**University of Porto**

**Faculty of Sport**

**Research Center of Physical Activity and Leisure**

# Chronic exercise mitigates Doxorubicin-induced cardiac and brain mitochondrial liabilities

Role for mitochondrial oxidative stress, apoptotic and quality control signaling.

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**Supervisors.** Professor Doutor António Ascensão

Professor Doutor José Magalhães

Inês Marques Aleixo

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**KEY WORDS:** Physical exercise, Adriamycin, Heart, Brain cortex, Cerebellum, Mitochondrial Bioenergetics, Apoptosis, Oxidative stress, Quality control

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ADP, adenosine diphosphate; AIF, apoptosis inducing factor; ATP, adenosine triphosphate; AD, Alzheimer disease; BDNF, brain-derived neurotrophic factor; $Ca^{2+}$, calcium; CAT, catalase; CypD, cyclophilin D; Cu/Zn-SOD, Copper/zinc superoxide dismutase; DOX, doxorubicin; Drp1, dynamin-related protein 1; ETC, Electron transport chain; FCCP, Carbonyl cyanide p-[trifluoromethoxy]-phenyl-hydrazone; Fis1, fission 1; GPx, Glutathione peroxidase; GSH, Glutathione; GSSH, Glutathione disulfide; HSPs, heat shock proteins; IDH2, isocitrate dehydrogenase 2; Mn-SOD, Manganese superoxide dismutase; mPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; Mfn, mitofusin; Opa1, optic atrophy type 1; OXPHOS, oxidative phosphorylation; PD, Parkinson Disease; PGC-1$\alpha$, peroxisome proliferator-activated receptor-$\gamma$ co-activator 1- $\alpha$, RCR, Respiratory control ratio; ROS, Reactive oxygen species; SOD, Superoxide dismutase; SIRT1, silent information regulator 1; SIRT3, silent information regulator 3; TCA, tricarboxylic acid cycle; TFAM, mitochondrial transcription factor A; UCPs, uncoupling proteins; $\Delta\psi m$, Transmembrane electric potential; TNFα, tumor necrosis factor-α; 8-OHdG, 8-hydroxydeoxyguanosine.

**Abstract**

Doxorubicin (DOX) also known as Adriamycin, is one of the most prominent and efficient anticancer drugs clinically available for the treatment of several tumors. However, the treatment with DOX is commonly associated with toxicity of non-target organs, including the heart and brain, being the cumulative and irreversible cardiomyopathy the most studied side effect. Although the full mechanisms behind DOX toxicity remain unclear, it has been associated with increased oxidative stress and mitochondrial bioenergetics disruption. Considering this, and the important role of mitochondria in the control of several physiological processes, including calcium homeostasis, cell fate and renewal, the study of mitochondrial-related disturbances is considered an important model to study DOX-induced cellular toxicity.

In an attempt to counteract DOX side effects, the efficiency of numerous adjunct therapies has been investigated. Physical exercise has been proposed as a non-pharmacological strategy to counteract DOX-induced toxicity, including mitochondrial dysfunction. Despite the reasonable amount of work performed, there are still some unsolved questions associated with types of treatment and exercise models in the set of cardio and neuroprotection targeting mitochondria. Additionally, the modulator effect of exercise performed before and particularly during the course of DOX treatment in cardiac, brain cortex and cerebellum mitochondrial bioenergetics, oxidative stress, apoptotic, dynamics and quality control signaling was never explored so far.

The present dissertation, comprises the theoretical background with two reviews aiming at understanding the mitochondrial-related molecular mechanisms underlying the protective effect of chronic exercise against DOX-induced cardiotoxicity (Chapter 2.1) and to discuss the role of physical exercise in the modulation of the mitochondrial-mediated mechanisms involved in neuroprotection (Chapter 2.2). The experimental section includes four original studies as follows: two intending to analyze the effect of two different long-term exercise models performed before and during sub-chronic DOX-treatment on cardiac mitochondrial bioenergetics, biogenesis and oxidative stress markers (Chapter 3.2) and on heart

mitochondrial susceptibility to permeability transition pore (mPTP) opening, apoptotic and autophagic signaling and mitochondrial dynamics (Chapter 3.3); the further two experimental studies were performed focusing on the effects of the same distinct exercise modalities on behavioral performance, and brain cortex and cerebellum mitochondrial bioenergetics, oxidative stress, mitochondrial permeability transition, apoptotic signaling and quality control (Chapter 3.4); similar behavioural, functional and molecular markers/endpoints were analyzed in sub-chronically DOX-treated animals in order to examine whether chronic exercise mitigates DOX-induced alterations in cognition, brain and cerebellum mitochondrial bioenergetics and associated mechanisms (Chapter 3.5).

