals, suggesting that there might be more damaged mitochondria explaining the impairment in State 3 for both complexes in VSED animals.

In our work we also tried to understand how sedentariness would affect cardiac muscle and cardiac mitochondria. The vast majority of variables analyzed were not affected or impaired by sedentariness in six months old rats. Nonetheless, it is important to refer that CS activity decreased in VEXE animals, GSSG content also increased and GSH decreased when compared with VSED animals. SOD and Bax expressions decreased in exercised animals (VEXE) when compared with sedentary animals (VSED). HSP70 highly increased in VEXE animals but once it presents high standard deviation, this result should be analyzed with caution. Nonetheless this results is in accordance with the literature (Gielen et al., 2010), demonstrating that voluntary physical activity improves the stress resistance. Results found for GSH, GSSG, SOD and Bax quantities would indicate less oxidative environment inside cardiac cells in exercised animals, meaning that sedentariness is favoring the oxidative stress inside the cell. As was referred elsewhere (Figueiredo, Ferreira, et al., 2008), measurement of CS activity is a better marker for the mitochondrial fraction in suspension, and our results indicate that VEXE animals had lower level of intact mitochondria. These differences between both groups were not found in morphological data because were not seen any changes in VEXE cardiac cells morphology or collagen deposition when compared with VSED animals.

The results for CS activity for VEXE animals would let us expect impairment in the mitochondrial functionality, accessed by mitochondria respiration, but results for complex I were in the opposite direction. The functional analysis of cardiac muscle mitochondria showed higher levels of State 3 complex I-linked substrates for VEXE animals comparing to VSED animals, meaning that the

maximal rate of mitochondria oxygen consumption was impaired by sedentariness. This is in accordance with previous research in skeletal muscle, where State 3 was also impaired by exacerbated sedentariness (Figueiredo, Powers, Ferreira, Amado, et al., 2009). State 4 for the same complex was also increased in VEXE animals. RCR for complex I-linked substrate was not different in both groups. State 3, State 4 or RCR for complex II-linked substrates were not altered in VEXE animals, meaning that this complex is not affected by sedentariness. Figueiredo and colleagues (Figueiredo, Powers, Ferreira, Amado, et al., 2009) in their work in mice skeletal muscle found a connection between sedentary behavior and impairment in complex II-linked substrates analysis. We failed to demonstrate that, but this may be explained either because cardiac muscle mitochondria might react differently to the sedentary behavior or, and more probable reason, because Figueiredo et. al. (Figueiredo, Powers, Ferreira, Amado, et al., 2009) studied 18 months old active and inactive mice while we studied 6 months old active and inactive mice.

III. Acute and late effects of Doxorubicin

One of the crucial steps of our work was to understand the effects of the cardiotoxic drug doxorubicin on Wistar rats, right after the drug administration and late in time. To do that we had two groups under doxorubicin treatment, DOX and DSED, which were sacrificed with two months of interval, being the results compared with two control groups with the same two months of interval in their sacrifice, V and VSED.

Animals under doxorubicin (from DOX and DSED groups) showed signs of toxicity in the cardiac muscle with the involvement of mitochondria. Data from optical and electron microscopy from DOX and DSED animals, when compared with data from V and VSED, showed immediate and late morphological alterations in the cardiac muscle with a great heterogeneity of mitochondrial shape and sizes as a response to the stress provoked by doxorubicin. Beyond this qualitative analysis, the quantitative morphometric exploration clearly

showed that animals from DOX and DSED groups had dramatically high cardiac muscle damage. Parameters measured using optical microscopy under H&E staining, such as cellular degeneration, inflammation, necrosis or loss of tissue organization were all increased in these two groups when compared to vehicle animals, thus cardiac muscle in doxorubicin animals showed signs of lesion and impairment. Similar results of cytoplasmic vacuolization and morphological impairment of cardiac muscle were found in previous research (Trivedi et al., 2011; Yi et al., 2006; Zhou et al., 2001). Animals under doxorubicin treatment (DOX and DSED) showed clear advanced cardiac muscle damage as we may see for the results on table 5 and in the images 12 and 13 of H&E staining and large collagen deposition as shown in figures 17 and 18, meaning tissue reparative process. As it was expected, the results of these two groups show that doxorubicin had a deleterious effect on cardiac muscle that lasted long in time. Our results indicate the presence of fibrotic tissue and damaged cells in inflammatory and healing process. The inflammatory process triggered by the stress stimulus involves a series of biochemical responses inside the cell in order to heal or ultimately to eliminate the damaged cells (Frangogiannis et al., 2002). One of the first changes in cells in response to a harming stimulus is the increase in reactive oxygen species in the cell environment. These molecules may act as signaling molecules, indicating a deleterious situation and thus triggering the inflammatory process trough the activation of the cytokines cascade (Finkel, 2011; Frangogiannis et al., 2002; Lefer & Granger, 2000), but as they are very reactive species and tend to react with some very important biomolecules as DNA, proteins or lipids, need to be neutralized very quickly (Finkel, 2001, 2011). The antioxidant system is a complex group of enzymes acting inside the cell to maintain the redox equilibrium and under stress conditions cells activate a defense system either to maintain the cell environment or to self destroy when repair is no longer available (Hensley et al., 2000).

Our data shows that antioxidant enzymes react differently to the stress provoked by doxorubicin. GPX seems to react firstly to the stress as its activity

increased in DOX animals when compared to V animals, returning back with time to the normal values, as we may see from the results of DSED group that were very similar to vehicle animals. The expression of this protein in doxorubicin animals was similar to their controls and thus doxorubicin does not alter the expression pattern of this protein. GR does not follow the same pattern as its activity is highly increased in DSED animals and decreased in DOX animals. In parallel with these results, the expression of this protein increased in DSED animals, while in DOX it was similar to V animals. It appears that GR is an antioxidant defense that tends to decrease right after the deleterious stimulus and increases if the stress last long in time, if we compare results from DOX and DSED.

SOD activity increased with time in doxorubicin animals, but not as accentuated as was for vehicle animals (if we compare the activities for V and VSED animals). In older doxorubicin animals, the SOD activity was lower although its expression was impressively high. We may infer that cell synthesizes more SOD due to high levels of superoxide caused by doxorubicin presence, however the enzyme activation mechanisms seem to be impaired. Differences in expression and activity of same enzymes may be explained by the oxidative damage, confirmed by the morphological data that showed muscle alterations with necrotic zones and high collagen deposition.

Other kind of important proteins when cell is under a stress situation are heat shock proteins that have the tendency to increase. We measured HSP60 and HSP70 expressions that increased differently with doxorubicin administration. We may infer that HSP60 is a first aid, because it increased in DOX animals, and HSP70 only appears when stress perpetuates over time, as it increased in DSED animals.

When the healing process fails to repair the damage caused by doxorubicin, apoptosis of damaged cells is the last process in order to maintain the heart a functional organ. Bax results showed a slight increase in DOX animals compared with V animals, although results were not significant. Nevertheless, Bax expression in DSED decreased significantly, suggesting a less favorable

environment for apoptosis. These findings are in accordance with Arola and colleagues (Arola et al., 2000) which found after single dose of doxorubicin, that apoptosis was induced right after administration, returning back to baseline levels.

Qualitative analyses of the ultrastructure of cardiac muscle using TEM also showed differences between doxorubicin animals (DOX and DSED) and vehicle animals. While vehicle animals showed normal ultrastructure of the cells and mitochondria, animals under doxorubicin had structural changes as abnormal mitochondria with irregular shapes and sizes, containing undefined and apparently lamellar cristae. It is widely accepted that mitochondria are highly affected by doxorubicin, being the cardiotoxicity mostly linked with damaged mitochondria and alterations in mtDNA (D Lebrecht & U A Walker, 2007). Grids from DOX and DSED animals presented a very heterogeneous pool of mitochondria. Signs of edema and necrosis were also clear in DOX and DSED images, which are in concordance with the literature (Oliveira et al., 2004; Zhou et al., 2001). Doxorubicin-induced cardiotoxicity was clear in animals under doxorubicin, as acutely (DOX group) as lasted long in time (DSED). Beyond the altered morphology of mitochondria with undefined cristae in DOX and DSED groups, it must be highlighted the great amount of secondary lysosomes, suggesting an enhanced autophagy in an attempt to remove the damaged mitochondria present in this tissue. In fact, the levels of APG12 protein were highly increased in DSED animals, meaning that cells were acting to promote mitochondrial autophagy in order to remove damaged mitochondria. It is important to notice that the increased expression of this protein was only seen late in time, meaning that the mitochondrial turn-over tends to occur late in time.

The functional analysis of cardiac mitochondria of doxorubicin animals showed that complex I-linked substrates State 3 had the opposite evolution when compared to vehicle animals. While the State 3, rated to CS, increased in V animals and decreased in VSED, in the DOX group the values were as low as for BSL animals, increasing highly in DSED group. For state 4, rated to CS, when comparing DOX with V and DSED with VSED, the values were similar,

meaning that doxorubicin had no effect on complex I-linked substrate state 4, and was only felt the effect of age, meaning that state 4 increased with age. Although differences between DOX and DSED were only seen in State 3, RCR values were equal between these two groups, both lower to those of V animals and similar to those of VSED animals. For complex II-linked substrate, only State 3 was impaired in DOX animals when compared to V animals. State 4 for DOX animals was similar to V animals and thus RCR for DOX animals decreased when compared with V animals' values. Our results indicate that the administration of seven low doses of doxorubicin did not impaired mitochondrial respiratory function as it does a single high dose (A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005).