Generally, exercise modalities improved cardiac and brain mitochondrial function as seen by oxygen consumption and transmembrane electric potential endpoints, increased the mitochondrial resistance to permeability transition and oxidative damage. It is possible that exercise-induced modulation in some metabolic sensors including SIRT3 and PGC1a as well as apoptotic, dynamic and auto(mito)phagic signaling markers (i.e., Bax/Bcl-2, Mfn1/2, OPA1, DRP1, Beclin, LC3II, PINK1 and Parkin) could contributed to the to augment the plasticity of cardiac and brain mitochondrial network in an attempt to mitigate the deleterious effects caused by sub-chronic DOX treatment.

The data support the notion that chronic exercise mitigates DOX-induced cardiac, brain cortex and cerebellum mitochondrial toxicity. Alterations in mitochondrial bioenergetic, biogenesis and quality control as well as in oxidative damage, mPTP and apoptotic signalling induced by sub-chronic DOX-treatment were observed in the studied tissues. Importantly, both exercise models performed before and during the course of treatment seem to positively impact cardiac, brain cortex and cerebellum mitochondria mitigating mitochondrial deleterious effects caused by DOX treatment.

**KEY WORDS:** Physical exercise, Adriamycin, Heart, Brain cortex, Cerebellum, Mitochondrial Bioenergetics, Oxidative stress, Apoptosis, Quality control

**Resumo**

A Doxorrubicina (DOX), também conhecida como Adriamicina, é um dos fármacos antineoplásicos clinicamente disponíveis mais eficazes para o tratamento de diversos tumores. Contudo, o tratamento com DOX está geralmente associado com a toxicidade em órgãos não-alvo, incluindo o coração e o cérebro, sendo a cardiomiopatia cumulativa e irreversível o efeito colateral mais estudado. Embora os mecanismos associados à toxicidade induzida pela DOX permaneçam ainda por esclarecer completamente, a literatura disponível aponta o aumento do stresse oxidativo e a disrupção da bioenergética mitocondrial. Assim, e considerando o papel importante das mitocôndrias no controlo de diversos processos fisiológicos, incluindo a homeostasia do cálcio, renovação e morte celulares, o estudo das alterações mitocondriais tem sido um importante modelo para estudar a toxicidade celular induzida pela DOX.

Na tentativa de contrariar os efeitos secundários da DOX, tem sido investigada a eficiência de numerosas terapias coadjuvantes. O exercício físico tem sido proposto como uma estratégia não farmacológica para neutralizar a toxicidade induzida pela DOX, incluindo a disfunção mitocondrial. Apesar da considerável quantidade de trabalho realizado, existem ainda algumas questões por esclarecer associadas ao efeito dos diferentes tipos de tratamento/modelos de exercício na tolerância das mitocôndrias cardíacas e cerebrais aos efeitos da DOX. Adicionalmente, o efeito modulador de exercício realizado antes e particularmente durante o tratamento com DOX na bioenergética mitocondrial, stresse oxidativo, apoptose e na sinalização da dinâmica e controle de qualidade no tecido cardíaco, córtex cerebral e cerebelo não foi ainda analisado.

A presente dissertação compreende a fundamentação teórica com duas revisões que visam a compreensão dos mecanismos moleculares mitocondriais subjacentes ao efeito protetor do exercício crónico contra a cardiotoxicidade induzida pela DOX (Capítulo 2.1) e a discussão do papel do exercício físico na modulação dos mecanismos mediados pelas mitocondriais envolvidos na neuroprotecção (Capítulo 2.2). A seção experimental inclui

quatro estudos originais: dois com a intenção de analisar o efeito de dois modelos diferentes de exercício crónico realizados antes e durante o tratamento sub-crónico com DOX na bioenergética mitocondrial cardíaca, biogénese e marcadores de stresse oxidativo (Capítulo 3.2) e na susceptibilidade para a abertura do poro de permeabilidade transitória mitocondrial (PPTm), na sinalização apoptótica e autofágica e na dinâmica mitocondrial no coração (Capítulo 3.3); os outros dois estudos experimentais foram realizados focando o efeito dos mesmos modelos de exercício no desempenho comportamental e na bioenergética mitocondrial, stresse oxidativo, PPTm, na sinalização apoptótica e controle de qualidade no córtex cerebral e cerebelo (Capítulo 3.4); Finalmente estes mesmos marcadores/*endpoints* comportamentais, funcionais e moleculares foram analisados de forma semelhante em animais tratados sub-cronicamente com o propósito de examinar se o exercício crónico atenua as alterações induzidas pela DOX na cognição, na bioenergética mitocondrial do cérebro e cerebelo e nos respetivos mecanismos associados à mitocôndria (Capítulo 3.5).

De uma forma geral, a realização de exercício crónico promoveu melhorias na funcionalidade mitocondrial cardíaca e cerebral observadas através da análise de parâmetros associados ao consumo de oxigénio e ao potencial elétrico trasmembranar, promoveu incremento da resistência das mitocôndrias à indução de permeabilidade transitória e do stress oxidativo. É possível que a modelação via exercício de alguns sensors metabólicos como a SIRT3 e a PGC1a, bem como de marcadores de sinalização apoptótica, de dinâmica e de auto(mito)fagia (i.e., Bax/Bcl-2, Mfn1/2, DRP1, Beclin, LC3II, PINK1 e Parkin) possam ter contribuido para aumentar a plasticidade da rede mitocondrial cardiac e cerebral, mitigando os efeitos deleterios do tratamento sub-crónico com DOX.

Os resultados obtidos apoiam a ideia de que o exercício crónico mitiga a toxicidade mitocondrial induzida pela DOX no coração, córtex cerebral e cerebelo. Foram observadas alterações nos tecidos estudados na bioenergética, biogénese e controlo de qualidade mitocondrial, bem como nos danos oxidativos, PPTm e sinalização apoptótica decorrentes do tratamento sub-crónico com DOX. Ambos os modelos de exercício realizados antes e

durante o tratamento parecem ter um impacto positivo na mitocôndrias cardíacas, do córtex cerebral e do cerebelo, assim como atenuar os efeitos deletérios causados pelo tratamento com DOX nas mitocôndrias.

**PALAVRAS CHAVE:** Exercício físico, Adriamicina, Coração, Córtex cerebral, Cerebelo, Bioenergética mitocondrial, Stress oxidativo, Apoptose, Controlo de Qualidade.

# 1.

# GENERAL INTRODUCTION

**A brief analysis of DOX-induce mitochondrial toxicity**

Doxorubicin (DOX, also known as Adriamycin) is a potent antineoplasic agent used to treat several types of solid tumors and hematological malignancies, including breast, bile ducts, prostate, uterus, ovary, oesophagus, stomach and liver tumors, childhood solid tumors, osteosarcomas and soft tissue sarcomas, Kaposi's sarcoma, as well as acute myeloblastic and lymphoblastic leukaemia and Wilms Tumor (Breslow et al., 2004; Danesi et al., 2002; Gewirtz, 1999; Gruber et al., 2004; Petrioli et al., 2008). However, it is well established that some potent and highly effective chemotherapeutic agents, of which DOX is a clear example, cause a variety of side effects that limit a more effective use in the reduction or elimination of tumor cell growth. Specifically, the clinical use of the broadly successful antineoplastic DOX is limited by the occurrence of a dose-related cardiac toxicity that results in life-threatening cardiomyopathy. Additionally, adverse effects of DOX treatment to other tissues, including the brain and liver, have also been studied since neurological disturbances, changes in cognitive function and compromised liver function are a common reported side effect of chemotherapy (Ahles et al., 2002; Barogi et al., 2000; Bender et al., 2006; Brezden et al., 2000; Catala et al., 2007; Ferguson et al., 2007; Hermelink et al., 2007; Jansen et al., 2008; Jenkins et al., 2006; Raffa, 2011; Santos et al., 2002; Valls-Belles et al., 2008).

The exact mechanisms of DOX deleterious effects are complex and still somewhat unclear. However, they have been associated, at least in part, with an increased production of reactive oxygen species (ROS) leading to oxidative damage and impairments in major mitochondrial functions (Carvalho et al., 2009; Carvalho et al., 2014; Pereira et al., 2011). Importantly, some differences have been reported concerning the mechanisms behind DOX-induced mitochondrial dysfunction depending to the studied tissue.

The cardiac mitochondria seem to be particularly sensitive to DOX toxicity. Among others, it has been proposed that the elevated affinity of DOX to cardiolipin, a major phospholipid component of the inner mitochondrial membrane, and the decreased antioxidant capacity

and higher mitochondrial content of cardiac tissue compared to other tissues, justify the lower heart capability of dealing with the increased oxidative stress induced by DOX (Carvalho et al., 2009; Wallace, 2003, 2007).

Generally, mitochondrial involvement in DOX-induced cardiotoxicity could be explained by the activation of DOX molecule into a more reactive semi-quinone at mitochondrial complex I, leading to the formation of superoxide anion and resulting in increased oxidative stress (Doroshow, 1983; Nohl, 1987; Nohl et al., 1998). Moreover, the controversial existence of a heart specific isoform of the NADH dehydrogenase (mitochondrial complex I) able to initiate DOX redox cycling and, consequently, promoting additional ROS formation, may be a critical step of DOX-induced deterioration of cardiac function and onset of chronic clinical cardiotoxicity (Nohl, 1987; Nohl et al., 1998). The increased oxidative stress associated with DOX toxicity leads to the depletion of mitochondrial reducing equivalents, impairment in oxidative phosphorylation with consequent decline in ATP levels, and interference with cellular calcium homeostasis (Wallace, 2003, 2007), including increased susceptibility to the mitochondrial permeability transition pore (mPTP) (Oliveira et al., 2004; Oliveira and Wallace, 2006; Santos et al., 2002; Solem and Wallace, 1993; Solem et al., 1994; Zhou et al., 2001). All together, these mechanisms can further lead to an increase in apoptotic signaling and may interfere with mitochondrial network and quality control (Carvalho et al., 2014; Pereira et al., 2011).