As already discussed, the mitochondrial isolation process might have been imperfect, what might have bias the results for mitochondrial respiratory capacity, a possible reason for the strange results found for RCR of complex-I and complex-II linked substrates. This hypothesis is supported by the electron microscopy results where it may be seen heterogeneity among the mitochondria in the cardiac muscle. We have also to consider the interindividual differences as a contributing factor to explain the unexpected results, as evidenced by the high standard deviations of the mean.

IV. Sedentary behavior perpetuates doxorubicin-induced cardiotoxicity

The main goal of the present study was to understand the effect of sedentariness on the cardiac muscle and especially on heart mitochondria of animals under stress provoked by doxorubicin. In order to do that we performed several experiments to assess the morphology and the biochemical changes on cells and mitochondria provoked by doxorubicin in sedentary animals and in active animals.

The histological results revealing cardiac muscle damage seen in DOX animals,

which stayed long in time in DSED group, were almost absent in DEXE animals that showed almost a normal morphology. The results for collagen deposition were also in accordance with the morphological data. The percentage of collagen deposition was lower in DEXE animals when compared with DOX and DSED animals, meaning that fibrosis was almost gone and tissues were almost repaired. The results from these three groups indicate that sedentariness maintains heart muscle damage and favors the collagen deposition in time, while voluntary physical activity helped to restore the cardiac muscle morphology to its normal morphology, as we may see when comparing the results for DSED and DEXE groups. When comparing DOX and DSED with DEXE we may infer that damage was present after doxorubicin administration and lasted long in time, with the post-doxorubicin physical activity favoring the regeneration of the tissue. Although with time it may be seen some regenerative changes in the animals under doxorubicin, sedentariness perpetuates the damage caused by doxorubicin in cardiac muscle, while voluntary activity increases highly the regeneration in the cardiac muscle, as it may be inferred also by the results of the collagen deposition in DEXE animals.

The pattern seen in the morphological data is also observed for some biochemical results. Voluntary physical activity appears to diminish the stress caused by doxorubicin once HSP70 expression is only increased in DSED animals and not in DEXE group, which expressed values close to DOX animals. As these heat shock proteins intervenes in the proteins' folding process, lower values of HSP60 and HSP70, in DSED and in DEXE groups respectively, maybe the reason why inactivated SOD accumulates in the cell but its activity is reduced in these two groups. Other reason to explain these results is the great heterogeneity of cardiomyocytes that can be seen in the optical microscopy. This heterogeneity may influence the biochemical results, as the expression and the activities of some enzymes, once these variables were assessed in the homogenized heart, with the mixture of damaged and healthy cells.

Bax decreased in DSED and DEXE groups but increased in DOX animals. It seems that apoptosis appears firstly to destroy the damaged cells preventing

the stress to perpetuate, however with time this programmed cell death response tends to decrease. As discussed earlier in this work, apoptosis seems to be more active right after the stress stimulus, and with time it tends to decrease. Ascensão (A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005) studied the cardiac protective effect of exercise against post-doxorubicin stress and found that trained animals were less prone to apoptosis, but our results may indicate that time is the main effect to diminish Bax expression and thus apoptosis, rather than exercise.

When analyzing the ultrastructure of the cardiac muscle and the mitochondria pool, we see that for DEXE group, when compared to DOX and DSED, even though damage mitochondria were still present, it can be detected a regenerative pattern because the changes were less pronounced. Exercise seems to activate the regeneration of the mitochondria pool, has we may infer for the absence of heterogeneity in the mitochondria and their normal shape, seen in figure 21. Nonetheless this reparative pattern, it is clear the heterogeneous mixture of different mitochondria, were it may be seen healthy organelles side to side with damage ones, with various shapes and sizes, what affected and biased the results of the mitochondrial functionality.

The renewal of the mitochondrial pool promoted by exercise is probably due to the increase in autophagy of the damaged mitochondria. Results from the expression of APG12 protein support this idea. Animals from the group DEXE had values of expression of this protein incredibly high when compared with all other groups, meaning that autophagy was very active in these animals. Group DSED had also increased expression of this protein but not as impressive as for DEXE, meaning that exercise probably accelerated autophagy process by increasing the expression of APG12. This result is very interesting because may be the answer why the mitochondrial pool in the TEM analysis for DEXE showed less damaged mitochondria than for DOX and DSED groups. Exercise could also have improved the pool of mitochondria by increasing autophagy and thus mitochondria that divide and create new ones are only the healthy, altering the quality of the mitochondria when compared to other two-doxorubicin groups.

Functional analysis of cardiac muscle mitochondria for complex I and complex II showed no differences between DEXE and VEXE, except for State 4 rated to CS for both complexes, meaning that doxorubicin did not impaired the mitochondrial respiratory function. Comparing DEXE to DSED results, for complex I-linked substrates there are no differences between both groups. In complex II-linked substrates results only for State 4 rated to CS there exist differences. In this case sedentariness did not seem to affect negatively this parameter, as no differences were seen. It is known that exercise has a protective effect if it is applied before the drug administration in mitochondrial respiratory function (A. Ascensao, J. Magalhaes, J. M. Soares, et al., 2005), but we failed to achieve the same results. We may infer that the mitochondrial DNA damage caused by doxorubicin was irreparable and although values for APG12 were incredible high for DEXE animals, autophagy process is still insufficient to alter the balance between damage and undamaged mitochondria. Exercise before doxorubicin increases cell defenses while exercise after the stress stimulus will help in the renewal of the mitochondrial pool, but latter in time.

Important to refer that doxorubicin did not impaired the physical fitness of the animals, as we may see by the results of the distance run by vehicle and doxorubicin animals with access to the free wheel during the two months of the protocol. Although it would be expected that the stress caused by the administration of doxorubicin would affect the physical capacity of animals that would chose not running, that was not seen in our data. In fact, voluntary exercised animals act as the real control for sedentary behavior. The physical activity pattern of rats with access to the free wheel would mimic their activity in the nature, thus animals that were confined to the rodent cage are forced to be inactive. We may infer that the sedentary behavior is a prejudicial behavior because it perpetuates the effects caused by doxorubicin in the cardiac muscle.

Transferring this results to cancer survivors, it should be advised an active life rather than a sedentary life as it was thought before (Fong et al., 2012). While in the past cancer survivors were advised to stay inactive after the treatment because it was thought that the cancer treatments were so strong that would let

them very weak, nowadays this seems to be a wrong idea, and physical activity prescribed by exercise professionals and under supervision is recommended for healthy.



Conclusions and Final Considerations

Based on the analysis of our data it is possible to conclude that doxorubicin chronically administered at low doses, in order to mimic the cancer treatment, causes cardiac muscle damage at the morphological level, altering the structure of the cells and also at biochemical level, altering the activity and expression of some antioxidant enzymes that will lead to further toxicity. We may also conclude that cardiotoxicity induced by doxorubicin is associated with alterations in the mitochondria pool.

Our results also allow stating that sedentariness is prejudicial for the doxorubicin-induced heart toxicity, as it perpetuates the harmful effects provoked by doxorubicin. Our data also showed that physically active doxorubicin rats had an improved cardiomyocytes mitochondrial turnover, altering the mitochondrial pool from damaged mitochondria to almost a normal pool of mitochondria. We may infer that continuous and monitored exercise throughout life is necessary to keep the normal mitochondrial pool and to improve cardiac muscle morphology and biochemistry.

The morphological data of cardiac muscle showed an heterogeneity of cardiomyocytes inside the muscle, supporting the concept that cells are not equally susceptible and affected by doxorubicin, with apparently healthy cells coexisting with damaged ones. The ultrastructural analysis also revealed differently affected mitochondria, possible due to the presence of heteroplasmic mitDNA inside the damaged cells. As it is known, mitDNA is highly susceptible to doxorubicin effect, but doxorubicin does not affect every molecule and every organelle in the same way, creating a heterogeneous pool of mitochondria in the cardiac muscle.

For future research it would be interesting to study deeper the mitochondria, examining proteins encoded by mitochondrial DNA to understand how doxorubicin affects mitDNA in contrast to nuclear DNA proteins, as nuclear DNA is not so affected as mitDNA by doxorubicin.

To understand better the biochemical processes behind the morphological changes it would be interesting to study the mitochondrial DNA dipper to find out how mitochondrial heteroplasmy affects the functionality of the cells.

Beyond studying mitochondrial proteins coded by mitochondrial DNA as COXI in comparison with mitochondrial proteins coded by nuclear DNA as COXIV, it would be interesting to perform mitochondrial DNA sequencing to better understand where and how mitochondrial DNA changes occur.

It would also be of high importance to understand how aerobic exercise training performed in treadmill affects the cardiac muscle and the mitochondrial pool of animals that underwent doxorubicin treatment.