Despite the relatively well-known side effects of DOX treatment in the heart, little is known about its effects in the brain. DOX does not cross the blood-brain barrier (Bigotte and Olsson, 1982a; Tangpong et al., 2006); therefore it has been proposed that DOX-induced brain mitochondrial dysfunction is indirect and apart from cellular penetration by the drug and from redox cycling. Although presently unclear, it has been proposed that the chemotherapy-related neurotoxicity is mediated by cytokines as DOX increases the systemic production of tumor necrosis factor-α (TNFα) (Aluise et al., 2011; Tangpong et al., 2006), which can migrate into the brain and stimulate locally its production (Seruga et al., 2008). The elevation

of these cytokines in brain leads to oxidative stress, mitochondrial dysfunction and neuronal death, processes that may contribute to the cognitive dysfunction often observed in patients undergoing chemotherapy (Joshi et al., 2010; Tangpong et al., 2006; Tangpong et al., 2007). This systemic and consequently brain pro-inflammatory environment caused by DOX induces oxidative damage driven by several cellular sources, including mitochondria and eventually lead to the increased susceptibility to mPTP and apoptosis (Cardoso et al., 2008). These mechanisms seem to be central to explain DOX-induced brain mitochondrial toxicity.

Since DOX has a wide clinical application, researchers have investigated efficient adjunct therapies in an attempt to counteract DOX-side effects. Given the above mentioned mechanisms of cardiac and brain toxicity associated with DOX, antioxidant based therapies including those targeting mitochondria, as well as physical exercise, a non-pharmacological tool known to positively modulate biochemical, morphological and functional features in skeletal, cardiac, liver and brain tissues (Ascensao and Magalhaes, 2006; Ascensao et al., 2007; Ascensao et al., 2011a; Ascensao et al., 2013; Goncalves et al., 2013; Lumini et al., 2008) have been proposed to counteract DOX side effects, particularly in the cardiovascular function.

**Exercise protection against mitochondria-related pathophysiological conditions**

In addition to improving health wellness, quality of life and physical fatigue, exercise has also been associated with health benefits against several chronic pathologies including metabolic disorders, pulmonary and cardiovascular diseases, ageing-related systemic, organ and tissue impairments and is also recommended as an additional therapy to patients with neurodegenerative diseases or cancer (Ascensao et al., 2011b; Deslandes et al., 2009; Warburton et al., 2006). Moreover, exercise is also able to protect against toxicity of some pharmacological drugs, including DOX (Ascensao et al., 2011b; Ascensao et al., 2012).

It is however noteworthy that the precise mechanisms behind exercise-related protective tissue phenotypes are not fully understood so far. Exercise-induced adaptations depends and requires the coordination of multiple cellular events and one of the most important modulatory effects at subcellular level probably occurs in the mitochondrion/mitochondrial network. Indeed, given the pivotal role of mitochondria providing the energy required for daily life activities, it is not surprising that modulation/adaptations in these organelles have been associated with physical exercise engagement. Moreover, mitochondria act not only as energy providers, but have also other vital functions in the cells, including the control of redox status and pH, calcium homeostasis and cellular signaling, and also determine cell fate through initiation or amplification of several cell death pathways (Cho et al., 2010; Kubli and Gustafsson, 2012). Some of these mitochondrial-related pathways appear to be compromised in ageing and during pathological conditions (Conley et al., 2007; Navarro and Boveris, 2007), during a large variety of metabolic disorders, including obesity, severe hyperglycemia (Bruce et al., 2006; Lumini-Oliveira et al., 2011; Wells et al., 2008) and type 2 diabetes (Hojlund et al., 2008; Lowell and Shulman, 2005), in many age related neurodegenerative diseases (Aliev et al., 2009; Calabrese et al., 2001; Chaturvedi and Flint Beal, 2013) and cardiomyopathies (Meyers et al., 2013; Wallace, 2000; Wallace, 2003; Walters et al., 2012), including those caused by DOX as detailed above. Therefore, it is expected that mitochondria may have a central role to explain the cross-tolerance phenomena between exercise and some pathophysiological conditions. For instances, cardioprotection induced by physical exercise incorporate an increased tolerance of cardiac tissue and mitochondria against the harmful effects of deleterious conditions that occur, at least in part, at the mitochondrial level. In the present thesis, an update theoretical section highlighting the role of mitochondria on exercise induced cardioprotection against DOX treatment is developed in Chapter 2.1. Similarly, as DOX induced alterations in brain function involving mitochondria in the process and physical exercise also improve cognitive and other brain functions through mitochondrial-related paths, the central role of mitochondria underlying brain protection afforded by exercise is extensively reviewed in Chapter 2.2.

**Exercise mitigates DOX-induced mitochondrial toxicity: relevance for the present work**

Research on the role of exercise and physical activity against DOX side effects, including mitochondrial dysfunction has been a topic of considerable interest over the last years (Ascensao et al., 2005a; Ascensao et al., 2005b; Ascensao et al., 2006a; Ascensao et al., 2006b; Ascensao et al., 2007; Ascensao et al., 2011a; Ascensao et al., 2011b; Ascensao et al., 2012; Chicco et al., 2006a; Chicco et al., 2006b; Dolinsky et al., 2013). Particular attention has been attributed to exercise type, timings, intensities and volumes needed to optimize cardiovascular benefits required to antagonize DOX side effects. The role of moderate exercise training following the diagnosis of the disease, either during treatment or after the cessation of chemotherapy has been investigated. It has been suggested that exercise is a feasible and safe supportive intervention, which increases the quality of life, muscular strength and reduces fatigue indexes in cancer patients (Doyle et al., 2006; Jones and Demark-Wahnefried, 2006; Lucia et al., 2003). Moreover, concerning exercise intensity, studies evaluating the efficacy of exercise prescriptions also reveal that high-intensity aerobic interval training is a safe and relatively well tolerated adjunct therapy in women undergoing anthracycline-containing chemotherapy (Hornsby et al., 2013).

Being exercise is a non-pharmacological therapy that promises significant heath improvements following cancer diagnosis and therapy with minimal adverse effects, one next logical question is whether exercise triggers biological mechanisms in non-target tissues sparing them from side effects resulting from anti-cancer treatment.

Previous work published by our group report that both acute (Ascensao et al., 2011a) and chronic exercise models (Ascensao et al., 2005a; Ascensao et al., 2005b; Ascensao et al., 2006b; Ascensao et al., 2006a) were able to positively modulate cardiac function of rats treated with a single DOX bolus, resulting in improved mitochondrial integrity and bioenergetics. However, mitochondrial mechanisms underlying the protective phenotype induced by chronic exercise during sub-chronic DOX administration were elusive. Therefore, we conducted experimental studies (Chapter 3.2 and 3.3) to better comprehend the effects of

two long-term exercise models, voluntary physical activity and forced treadmill training performed before and during sub-chronic cumulative DOX administration treatment against the perturbations in heart mitochondrial oxidative phosphorylation capacity and pro-oxidant redox modifications, alterations in mPTP susceptibility and apoptotic signaling. Moreover, in Chapter 3.2., a further step into the understanding of the molecular mechanisms related to exercise-induced cardioprotection against DOX is attempted by analyzing whether these cross-tolerance effects are also associated with alterations in the molecular markers of mitochondrial dynamics and quality control.

Although DOX-induced cardiotoxicity has been a major concern, recent therapeutic or preventive measures to counteract chemotherapy-induced cognitive impairments, including physical exercise, are receiving increasing attention (Fardell et al., 2011). Therefore, we here performed (Chapter 3.4) an experimental study to elucidate whether both models of physical exercise *per se* (forced endurance treadmill training and voluntary free wheel running activity) modulate brain cortex and cerebellum mitochondrial bioenergetics by measuring oxygen consumption, transmembrane electric potential and calcium-induced mPTP induction, as well as redox- and apoptotic-associated markers, research topics underexplored so far. Indeed, exercise has been reported to play a significant role in brain and cerebellum mitochondrial machinery improvements and likely serves to attenuate mitochondrial dysfunction that characterizes, for instances, aging and neurodegenerative diseases. However, the effects of physical exercise on the susceptibility to mPTP opening and mitochondrial quality control on brain and cerebellum mitochondria were not clarified before. Moreover, whether exercise-induced brain mitochondrial improvements are exercise model and/or intensity dependent are also addressed (Chapter 3.4).

Finally, considering the opposite effects of DOX treatment and physical exercise on mitochondrial activity, the next question was to analyze whether exercise counteracted DOX-induced brain cortex and cerebellum mitochondrial toxicity, which was never addressed before. Thus, whether brain cortex and cerebellum mitochondrial modifications associated

with cumulative sub-chronic DOX administration, including perturbations in mitochondrial oxidative phosphorylation capacity, modifications in redox sate, in mPTP susceptibility, apoptotic signaling and mitochondrial quality control were modulated by long-term physical exercise performed before and during during treatments was the focus of the Chapter 3.4.

**Aim of the present dissertation**

In the context of DOX-induced toxicity to non-target tissues, exercise promises to be an effective strategy to limit tissue and mitochondrial liabilities. However, although several reports pointed out the importance of this cross-tolerance phenomenon, more information is required regarding the mechanisms and the relationships between types of treatment and exercise models in the set of cardio and neuroprotection induced by exercise. Indeed, it is our belief that the understanding of the potential interaction between exercise, the concurrent administration of chemotherapy and the mechanisms related to decreased cardiac and brain toxicity during anti-cancer therapy targeting mitochondria are essential to fully understand the safety and efficient application of exercise models as examples of active life-styles and supportive interventions to minimize cancer treatment side effects.