References

Adachi, K., Fujiura, Y., Mayumi, F., Nozuhara, A., Sugiu, Y., Sakanashi, T., Hidaka, T., & Toshima, H. (1993). A deletion of mitochondrial DNA in murine doxorubicin-induced cardiotoxicity. *Biochem Biophys Res Commun, 195*(2), 945-951.

American Cancer Society. (November 2014). 2014, disponível em http://www.cancer.org/cancer/cancerbasics/what-is-cancer

Anderson, M. E. (1985). Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol, 113*, 548-555.

Arcamone, F., Cassinelli, G., Fantini, G., Grein, A., Orezzi, P., & Spalla, C. (1969). Adriamycin, 14-hydroxydaunomycin, a mew antitumor antibiotic from S. peucetius var. caesius. *Biotechnology and Bioengineering, 11*, 1101-1110.

Armenian, S. H., & Robison, L. L. (2013). Childhood cancer survivorship: an update on evolving paradigms for understanding pathogenesis and screening for therapy-related late effects. *Curr Opin Pediatr, 25*(1), 16-22.

Armstrong, G. T., Liu, Q., Yasui, Y., Neglia, J. P., Leisenring, W., Robison, L. L., & Mertens, A. C. (2009). Late mortality among 5-year survivors of childhood cancer: a summary from the Childhood Cancer Survivor Study. *J Clin Oncol, 27*(14), 2328-2338.

Armstrong, G. T., Stovall, M., & Robison, L. L. (2010). Long-term effects of radiation exposure among adult survivors of childhood cancer: results from the childhood cancer survivor study. *Radiat Res, 174*(6), 840-850.

Arola, O. J., Saraste, A., & Pulkki, K. (2000). Acute doxorubicin cardiotoxicity involves cardiomyocyte apoptosis. *Cancer Res, 60*, 1789-1792.

Ascensao, A., Ferreira, R., & Magalhaes, J. (2007). Exercise-induced cardioprotection--biochemical, morphological and functional evidence in whole tissue and isolated mitochondria. *Int J Cardiol, 117*, 16-30.

Ascensao, A., Ferreira, R., Oliveira, P. J., & Magalhaes, J. (2006). Effects of endurance training and acute doxorubicin treatment on rat heart mitochondrial alterations in duced by in vitro Anoxia-Reoxygenation. *Cardiovasc Toxicol, 06*, 159-171.

Ascensao, A., Lumini-Oliveira, J., Machado, N. G., Ferreira, R. M., Goncalves, I. O., Moreira, A. C., Marques, F., Sardao, V. A., Oliveira, P. J., & Magalhaes, J. (2011). Acute exercise protects against calcium-induced cardiac mitochondrial permeability transition pore opening in doxorubicin-treated rats. *Clin Sci (Lond), 120*(1), 37-49.

Ascensao, A., Lumini-Oliveira, J., Machado, N. G., Ferreira, R. M., Goncalves, I. O., Moreira, A. C., Marques, F., Sardao, V. A., Oliveira, P. J., & Magalhaes, J. (2011). Acute exercise protects against calcium-induced cardiac mitochondrial permeability transition pore opening in doxorubicin-treated rats. *Clin Sci (Lond), 120*, 37-49.

Ascensao, A., Magalhaes, J., Soares, J., Ferreira, R., Neuparth, M., Marques, F., Oliveira, J., & Duarte, J. (2005). Endurance training attenuates doxorubicin-induced cardiac oxidative damage in mice. *Int J Cardiol, 100*, 451-460.

Ascensao, A., Magalhaes, J., Soares, J., Ferreira, R., Neuparth, M., Marques, F., Oliveira, J., & Duarte, J. (2005). Endurance training attenuates doxorubicin-induced cardiac oxidative damage in mice. *Int J Cardiol, 100*(3), 451-460.

Ascensao, A., Magalhaes, J., Soares, J. M., Ferreira, R., Neuparth, M., Marques, F., Oliveira, P. J., & Duarte, J. A. (2005). Moderate endurance training prevents doxorubicin-induced in vivo mitochondriopathy and reduces the development of cardiac apoptosis. *Am J Physiol Heart Circ Physiol, 289*, H722-H731.

Ascensao, A., Magalhaes, J., Soares, J. M., Ferreira, R., Neuparth, M. J., Marques, F., Oliveira, P. J., & Duarte, J. A. (2006). Endurance training limits the functional alterations of rat heart mitochondria submitted to in vitro anoxia-reoxygenation. *Int J Cardiol, 109*, 169-178.

Ascensao, A., Oliveira, P. J., & Magalhaes, J. (2012). Exercise as a beneficial adjunct therapy during Doxorubicin treatment--role of mitochondria in cardioprotection. *Int J Cardiol, 156*, 4-10.

Ashley, N., & Poulton, J. (2009). Mitochondrial DNA is a direct target of anti-cancer anthracycline drugs. *Biochem Biophys Res Commun, 378*(3), 450-455.

Ashrafi, J., & Roshan, V. D. (2012). Is Short-term Exercise a Therapeutic Tool for Improvement of Cardioprotection Against DOX-induced Cardiotoxicity? An Experimental Controlled Protocol in Rats. *Asian Pacific Journal of Cancer Prevention, 13*, 4025-4030.

Bagchi, D., Bagchi, M., Hassoun, E. A., Kelly, J., & Stohs, S. J. (1995). Adriamycin-induced hepatic and myocardial lipid peroxidation and DNA damage, and enhanced excretion of urinary lipid metabolites in rats. *Toxicology, 95*, 1-9.

Ballinger, S. W. (2005). Mitochondrial dysfunction in cardiovascular disease. *Free Radic Biol Med, 38*(10), 1278-1295.

Baumann, F. T., Bloch, W., & Beulertz, J. (2013). Clinical exercise interventions in pediatric oncology: a systematic review. *Pediatr Res, 74*(4), 366-374.

Bonadonna, G., Monfardini, S., Lena, M., & Fossati-Bellani, F. (1969). Clinical evaluation of adriamycin, a new antitumor ntibiotic. *British Medical Journal, 3*, 503-506.

Braam, K. I., van der Torre, P., Takken, T., Veening, M. A., van Dulmen-den Broeder, E., & Kaspers, G. J. (2013). Physical exercise training interventions for children and young adults during and after treatment for childhood cancer. *Cochrane Database Syst Rev, 4*, CD008796.

Carlberg, I., & Mannervik, B. (1985). Glutathione reductase. *Methods Enzymol, 113*, 484-490.

Chen, N., & Debnath, J. (2010). Autophagy and tumorigenesis. *FEBS Lett, 584*(7), 1427-1435.

Chicco, A. J., Hydock, D. S., Schneider, C. M., & Hayward, R. (2005). Low-intensity exercise trainning during doxorubicin treatment protects against cardiotoxicity. *J Appl Physiol, 100*, 519-527.

Chicco, A. J., Schneider, C. M., & Hayward, R. (2005). Voluntary exercise protects against acute doxorubicin cardiotoxicity in the isolated perfused rat heart. *Am J Physiol Regul Integr Comp Physiol, 289*, R424-R431.

Chicco, A. J., Schneider, C. M., & Hayward, R. (2006). Exercise training attenuates acute doxorubicin-induce cardiac dysfunction. *J Cardiovasc Pharmacol, 47*, 182-189.

Chicco, A. J., Schneider, C. M., & Hayward, R. (2006). Exercise training attenuates acute doxorubicin-induce cardiac dysfunction. *J Cardiovasc Pharmacol, 47*(2), 182-189.

Childs, A. C., Phaneuf, S., & Dirks, A. (2002). Doxorubicin treatment in Vivo causes cytochrome c release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. *Cancer Res, 62*, 4592-4598.

Chomyn, A., & Attardi, G. (2003). MtDNA mutations in aging and apoptosis. *Biochemical and Biophysical Research Communications, 304*, 519-529.

Coore, H. G., Denton, R. M., Martin, B. R., & Randle, P. J. (1971). Regulation of adipose tissue pyruvate dehydrogenase by insulin and other hormones. *Biochem J, 125*(1), 115-127.

De Caro, E., Smeraldi, A., Trocchio, G., Calevo, M., Hanau, G., & Pongiglione, G. (2011). Subclinical cardiac dysfunction and exercise performance in childhood cancer survivors. *Pediatr Blood Cancer, 56*(1), 122-126.

de Moor, J. S., Mariotto, A. B., Parry, C., Alfano, C. M., Padgett, L., Kent, E. E., Forsythe, L., Scoppa, S., Hachey, M., & Rowland, J. H. (2013). Cancer survivors in the United States: prevalence across the survivorship trajectory and implications for care. *Cancer Epidemiol Biomarkers Prev, 22*(4), 561-570.

de Vathaire, F., Francois, P., Hill, C., Schweisguth, O., Rodary, C., Sarrazin, D., Oberlin, O., Beurtheret, C., Dutreix, A., & Flamant, R. (1989). Role of radiotherapy and chemotherapy in the risk of second malignant neoplasms after cancer in childhood. *Br J Cancer, 59*(5), 792-796.

Debatin, K. M., & Krammer, P. H. (2004). Death receptors in chemotherapy and cancer. *Oncogene, 23*(16), 2950-2966.

Demark-Wahnefried, W., Rock, C. L., Patrick, K., & Byers, T. (2008). Lifestyle interventions to reduce cancer risk and improve outcomes. *Am Fam Physician, 77*(11), 1573-1578.

Diller, L., Chow, E. J., Gurney, J. G., Hudson, M. M., Kadin-Lottick, N. S., Kawashima, T. I., Leisenring, W. M., Meacham, L. R., Mertens, A. C., Mulrooney, D. A., Oeffinger, K. C., Packer, R. J., Robison, L. L., & Sklar, C. A. (2009). Chronic disease in the Childhood Cancer Survivor Study cohort: a review of published findings. *J Clin Oncol, 27*(14), 2339-2355.

Dinis-Oliveira, R. J., Sousa, C., Remiao, F., Duarte, J. A., Navarro, A. S., Bastos, M. L., & Carvalho, F. (2007). Full survival of paraquat-exposed rats after treatment with sodium salicylate. *Free Radic Biol Med, 42*(7), 1017-1028.

Ellison, G. M., Waring, C. D., Vicinanza, C., & Torella, D. (2012). Physiological cardiac remodelling in response to endurance exercise training: cellular and molecular mechanisms. *Heart, 98*(1), 5-10.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol, 35*(4), 495-516.

Estabrook, R. W. (1976). Mitochondrial respiratory control and the polarographic measurement of ADP: O ratios. *Methods in Enzymology, 10*, 41-47.

Ettinghausen, S., Bonow, R., Palmeri, S., Seipp, C., Steinberg, S., White, D., & Rosenberg, S. (1986). Prospective study of cardiomyopathy induced by

adjuvant doxorubicin therapy in patients with soft-tissue sarcomas. *Arch Surg, 121*, 1445-1451.

Figueiredo, P. A., Appell Coriolano, H. J., & Duarte, J. A. (2014). Cardiac regeneration and cellular therapy: is there a benefit of exercise? *Int J Sports Med, 35*(3), 181-190.

Figueiredo, P. A., Ferreira, R. M., Appell, H. J., & Duarte, J. A. (2008). Age-induced morphological, biochemical, and functional alterations in isolated mitochondria from murine skeletal muscle. *J Gerontol A Biol Sci Med Sci, 63*, 350-359.

Figueiredo, P. A., Mota, M. P., Appell, H. J., & Duarte, J. A. (2008). The role of mitochondria in aging of skeletal muscle. *Biogerontology, 9*, 67-84.

Figueiredo, P. A., Powers, S. K., Ferreira, R. M., Amado, F., Appell, H. J., & Duarte, J. A. (2009). Impact of lifelong sedentary behavior on mitochondrial function of mice skeletal muscle. *J Gerontol A Biol Sci Med Sci, 64*, 927-939.

Figueiredo, P. A., Powers, S. K., Ferreira, R. M., Appell, H. J., & Duarte, J. A. (2009). Aging impairs skeletal muscle mitochondrial bioenergetic function. *J Gerontol A Biol Sci Med Sci, 64*, 21-33.

Finkel, T. (2001). Reactive oxygen species and signal transduction. *IUBMB Life, 52*(1-2), 3-6.

Finkel, T. (2011). Signal transduction by reactive oxygen species. *J Cell Biol, 194*(1), 7-15.

Fong, D. Y., Ho, J. W., Hui, B. P., Lee, A. M., Macfarlane, D. J., Leung, S. S., Cerin, E., Chan, W. Y., Leung, I. P., Lam, S. H., Taylor, A. J., & Cheng, K. K. (2012). Physical activity for cancer survivors: meta-analysis of randomised controlled trials. *BMJ, 344*, e70.

Frangogiannis, N. G., Smith, C. W., & Entman, M. L. (2002). The inflammatory response in myocardial infarction. *Cardiovasc Res, 53*(1), 31-47.

Fridovich, I. (1997). Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *J Biol Chem, 272*(30), 18515-18517.

Friedman, M. A., Bozdech, M. J., Billingham, M. E., & Rider, A. K. (1978). Doxorubicin cardiotoxicity: serial endomyocardial biopsies and systolic time intervals. *JAMA, 240*(15), 1603-1606.

Fulbright, J. M., Huh, W., Anderson, P., & Chandra, J. (2010). Can anthracycline therapy for pediatric malignancies be less cardiotoxic? *Curr Oncol Rep, 12*(6), 411-419.

Garwicz, S., Anderson, H., Olsen, J. H., Dollner, H., Hertz, H., Jonmundsson, G., Langmark, F., Lanning, M., Moller, T., Sankila, R., & Tulinius, H. (2000). Second malignant neoplasms after cancer in childhood and adolescence: a population-based case-control study in the 5 Nordic countries. The Nordic Society for Pediatric Hematology and Oncology. The Association of the Nordic Cancer Registries. *Int J Cancer, 88*(4), 672-678.

Gatta, G., Capocaccia, R., Coleman, M. P., Ries, L. A., & Berrino, F. (2002). Childhood cancer survival in Europe and the United States. *Cancer, 95*(8), 1767-1772.

Gatta, G., Capocaccia, R., Stiller, C., Kaatsch, P., Berrino, F., Terenziani, M., & Group, E. W. (2005). Childhood cancer survival trends in Europe: a EUROCARE Working Group study. *J Clin Oncol, 23*(16), 3742-3751.

Ghibu, S., Delemasure, S., Richard, C., Guilland, J. C., Martin, L., Gambert, S., Rochette, L., & Vergely, C. (2012). General oxidative stress during doxorubicin-induced cardiotoxicity in rats: absence of cardioprotection and low antioxidant efficiency of alpha-lipoic acid. *Biochimie, 94*(4), 932-939.

Giantris, A., Abdurrahman, L., Hinkle, A., Asselin, B., & Lipshultz, S. E. (1998). Anthracycline-induced cardiotoxicity in children and young adults. *Critical Reviews in Oncology/Hematology, 27*, 53-68.

Gielen, S., Schuler, G., & Adams, V. (2010). Cardiovascular effects of exercise training: molecular mechanisms. *Circulation, 122*, 1221-1238.

Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol, 221*(1), 3-12.

Gornall, A. G., Bardawill, C. J., & David, M. M. (1949). Determination of Serum proteins by means of the biuret reaction. *Journal of Biological Chemistry*.

Gul, M., Demircan, B., Taysi, S., Oztasan, N., Gumustekin, K., Siktar, E., Polat, M. F., Akar, S., Akcay, F., & Dane, S. (2006). Effects of endurance training and acute exhaustive exercise on antioxidant defense mechanisms in rat heart. *Comp Biochem Physiol A Mol Integr Physiol, 143*(2), 239-245.

Gurney, J. G., Ness, K. K., Stovall, M., Wolden, S., Punyko, J. A., Neglia, J. P., Mertens, A. C., Packer, R. J., Robison, L. L., & Sklar, C. A. (2003). Final height and body mass index among adult survivors of childhood brain cancer: childhood cancer survivor study. *J Clin Endocrinol Metab, 88*(10), 4731-4739.

Hayward, R., Lien, C. Y., Jensen, B. T., Hydock, D. S., & Schneider, C. M. (2012). Exercise training mitigates anthracycline-induced chronic cardiotoxicity in a juvenile rat model. *Pediatr Blood Cancer, 59*, 149-154.

Hayward, R., Lien, C. Y., Jensen, B. T., Hydock, D. S., & Schneider, C. M. (2012). Exercise training mitigates anthracycline-induced chronic cardiotoxicity in a juvenile rat model. *Pediatr Blood Cancer, 59*(1), 149-154.

Hensley, K., Robinson, K. A., Gabbita, S. P., Salsman, S., & Floyd, R. A. (2000). Reactive oxygen species, cell signaling, and cell injury. *Free Radic Biol Med, 28*(10), 1456-1462.

Homer, C. R., Richmond, A. L., Rebert, N. A., Achkar, J. P., & McDonald, C. (2010). ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. *Gastroenterology, 139*(5), 1630-1641, 1641 e1631-1632.

Huang, T. T., & Ness, K. K. (2011). Exercise interventions in children with cancer: a review. *Int J Pediatr, 2011*, 461512.

Hudson, M. M., Mertens, A. C., Yasui, Y., Hobbie, W., Chen, H., Gurney, J. G., Yeazel, M., Recklitis, C. J., Marina, N., Robison, L. R., Oeffinger, K. C., & Childhood Cancer Survivor Study, I. (2003). Health status of adult long-term survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. *JAMA, 290*(12), 1583-1592.

Hudson, M. M., Mulrooney, D. A., Bowers, D. C., Sklar, C. A., Green, D. M., Donaldson, S. S., Oeffinger, K. C., Neglia, J. P., Meadows, A. T., & Robison, L. L. (2009). High-risk populations identified in Childhood Cancer Survivor Study investigations: implications for risk-based surveillance. *J Clin Oncol, 27*(14), 2405-2414.

Hydock, D. S., Lien, C. Y., Jensen, B. T., Parry, T. L., Schneider, C. M., & Hayward, R. (2012). Rehabilitative exercise in a rat model of doxorubicin cardiotoxicity. *Exp Biol Med (Maywood), 237*, 1483-1492.