Therefore, the present work can contribute to extend the knowledge in this particular area representing a step forward to understand mitochondrial-related effects of exercise combined with DOX treatment. Hence, the main objectives of the present thesis were to analyze the effect of two long-term exercise models of physical exercise (endurance treadmill training and free-wheel voluntary physical activity) performed before and during the course of treatment, against heart and brain mitochondrial dysfunction induced by sub-chronic treatment of DOX.

The theoretical background attempts to briefly review the known mechanisms by which exercise can counteract DOX induced heart mitochondrial toxicity highlighting the timings of

exercise and DOX schedules (Chapter 2.1) and to provide a detailed analysis of how brain mitochondrial activity is modulated by exercise (Chapter 2.2).

It is our belief that limiting DOX-induced mitochondrial dysfunction may be one of the mechanisms explaining the protective benefits of chronic exercise against DOX side effects to non-target tissues. Therefore, to test this hypothesis, the following experimental studies were performed: the analyzes of the role of two models of chronic exercise on the alterations in cardiac mitochondrial bioenergetics, biogenesis, morphology, oxidative phosphorylation organization and activity, and oxidative stress induced by sub-chronic treatment of DOX (Chapter 3.2). Additionally, to better understand the mitochondrial mechanisms related to exercise-induced cardioprotection against sub-chronic DOX treatment, the study of calcium-induced mPTP, apoptotic and auto(mito)phagic signaling and mitochondrial dynamic in the above mentioned conditions, was comprised (Chapter 3.3).

The adaptations induced by both models of exercise on the performance in behavioral tests and on brain and cerebellum mitochondrial bioenergetic function, mPTP opening, apoptotic signaling, oxidative status and mitochondrial quality control were analyzed (Chapter 3.4). The same experiments were performed in sub-chronic treated animals in order to examine if chronic exercise is able mitigate DOX-induced alterations in cognitive tests and in brain and cerebellum mitochondrial dysfunction (Chapter 3.5).

Further steps into the understanding of putative sensors/biological markers associated with the referred protective phenotypes that were common to the experimental studies here presented were the measurement and analysis of proteins in the context of this combination treatment. These include, for example, the expression of proteins associated with the regulation of redox environment involving mitochondria such as SIRT3 and p66shc; TFAM, PGC1α, OPA1, DRP1, Mnf 1 and Mnf 2 for mitochondrial biogenesis and dynamics; LC3, p62, Beclin, PINK 1 and Parkin for auto(mito)phagy flux control. Also, the expression of proteins associated with mitochondrial respiratory and phosphorylative activity such as OXPHOS subunits, ANT, cofilin and CypD were semiquantified.

# 2.

# THEORETICAL BACKGROUND

# 2.1

## Exercise mitigates Doxorubicin-induced heart mitocondrionopathy: An update

Marques-Aleixo I[1], Estela Santos-Alves[1], Paulo J Oliveira[2], José Magalhães[1], António Ascensão[1]

[1]CIAFEL - Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Portugal

[2]CNC - Centre for Neuroscience and Cell Biology, Department of Life Sciences, University of Coimbra, Portugal

**ABSTRACT**

Doxorubicin (DOX) is a widely used antineoplastic agent to treat a wide range of cancers, including hematological malignancies, soft tissue sarcomas and solid tumors. However, DOX exhibit a dose-related cardiac toxicity that results in life-threatening cardiomyopathy. This toxicity seems to be related, at least in part, to mitochondrial structural, molecular and functional impairments. This review focuses on the current understanding of the mitochondrial-related molecular mechanisms underlying the protective effect of chronic exercise against DOX-induced cardiotoxicity. Recent cross-tolerance studies of chronic exercise against DOX-induced cardiac and mitochondrial dysfunction comprise exercise periods during and after sub-chronic DOX treatment schedules, which is also a matter of concern in this review. In addition to the redox alterations, mitochondrial bioenergetics and related apoptotic issues, here we analyze recent developments regarding the possible role of exercise in the modulation of the delicate balance of cardiac mitochondrial dynamics and autophagy molecular regulators in rodents submitted to DOX, which may possibly represent important cellular targets by exercise.