Hydock, D. S., Lien, C. Y., Jensen, B. T., Parry, T. L., Schneider, C. M., & Hayward, R. (2012). Rehabilitative exercise in a rat model of doxorubicin cardiotoxicity. *Exp Biol Med (Maywood), 237*(12), 1483-1492.

Hydock, D. S., Lien, C. Y., Jensen, B. T., Schneider, C. M., & Hayward, R. (2011). Exercise preconditioning provides long-term protection against early chronic doxorubicin cardiotoxicity. *Integr Cancer Ther, 10*, 47-57.

Hydock, D. S., Lien, C. Y., Jensen, B. T., Schneider, C. M., & Hayward, R. (2011). Exercise preconditioning provides long-term protection against early chronic doxorubicin cardiotoxicity. *Integr Cancer Ther, 10*(1), 47-57.

Hydock, D. S., Lien, C. Y., Schneider, C. M., & Hayward, R. (2008). Exercise preconditioning protects against doxorubicin-induced cardiac dysfunction. *Med Sci Sports Exerc, 40*, 808-817.

Hydock, D. S., Lien, C. Y., Schneider, C. M., & Hayward, R. (2008). Exercise preconditioning protects against doxorubicin-induced cardiac dysfunction. *Med Sci Sports Exerc, 40*(5), 808-817.

Hydock, D. S., Parry, T. L., Jensen, B. T., Lien, C. Y., Schneider, C. M., & Hayward, R. (2011). Effects of endurance training on combined goserelin acetate and doxorubicin treatment-induced cardiac dysfunction. *Cancer Chemother Pharmacol, 68*, 685-692.

Hydock, D. S., Wonders, K. Y., Schneider, C. M., & Hayward, R. (2009). Voluntary wheel running in rats receiving doxorubicin: effects on running activity and cardiac myosin heavy chain. *Anticancer Research, 29*, 4401-4408.

Ichikawa, Y., Ghanefar, M., Bayeva, M., Wu, R. X., Khechaduri, A., Prasad, S. V. N., Mutharasan, R. K., Naik, T. J., & Ardehali, H. (2014). Cardiotoxicity of doxorubicin is mediated through mitochondrial iron accumulation. *Journal of Clinical Investigation, 124*(2), 617-630.

Jacobs, R. A., & Lundby, C. (2013). Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes. *J Appl Physiol, 114*(3), 344-350.

Jain, D. (2000). Cardiotoxicity of doxorubicin and other anthracycline derivatives. *Journal of Nuclear Cardiology, 7*, 53-62.

Jeppesen, T. D., Schwartz, M., Olsen, D. B., Wibrand, F., Krag, T., Duno, M., Hauerslev, S., & Vissing, J. (2006). Aerobic training is safe and improves exercise capacity in patients with mitochondrial myopathy. *Brain, 129*(Pt 12), 3402-3412.

Johnston, A. P., De Lisio, M., & Parise, G. (2008). Resistance training, sarcopenia, and the mitochondrial theory of aging. *Appl Physiol Nutr Metab, 33*, 191-199.

Jones, S. E., Durie, B. G. M., & Salmon, S. E. (1975). Combination Chemotherapy with adriamycin and cyclophosphamide for advance breast cancer. *Cancer, 36*(1), 90-97.

Judge, S., Jang, Y. M., Smith, A., Hagen, T., & Leeuwenburgh, C. (2005). Age-associated increses in oxidative stress and antioxidsant enzyme activities in

cardiac interfibrillar mitochondria: implications for the mitochondrial theory of aging. *FASEB j.*

Judge, S., Jang, Y. M., Smith, A., Selman, C., Philips, T., Speakman, J. R., Hagen, T., & Leeuwenburgh, C. (2005). Exercise by lifelong voluntary wheel running reduces suvsarcolemmal and interfibrillar mitochondrial hydrogen peroxide production in the heart. *Am J Physiol Regul Integr Comp Physiol, 289*, R1564-R1572.

Kanter, M. M., Hamlin, R. L., Unverferth, D. V., Davis, H. W., & Merola, A. J. (1985). Effect of exercise training on antioxidant enzymes and cardiotoxicity of doxorubicin. *J Appl Physiol, 59*(4), 1298-1303.

Katzmarzyk, P. T., Church, T. S., Craig, C. L., & Bouchard, C. (2009). Sitting time and mortality from all causes, cardiovascular disease, and cancer. *Med Sci Sports Exerc, 41*(5), 998-1005.

Kaur, A., Kaur, M., Sharma, R., & Kumar, S. (2012). Doxorubicin: a critial review on toxicity. *Journal of Pharmacy Research, 5*(5), 2890-2894.

Kavazis, A. N., Smuder, A. J., Min, K., Tumer, N., & Powers, S. K. (2010). Short-term exercise training protects against doxorubicin-induced cardiac mitochondrial damage independent of HSP72. *Am J Physiol Heart Circ Physiol, 299*, H1515-H1524.

Kenney, L. B., Nancarrow, C. M., Najita, J., Vrooman, L. M., Rothwell, M., Recklitis, C., Li, F. P., & Diller, L. (2010). Health status of the oldest adult survivors of cancer during childhood. *Cancer, 116*(2), 497-505.

Klettner, A., Kauppinen, A., Blasiak, J., Roider, J., Salminen, A., & Kaarniranta, K. (2013). Cellular and molecular mechanisms of age-related macular degeneration: from impaired autophagy to neovascularization. *Int J Biochem Cell Biol, 45*(7), 1457-1467.

Kumar, S. N., Konorev, E. A., Aggarwal, D., & Kalyanaraman, B. (2011). Analysis of proteome changes in doxorubicin-treated adult rat cardiomyocyte. *J Proteomics, 74*(5), 683-697.

Kwak, H. B., Song, W., & Lawler, J. M. (2006). Exercise training attenuates age-induced elevation in Bax/Bcl-2 ratio, apoptosis, and remodeling in the rat heart. *FASEB J, 20*(6), 791-793.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680-685.

Landier, W., Bhatia, S., Eshelman, D. A., Forte, K. J., Sweeney, T., Hester, A. L., Darling, J., Armstrong, F. D., Blatt, J., Constine, L. S., Freeman, C. R., Friedman, D. L., Green, D. M., Marina, N., Meadows, A. T., Neglia, J. P., Oeffinger, K. C., Robison, L. L., Ruccione, K. S., Sklar, C. A., & Hudson, M. M. (2004). Development of risk-based guidelines for pediatric cancer survivors: the Children's Oncology Group Long-Term Follow-Up Guidelines from the Children's Oncology Group Late Effects Committee and Nursing Discipline. *J Clin Oncol, 22*(24), 4979-4990.

Lanza, I. R., Short, D. K., Short, K. R., Raghavakaimal, S., Basu, R., Joyner, M. J., McConnell, J. P., & Nair, K. S. (2008). Endurance exercise as a countermeasure for aging. *Diabetes, 57*, 2933-2942.

Lanza, I. R., Short, D. K., Short, K. R., Raghavakaimal, S., Basu, R., Joyner, M. J., McConnell, J. P., & Nair, K. S. (2008). Endurance exercise as a countermeasure for aging. *Diabetes, 57*(11), 2933-2942.

Larsson, N. G. (2010). Somatic mitochondrial DNA mutations in mammalian aging. *Annu Rev Biochem, 79*, 683-706.

Lebrecht, D. (2004). Mitochondrial DNA and its respiratory chain products are defective in doxorubicin nephrosis. *Nephrology Dialysis Transplantation, 19*, 329-336.

Lebrecht, D. (2004). Mitochondrial DNA and its respiratory chain products are defective in doxorubicin nephrosis. *Nephrology Dialysis Transplantation, 19*(2), 329-336.

Lebrecht, D., Kokkori, A., Ketelsen, U. P., Setzer, B., & Walker, U. A. (2005). Tissue-specific mtDNA lesions and radical-associated mitochondrial dysfunction in human hearts exposed to doxorubicin. *J Pathol, 207*(4), 436-444.

Lebrecht, D., Kokkori, A., Ketelsen, U. P., Setzer, B., & Walker, U. A. (2005). Tissue-specific mtDNA lesions and radical-associated mitochondrial dysfunction in human hearts exposed to doxorubicin. *J Pathol, 207*, 436-444.

Lebrecht, D., Setzer, B., Ketelsen, U. P., Haberstroh, J., & Walker, U. A. (2003). Time-dependent and tissue-specific accumulation of mtDNA and respiratory chain defects in chronic doxorubicin cardiomyopathy. *Circulation, 108*(19), 2423-2429.

Lebrecht, D., Setzer, B., Ketelsen, U. P., Haberstroh, J., & Walker, U. A. (2003). Time-dependent and tissue-specific accumulation of mtDNA and respiratory chain defects in chronic doxorubicin cardiomyopathy. *Circulation, 108*, 2423-2429.

Lebrecht, D., & Walker, U. A. (2007). Role of mtDNA lesions in anthracycline cardiotoxicity. *Cardiovasc Toxicol, 7*(2), 108-113.

Lebrecht, D., & Walker, U. A. (2007). Role of mtDNA lesions in anthracycline cardiotoxicity. *Cardiovasc Toxicol, 7*, 108-113.

Lefer, D. J., & Granger, D. N. (2000). Oxidative stress and cardiac disease. *Am J Med, 109*(4), 315-323.

Lemieux, H., Vazquez, E. J., Fujioka, H., & Hoppel, C. L. (2010). Decrease in mitochondrial function in rat cardiac permeabilized fibers correlates with the aging phenotype. *J Gerontol A Biol Sci Med Sci, 65*, 1157-1164.

Lennon, S. L., Quindry, J., Hamilton, K. L., French, J., Staib, J., Mehta, J. L., & Powers, S. K. (2004). Loss of exercise-induced cardioprotection after cessation of exercise. *J Appl Physiol, 96*(4), 1299-1305.

Levine, B., & Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell, 132*(1), 27-42.

Li, M., Schonberg, A., Schaefer, M., Schroeder, R., Nasidze, I., & Stoneking, M. (2010). Detecting heteroplasmy from high-throughput sequencing of complete human mitochondrial DNA genomes. *Am J Hum Genet, 87*, 237-249.

Libonati, J. R. (2013). Cardiac Effects of Exercise Training in Hypertension. *ISRN Hypertension, 2013*, 1-9.

Lipshultz, S. E., Adams, M. J., Colan, S. D., Constine, L. S., Herman, E. H., Hsu, D. T., Hudson, M. M., Kremer, L. C., Landy, D. C., Miller, T. L., Oeffinger, K. C.,

Rosenthal, D. N., Sable, C. A., Sallan, S. E., Singh, G. K., Steinberger, J., Cochran, T. R., Wilkinson, J. D., American Heart Association Congenital Heart Defects Committee of the Council on Cardiovascular Disease in the Young, C. o. B. C. S. C. o. C., & Stroke Nursing, C. o. C. R. (2013). Long-term cardiovascular toxicity in children, adolescents, and young adults who receive cancer therapy: pathophysiology, course, monitoring, management, prevention, and research directions: a scientific statement from the American Heart Association. *Circulation, 128*(17), 1927-1995.

Lipshultz, S. E., Alvarez, J. A., & Scully, R. E. (2008). Anthracycline associated cardiotoxicity in survivors of childhood cancer. *Heart, 94*, 525-533.

Lipshultz, S. E., Landy, D. C., Lopez-Mitnik, G., Lipsitz, S. R., Hinkle, A. S., Constine, L. S., French, C. A., Rovitelli, A. M., Proukou, C., Adams, M. J., & Miller, T. L. (2012). Cardiovascular status of childhood cancer survivors exposed and unexposed to cardiotoxic therapy. *J Clin Oncol, 30*(10), 1050-1057.

Lu, Q., Krull, K. R., Leisenring, W., Owen, J. E., Kawashima, T., Tsao, J. C., Zebrack, B., Mertens, A., Armstrong, G. T., Stovall, M., Robison, L. L., & Zeltzer, L. K. (2011). Pain in long-term adult survivors of childhood cancers and their siblings: a report from the Childhood Cancer Survivor Study. *Pain, 152*(11), 2616-2624.

Machado, N. G., Baldeiras, I., Pereira, G. C., Pereira, S. P., & Oliveira, P. J. (2010). Sub-chronic administration of doxorubicin to Wistar rats results in oxidative stress and unaltered apoptotic signaling in the lung. *Chem Biol Interact, 188*, 478-486.

Mandavilli, B. S., Santos, J. H., & Van Houten, B. (2002). Mitochondrial DNA repair and aging. *Mutat Res, 509*(1-2), 127-151.

Marcus, A. D. (2013). Childhood Cancer's Health Woes Persist for Years. *The Wall Street Journal*(U.S. edition), A3.

Martins, R. A., Minari, A. L., Chaves, M. D., dos Santos, R. W., Barbisan, L. F., & Ribeiro, D. A. (2012). Exercise preconditioning modulates genotoxicity induced by doxorubicin in multiple organs of rats. *Cell Biochem Funct, 30*, 293-296.

Martins, R. A., Minari, A. L., Chaves, M. D., dos Santos, R. W., Barbisan, L. F., & Ribeiro, D. A. (2012). Exercise preconditioning modulates genotoxicity induced by doxorubicin in multiple organs of rats. *Cell Biochem Funct, 30*(4), 293-296.

Matsuura, C., Brunini, T. M., Carvalho, L. C., Resende, A. C., Carvalho, J. J., de Castro, J. P., & Mendes-Ribeiro, A. C. (2010). Exercise training in doxorubicin-induced heart failure: effects on the L-arginine-NO pathway and vascular reactivity. *J Am Soc Hypertens, 4*, 7-13.

Melo, S. A., Sugimoto, H., O'Connell, J. T., Kato, N., Villanueva, A., Vidal, A., Qiu, L., Vitkin, E., Perelman, L. T., Melo, C. A., Lucci, A., Ivan, C., Calin, G. A., & Kalluri, R. (2014). Cancer Exosomes Perform Cell-Independent MicroRNA Biogenesis and Promote Tumorigenesis. *Cancer Cell, 26*(5), 707-721.

Mertens, A. C., Liu, Q., Neglia, J. P., Wasilewski, K., Leisenring, W., Armstrong, G. T., Robison, L. L., & Yasui, Y. (2008). Cause-specific late mortality among 5-year

survivors of childhood cancer: the Childhood Cancer Survivor Study. *J Natl Cancer Inst, 100*(19), 1368-1379.

Michel, G., von der Weid, N. X., Zwahlen, M., Adam, M., Rebholz, C. E., Kuehni, C. E., Swiss Childhood Cancer, R., & Swiss Paediatric Oncology Group Scientific, C. (2007). The Swiss Childhood Cancer Registry: rationale, organisation and results for the years 2001-2005. *Swiss Med Wkly, 137*(35-36), 502-509.

Minotti, G., Cairo, G., & Monti, E. (1999). Role of iron in anthracycline cardiotoxicity: new tunes for an old song? *FASEB j., 13*, 199-212.

Minotti, G., Ronchi, R., Salvatorelli, E., Menna, P., & Cairo, G. (2001). Doxorubicin irreversibly inactivates iron regulatory proteins 1 and 2 in cardiomyocytes: evidence for distinct metabolic pathways and implications for iron-mediated cardiotoxicity of antitumor therapy. *Cancer Res, 61*(23), 8422-8428.

Minow, R. A., Benjamin, R. S., Lee, E. T., & Gottlieb, J. A. (1977). Adriamycin cardiomyopathy- risk factors. *Cancer, 39*, 1397-1402.

Mishra, S. I., Scherer, R. W., Geigle, P. M., Berlanstein, D. R., Topaloglu, O., Gotay, C. C., & Snyder, C. (2012). Exercise interventions on health-related quality of life for cancer survivors. *Cochrane Database Syst Rev, 8*, CD007566.

Mishra, S. I., Scherer, R. W., Snyder, C., Geigle, P., & Gotay, C. (2014). Are exercise programs effective for improving health-related quality of life among cancer survivors? A systematic review and meta-analysis. *Oncol Nurs Forum, 41*(6), E326-342.

Mishra, S. I., Scherer, R. W., Snyder, C., Geigle, P. M., Berlanstein, D. R., & Topaloglu, O. (2012). Exercise interventions on health-related quality of life for people with cancer during active treatment. *Clin Otolaryngol, 37*(5), 390-392.

Mizushima, N., Levine, B., Cuervo, A. M., & Klionsky, D. J. (2008). Autophagy fights disease through cellular self-digestion. *Nature, 451*(7182), 1069-1075.

Mizushima, N., Yoshimori, T., & Ohsumi, Y. (2003). Role of the Apg12 conjugation system in mammalian autophagy. *Int J Biochem Cell Biol, 35*(5), 553-561.

Mohamed, R. H., Karam, R. A., & Amer, M. G. (2011). Epicatechin attenuates doxorubicin-induced brain toxicity: critical role of TNF-alpha, iNOS and NF-kappaB. *Brain Res Bull, 86*(1-2), 22-28.

Nathan, P. C., Ford, J. S., Henderson, T. O., Hudson, M. M., Emmons, K. M., Casillas, J. N., Lown, E. A., Ness, K. K., & Oeffinger, K. C. (2009). Health behaviors, medical care, and interventions to promote healthy living in the Childhood Cancer Survivor Study cohort. *J Clin Oncol, 27*(14), 2363-2373.

National Cancer Institute. 2014, disponível em http://www.cancer.gov/cancertopics/cancerlibrary/what-is-cancer

Neglia, J. P., Friedman, D. L., Yasui, Y., Mertens, A. C., Hammond, S., Stovall, M., Donaldson, S. S., Meadows, A. T., & Robison, L. L. (2001). Second malignant neoplasms in five-year survivors of childhood cancer: childhood cancer survivor study. *J Natl Cancer Inst, 93*(8), 618-629.