**Keywords.** Adrimycin, cardiac injury, mitochondria, exercise

## Introduction

Doxorubicin (DOX, Adriamycin) is an anthracycline used in the treatment of a wide range of cancers, including hematological malignancies, many types of carcinoma, soft tissue sarcomas and has demonstrated significant activity against solid tumors. However, despite the well kwon efficiency of this antineoplastic agent, DOX clinical use is limited due to a dose-dependent development of cardiac toxicity and dysfunction, leading to congestive heart failure and ultimately death (Mazevet et al., 2013). Moreover, DOX-induced cardiomyocyte dysfunction is associated with increased levels of oxidative damage and apoptosis, involving mitochondria in the process (Berthiaume and Wallace, 2007; Carvalho et al., 2014; Pereira et al., 2011; Wallace, 2007). Recent data also suggest that DOX disturbs the proper balance between mitochondrial fusion and fission mechanisms that are essential for healthy cell and mitochondrial regulation (Marechal et al., 2011). Moreover, DOX may induces dyhomeostasis of cellular and mitochondrial quality control regulation (Lu et al., 2009; Zhang et al., 2009). Together, the shifts of these processes from regulatory and adaptive functions to disruptive unbalances are thought to contribute to the cardiac dysfunctional phenotype observed during and after DOX treatments and may represent therapeutic targets against the related side-effects.

The limitation of DOX clinical use compromises its effectiveness in the reduction or elimination of tumor cell growth. Therefore, an ongoing challenge in cancer treatment is to exploit the DOX anti-tumor effects, while minimize cardiac toxicity and the associated heart mitochondrial damage. One of the most studied non-pharmacological and promising strategies used to counteract DOX side effects is chronic physical exercise (Ascensao et al., 2011c; Ascensao et al., 2012). Considering the important role of mitochondrial dysfunction in DOX-related cardiotoxicity and, at the same time,

the beneficial role of physical exercise *per se* and in DOX treated animals regarding the cardiac mitochondrial modulation justify the increasing interest in these organelles in this cross tolerance phenomena.

The present review briefly highlights some recent published data showing the role of exercise, particularly chronic interventions, as a strategy to mitigate cumulative DOX-induced cardiac toxicity, targeting the mitochondrial-driven mechanisms including apoptotic signaling, mitochondrial dynamics and quality control.

**Exercise: what type, when and how much?**

Physical exercise as been associated with the modulation of important physiological mechanisms, up-regulating some important cardiac defense systems to antagonize the toxic effects caused by DOX treatment. When exercise is used as a preventive and/or therapeutic strategy to counteract the collateral effects of anticancer drugs, as well as in the context of cancer patients-related fatigue, a brief analysis of the different exercise models (type, timings, intensities and volumes) may be useful.

Reports exist showing a preconditioning-like effect of a single exercise bout against cardiac dysfunction and mitochondrionopathy characterizing DOX administration (Ascensao et al., 2011a; Wonders et al., 2008). Moreover, some authors frequently use very short-term exercise running programs comprising only five exercise days followed by the induction of cardiac pathological state including myocardial ischemia-reperfusion injury or DOX-related cardiac dysfunction (Kavazis et al., 2010; Lee et al., 2012; Smuder et al., 2013). However, despite useful to unravel potential mechanisms related to cardioprotection, these short duration pre-conditioning models preceding the diseased state are unlikely to result in prolonged functional and general health benefits

to the subjects with early and/or late dysfunction such as those characterizing subjects undergoing DOX-based chemotherapy. Therefore, chronic exercise models of long duration have been widely studied and include forced moderate or low intensity endurance treadmill training and voluntary running on free wheels.

DOX patients undergoing chemotherapy exhibit severe fatigue and considerable exercise intolerance, being therefore unable to exercise at high intensities. This may possibly limit the perspectives of exercise use as a co-adjuvant therapy in patients undergoing DOX treatment (Emter and Bowles, 2008). However, it has been suggested that a supervised aerobic training program comprising high-intensity aerobic interval training is a safe (and relatively well tolerated) during adjunct therapy in women undergoing anthracycline-containing chemotherapy (Hornsby et al., 2014). This interval-training model may present some similitude to voluntary free wheel running, in which rodents perform peak running periods interspersed by low intensity or resting. Although low-to moderate-intensity exercise is currently recommended and considered safe for patients undergoing DOX treatment (Hayes et al., 2009; Schmitz et al., 2010), these contradictory results/opinions justify the need to further investigate different exercise models to optimize cardiovascular benefits required to antagonize DOX side effects.