Nicolay, K., & Kruijff, B. (1987). Effects of adriamycin on respiratory chain activities in mitochondria from rat liver, rat heart and bovine heart. Evidence for a preferential inhibition of complexx III and IV. *Biochim Biophys Acta, 892*, 320-330.

Nie, S. X., & Gao, C. Q. (2014). Health behaviors and quality of life in Chinese survivors of cervical cancer: a retrospective study. *Onco Targets Ther, 7*, 627-632.

Nygaard, R., Garwicz, S., Haldorsen, T., Hertz, H., Jonmundsson, G. K., Lanning, M., & Moe, P. J. (1991). Second malignant neoplasms in patients treated for childhood leukemia. A population-based cohort study from the Nordic countries. The Nordic Society of Pediatric Oncology and Hematology (NOPHO). *Acta Paediatr Scand, 80*(12), 1220-1228.

Oeffinger, K. C., Mertens, A. C., Sklar, C. A., Kawashima, T., Hudson, M. M., Meadows, A. T., Friedman, D. L., Marina, N., Hobbie, W., Kadan-Lottick, N. S., Schwartz, C. L., Leisenring, W., Robison, L. L., & Childhood Cancer Survivor, S. (2006). Chronic health conditions in adult survivors of childhood cancer. *N Engl J Med, 355*(15), 1572-1582.

Oliveira, P. J., Bjork, J. A., Santos, M. S., Leino, R. L., Froberg, M. K., Moreno, A. J., & Wallace, K. B. (2004). Carvedilol-mediated antioxidant protection against doxorubicin-induced cardiac mitochondrial toxicity. *Toxicol Appl Pharmacol, 200*, 159-168.

Olsen, J. H., Garwicz, S., Hertz, H., Jonmundsson, G., Langmark, F., Lanning, M., Lie, S. O., Moe, P. J., Moller, T., Sankila, R., & et al. (1994). [Second cancer. Risk of a second malignant neoplasm in persons with cancer in childhood and adolescence]. *Ugeskr Laeger, 156*(32), 4565-4571.

Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., & Bao, J. K. (2012). Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Prolif, 45*(6), 487-498.

PAC2. (2013). PEOPLE AGAINST CHILDHOOD CANCER. disponível

Petrosillo, G., Matera, M., Moro, N., Ruggiero, F. M., & Paradies, G. (2009). Mitochondrial complex I dysfunction in rat heart with aging: critical role of reactive oxygen species and cardiolipin. *Free Radic Biol Med, 46*, 88-94.

Powers, S. K., Smuder, A. J., Kavazis, A. N., & Quindry, J. C. (2014). Mechanisms of exercise-induced cardioprotection. *Physiology (Bethesda), 29*(1), 27-38.

Raha, S., & Robinson, B. H. (2000). Mitochondria, oxygen free radicals, disease and ageing. *TIBS, 25*.

Reulen, R. C., Winter, D. L., Frobisher, C., Lancashire, E. R., Stiller, C. A., Jenney, M. E., Skinner, R., Stevens, M. C., Hawkins, M. M., & British Childhood Cancer Survivor Study Steering, G. (2010). Long-term cause-specific mortality among survivors of childhood cancer. *JAMA, 304*(2), 172-179.

Reulen, R. C., Winter, D. L., Lancashire, E. R., Zeegers, M. P., Jenney, M. E., Walters, S. J., Jenkinson, C., & Hawkins, M. M. (2007). Health-status of adult survivors of childhood cancer: a large-scale population-based study from the British Childhood Cancer Survivor Study. *Int J Cancer, 121*(3), 633-640.

Richard, C., Ghibu, S., Delemasure-Chalumeau, S., Guilland, J. C., Des Rosiers, C., Zeller, M., Cottin, Y., Rochette, L., & Vergely, C. (2011). Oxidative stress and myocardial gene alterations associated with Doxorubicin-induced cardiotoxicity in rats persist for 2 months after treatment cessation. *J Pharmacol Exp Ther, 339*(3), 807-814.

Richard, C., Lauzier, B., Delemasure, S., Talbot, S., Ghibu, S., Collin, B., Senecal, J., Menetrier, F., Vergely, C., Couture, R., & Rochette, L. (2008). Effects of angiotensin-1 converting enzyme inhibition on oxidative stress and bradykinin receptor expression during doxorubicin-induced cardiomyopathy in rats. *J Cardiovasc Pharmacol, 52*(3), 278-285.

Robison, L. L., Armstrong, G. T., Boice, J. D., Chow, E. J., Davies, S. M., Donaldson, S. S., Green, D. M., Hammond, S., Meadows, A. T., Mertens, A. C., Mulvihill, J. J., Nathan, P. C., Neglia, J. P., Packer, R. J., Rajaraman, P., Sklar, C. A., Stovall, M., Strong, L. C., Yasui, Y., & Zeltzer, L. K. (2009). The Childhood Cancer Survivor Study: a National Cancer Institute-supported resource for outcome and intervention research. *J Clin Oncol, 27*(14), 2308-2318.

Robison, L. L., Mertens, A. C., Boice, J. D., Breslow, N. E., Donaldson, S. S., Green, D. M., Li, F. P., Meadows, A. T., Mulvihill, J. J., Neglia, J. P., Nesbit, M. E., Packer, R. J., Potter, J. D., Sklar, C. A., Smith, M. A., Stovall, M., Strong, L. C., Yasui, Y., & Zeltzer, L. K. (2002). Study design and cohort characteristics of the Childhood Cancer Survivor Study: a multi-institutional collaborative project. *Med Pediatr Oncol, 38*(4), 229-239.

Rodriquez, D. C., Guidoni, E., D'Ambrosio, A., Pochiero, F., Caini, M., Guglielmucci, D., Lucherini, M., Carra, F., & Municchi, G. (2014). Abnormalities of the thyroid in survivors of childhood Hodgkin's disease. *Minerva Pediatr, 66*(4), 249-256.

Rossato, L. G., Costa, V. M., Dallegrave, E., Arbo, M., Dinis-Oliveira, R. J., Santos-Silva, A., Duarte, J. A., Bastos, M. D., Palmeira, C., & Remiao, F. (2014). Cumulative Mitoxantrone-Induced Haematological and Hepatic Adverse Effects in a Subchronic In vivo Study. *Basic & Clinical Pharmacology & Toxicology, 114*(3), 254-262.

Rossato, L. G., Costa, V. M., Dallegrave, E., Arbo, M., Silva, R., Ferreira, R., Amado, F., Dinis-Oliveira, R. J., Duarte, J. A., Bastos, M. D., Palmeira, C., & Remiao, F. (2014). Mitochondrial Cumulative Damage Induced by Mitoxantrone: Late Onset Cardiac Energetic Impairment. *Cardiovascular Toxicology, 14*(1), 30-40.

Saad, S. Y., Najjar, T. A., & Al-Rikabi, A. C. (2001). The preventive role of deferoxamine against acute doxorubicin-induced cardiac, renal and hepatic toxicity in rats. *Pharmacol Res, 43*(3), 211-218.

Santos, D. L., Moreno, A. J., Leino, R. L., Froberg, M. K., & Wallace, K. B. (2002). Carvedilol protects against doxorubicin-induced mitochondrial cardiomyopathy. *Toxicol Appl Pharmacol, 185*(3), 218-227.

Scott, J. M., Koelwyn, G. J., Hornsby, W. E., Khouri, M., Peppercorn, J., Douglas, P. S., & Jones, L. W. (2013). Exercise therapy as treatment for cardiovascular and oncologic disease after a diagnosis of early-stage cancer. *Semin Oncol, 40*(2), 218-228.

Shapira, J., Gotfried, M., Lishner, M., & Ravid, M. (1990). Reduced cardiotoxicity of doxorubicin by a 6-hour infusion regimen. *Cancer, 65*, 870-873.

Shi, Y., Moon, M., Dawood, S., McManus, B., & Liu, P. P. (2011). Mechanisms and management of doxorubicin cardiotoxicity. *Herz, 36*(4), 296-305.

Short, K. R., Vittone, J. L., Bigelow, M. L., Proctor, D. N., & Nair, K. S. (2004). Age and aerobic exercise training effects on whole body and muscle protein metabolism. *Am J Physiol Endocrinol Metab, 286*(1), E92-101.

Short, K. R., Vittone, J. L., Bigelow, M. L., Proctor, D. N., Rizza, R. A., Coenen-Schimke, J. M., & Nair, K. S. (2003). Impact of aerobic exercise training on age-related changes in insulin sensitivity and muscle oxidative capacity. *Diabetes, 52*.

Siciliano, G., Simoncini, C., Lo Gerfo, A., Orsucci, D., Ricci, G., & Mancuso, M. (2012). Effects of aerobic training on exercise-related oxidative stress in mitochondrial myopathies. *Neuromuscul Disord, 22 Suppl 3*, S172-177.

Simunek, T., Sterba, M., Popelova, O., Adamcova, M., Hrdina, R., & Gersi, V. (2009). Anthracycline-induced cardiotoxicity: Overview of studies examining the roles of oxidative stress and free cellular iron. *Pharmacol Rep, 61*, 154-171.

Singal, P. K., Iliskovic, N., Li, T., & Kumar, D. (1997). Adriamycin cardiomyopathy: pathophysiology and prevention. *FASEB j., 11*, 931-936.

Siu, P. M., Bryner, R. W., Martyn, J. K., & Alway, S. E. (2004). Apoptotic adaptations from exercise training in skeletal and cardiac muscles. *FASEB J, 18*(10), 1150-1152.

Sklar, C., Whitton, J., Mertens, A., Stovall, M., Green, D., Marina, N., Greffe, B., Wolden, S., & Robison, L. (2000). Abnormalities of the thyroid in survivors of Hodgkin's disease: data from the Childhood Cancer Survivor Study. *J Clin Endocrinol Metab, 85*(9), 3227-3232.

Smuder, A. J., Kavazis, A. N., Min, K., & Powers, S. K. (2013). Doxorubicin-induced markers of myocardial autophagic signaling in sedentary and exercise trained animals. *J Appl Physiol*.

Steinherz, L. J., Steinherz, P. G., Tan, C., Heller, G., & Murphy, L. (1991). Cardiac toxicity 4 to 20 years after completing anthracycline therapy. *JAMA, 266*(12), 1672-1677.

Sterba, M., Popelova, O., Vavrova, A., Jirkovsky, E., Kovarikova, P., Gersl, V., & Simunek, T. (2013). Oxidative stress, redox signaling, and metal chelation in anthracycline cardiotoxicity and pharmacological cardioprotection. *Antioxid Redox Signal, 18*(8), 899-929.

Suter, T. M., & Ewer, M. S. (2013). Cancer drugs and the heart: importance and management. *Eur Heart J, 34*, 1102-1111.

Sweat, F., Puchtler, H., & Rosenthal, S. I. (1964). Sirius Red F3ba as a Stain for Connective Tissue. *Arch Pathol, 78*, 69-72.

Taghian, A., de Vathaire, F., Terrier, P., Le, M., Auquier, A., Mouriesse, H., Grimaud, E., Sarrazin, D., & Tubiana, M. (1991). Long-term risk of sarcoma following radiation treatment for breast cancer. *Int J Radiat Oncol Biol Phys, 21*(2), 361-367.

Taivassalo, T., Gardner, J. L., Taylor, R. W., Schaefer, A. M., Newman, J., Barron, M. J., Haller, R. G., & Turnbull, D. M. (2006). Endurance training and detraining in mitochondrial myopathies due to single large-scale mtDNA deletions. *Brain, 129*(Pt 12), 3391-3401.

Taivassalo, T., Gardner, J. L., Taylor, R. W., Schaefer, A. M., Newman, J., Barron, M. J., Haller, R. G., & Turnbull, D. M. (2006). Endurance training and detraining in

mitochondrial myopathies due to single large-scale mtDNA deletions. *Brain, 129*, 3391-3401.

Tan, C., Tasaka, H., Yu, K. P., Murphy, M. L., & Karnofsky, D. A. (1967). Daunomycin, an antitumor antibiotic, in the treatment of neoplastic disease. *Cancer, 20*(3).

Tan, C., Tasaka, H., Yu, K. P., Murphy, M. L., & Karnofsky, D. A. (1967). Daunomycin, an antitumor antibiotic, in the treatment of neoplastic disease. *cancer, 20*.

Taylor, A. J., Croft, A. P., Palace, A. M., Winter, D. L., Reulen, R. C., Stiller, C. A., Stevens, M. C., & Hawkins, M. M. (2009). Risk of thyroid cancer in survivors of childhood cancer: results from the British Childhood Cancer Survivor Study. *Int J Cancer, 125*(10), 2400-2405.

Tester, W. J., Kinsella, T. J., Waller, B., Makuch, R. W., Kelley, P. A., Glatstein, E., & DeVita, V. T. (1984). Second malignant neoplasms complicating Hodgkin's disease: the National Cancer Institute experience. *J Clin Oncol, 2*(7), 762-769.

Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D., & Liu, L. F. (1984). Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science, 226*(4673), 466-468.

Tiana, L., Caib, Q., & Wei, H. (1998). alterations of antioxidant enzymes and oxidative damage to macromolecules in different organs of rats during aging. *Free Radical Biology and Medicine*.

Tokarska-Schlattner, M., Zaugg, M., Zuppinger, C., Wallimann, T., & Schlattner, U. (2006). New insights into doxorubicin-induced cardiotoxicity: the critical role of cellular energetics. *J Mol Cell Cardiol, 41*(3), 389-405.

Travis, L. B., Curtis, R. E., Storm, H., Hall, P., Holowaty, E., Van Leeuwen, F. E., Kohler, B. A., Pukkala, E., Lynch, C. F., Andersson, M., Bergfeldt, K., Clarke, E. A., Wiklund, T., Stoter, G., Gospodarowicz, M., Sturgeon, J., Fraumeni, J. F., Jr., & Boice, J. D., Jr. (1997). Risk of second malignant neoplasms among long-term survivors of testicular cancer. *J Natl Cancer Inst, 89*(19), 1429-1439.

Trivedi, P. P., Kushwaha, S., Tripathi, D. N., & Jena, G. B. (2011). Cardioprotective effects of hesperetin against doxorubicin-induced oxidative stress and DNA damage in rat. *Cardiovasc Toxicol, 11*, 215-225.

Tucker, M. A., Coleman, C. N., Cox, R. S., Varghese, A., & Rosenberg, S. A. (1988). Risk of second cancers after treatment for Hodgkin's disease. *N Engl J Med, 318*(2), 76-81.

Ventura-Clapier, R., Garnier, A., & Veksler, V. (2004). Energy metabolism in heart failure. *J Physiol, 555*(Pt 1), 1-13.

Wallace, K. B. (2003). Doxorubicin-induced cardiac mitochondrionopathy. *Pharmacol & Toxicology, 93*, 105-115.

Wei, Y. H., & Lee, H. C. (2002). Oxidative stress, mitochondrial dna mutation, and impairment of antioxidant enzymes in aging. *Exp Biol Med (Maywood), 227*, 671-682.

Werner, C., Hanhoun, M., Widmann, T., Kazakov, A., Semenov, A., Poss, J., Bauersachs, J., Thum, T., Pfreundschuh, M., Muller, P., Haendeler, J., Bohm, M., & Laufs, U. (2008). Effects of physical exercise on myocardial telomere-

regulating proteins, survival pathways, and apoptosis. *J Am Coll Cardiol, 52*(6), 470-482.

WHO. (2008). International Agency for Research on Cancer -GLOBOCAN [Versão eletrónica], disponível.

Wolin, K. Y., Dart, H., & Colditz, G. A. (2013). Eight ways to stay healthy after cancer: an evidence-based message. *Cancer Causes Control, 24*(5), 827-837.

Wolin, K. Y., Schwartz, A. L., Matthews, C. E., Courneya, K. S., & Schmitz, K. H. (2012). Implementing the exercise guidelines for cancer survivors. *J Support Oncol, 10*(5), 171-177.

Wonders, K. Y., Hydock, D. S., Greufe, S., Schneider, C. M., & Hayward, R. (2009). Endurance exercise training preserves cardiac function in rats receiving doxorubicin and the HER-2 inhibitor GW2974. *Cancer Chemother Pharmacol, 64*, 1105-1113.

World Child Cancer. (2013a).

World Child Cancer. (2013b). disponível

Yagmurca, M., Fadillioglu, E., Erdogan, H., Ucar, M., Sogut, S., & Irmak, M. K. (2003). Erdosteine prevents doxorubicin-induced cardiotoxicity in rats. *Pharmacol Res, 48*(4), 377-382.

Yakes, F. M., & Houten, B. V. (1997). Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc Natl Acad Sci U S A, 94*, 514-519.

Yan, Z., Lira, V. A., & Greene, N. P. (2012). Exercise training-induced regulation of mitochondrial quality. *Exerc Sport Sci Rev, 40*(3), 159-164.

Yang, J. H., Lee, H. C., Lin, K. J., & Wei, Y. H. (1994). A specific 4977-bp deletion of mitochondrial DNA in human ageing skin. *Arch Dermatol Res, 286*, 386-390.

Yang, Z., & Klionsky, D. J. (2010). Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol, 22*(2), 124-131.

Yasuda, K., Park, H. C., Ratliff, B., Addabbo, F., Hatzopoulos, A. K., Chander, P., & Goligorsky, M. S. (2010). Adriamycin nephropathy: a failure of endothelial progenitor cell-induced repair. *Am J Pathol, 176*(4), 1685-1695.

Yi, X., Bekeredjian, R., DeFilippis, N. J., Siddiquee, Z., Fernandez, E., & Shohet, R. V. (2006). Transcriptional analysis of doxorubicin-induced cardiotoxicity. *Am J Physiol Heart Circ Physiol, 290*, H1098-1102.

Zhang, Y. W., Shi, J., Li, Y. J., & Wei, L. (2009). Cardiomyocyte death in doxorubicin-induced cardiotoxicity. *Arch Immunol Ther Exp (Warsz), 57*(6), 435-445.

Zhou, S., Starkov, A., & Froberg, M. K. (2001). Cumulative and Irreversible cardiac mitochondrial dysfucntion Induced by Doxorubicin. *Cancer Res, 61*, 771-777.