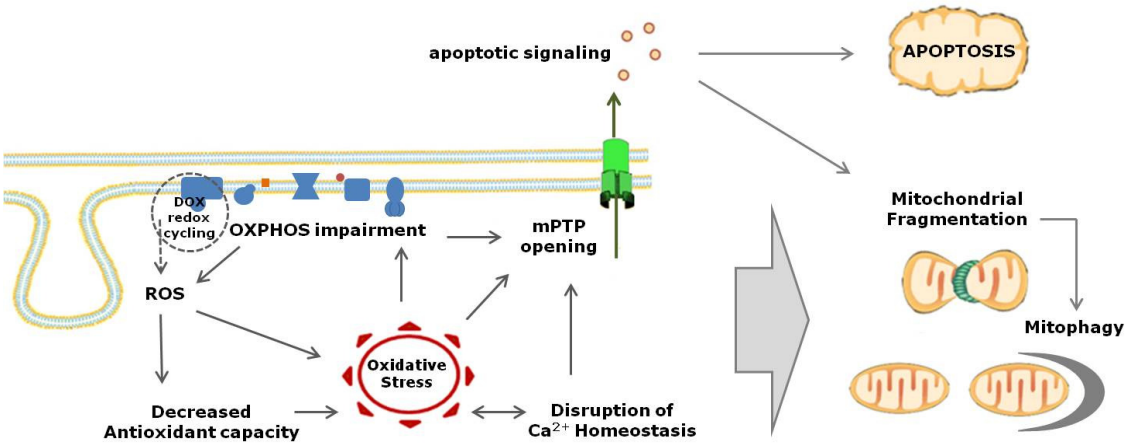
Besides exercise intensity, the timing of exercise and DOX treatment schedule (pre-conditioning, overlapping, rehabilitative), representing both preventive and/or therapeutic counter measurements, has also been a concern. The most explored model exercise preconditioning i. e., performed prior to DOX bolus, prevented DOX-induced impairments in LV systolic and diastolic function (Chicco et al., 2005, 2006b), the increased plasma cardiac troponin I (cTnI) levels (Ascensao et al., 2005a; Ascensao et al., 2006b) and attenuate the severe morphological signs of cardiac muscle injury (Ascensao et al., 2006b). Importantly, most studies addressing exercise

preconditioning used a single DOX bolus instead of a cumulative DOX treatment, as it resembles usual DOX treatments typically involving small doses administered over time to reach a cumulative dose. In an attempt to respond to this concern, Hydock and collaborators (2011) reported that 10 weeks of exercise preconditioning following sub-chronic DOX administration preserved *in vivo* and *ex vivo* cardiac function, suggesting that training status may be a determining factor in the degree of late-onset cardiotoxicity experienced by cancer patients undergoing treatment with DOX. However, understanding the potential benefits of chronic exercise as a rehabilitative strategy to counteract DOX side effects in untrained individuals is a current challenge. Chronic exercise performed during and/or after sub-chronic DOX treatment decreased sub-chronic DOX-induced histopathological myocyte damage (Kanter et al., 1985) and protected/attenuated cardiac hemodynamic alterations (Chicco et al., 2006a; Hayward et al., 2012b; Hydock et al., 2012b). Recently, our group reported that two different models of chronic exercise (voluntary and treadmill running) performed before and during sub-chronic DOX treatment prevented DOX-induced mitochondrial morphological disarrangements caused by DOX (Marques-Aleixo et al., a; Marques-Aleixo et al., b). What should be an additional concern is the effects of exercise in the efficacy of DOX in reducing tumor growth. However, data suggested that exercise provides a safe cardioprotective intervention without reducing the antitumor efficacy of DOX (Jones et al., 2005).

Collectively, these evidences support that chronic exercise can be a promising strategy to prevent or treat DOX cardiotoxic effects. Current knowledge proposes several putative molecular mechanisms underlying the protective benefits of chronic exercise against the adverse cardiac complications subsequent of DOX treatment, including those involving mitochondria and associated pathways, which are briefly described herein.

**Exercise mitigates DOX-induced cardiac mitochondrionopathy: modulation of redox state, apoptotic signaling and mitochondrial network**

Several mechanisms have been proposed to explain DOX-induced cardiomyophathy, suggesting that this is a multifactorial process. One of recognized mechanisms of DOX-induced cardiotoxicity is associated with an increase in oxidative stress, as DOX undergoes redox cycling at complex I of the electron transport chain, resulting in the formation of reactive oxygen species (ROS), as evidenced by increased oxidative stress markers, along with reduced levels of antioxidants (Davies and Doroshow, 1986; Doroshow and Davies, 1986). Several reviews have already covered that DOX-induced increased oxidative damage is associated mitochondrial bioenergetic disruption, interference with calcium homeostasis and enhanced apoptotic signaling thought the increased susceptibility to mitochondrial permeability transition pore (mPTP) (Ascensao et al., 2011c; Ascensao et al., 2012; Carvalho et al., 2014; Mazevet et al., 2013; Pereira et al., 2011). The most common pathways underlying DOX-induced cardiac mitochondrial toxicity are briefly summarized in Figure 1.




**Chapter 2.1 - Figure 1.** Mechanisms of DOX-induced cardiac mitochondrial toxicity. DOX is reduced by complex I, forming a highly reactive semiquinone, initiating a redox cycle after reacting with oxygen and releasing ROS in the process. DOX-increased ROS generation compromise antioxidant machinery and damages to lipids, proteins and nucleic acids, which results in an inhibition of oxidative phosphorylation, mitochondrial depolarization, ATP depletion, loss of calcium ($Ca^{2+}$) loading capacity, mPTP susceptibility and release of pro-apoptotic proteins. Together, these events may also result mitochondrial fragmentation and contribute to an increased auto(mito)phagy signaling.

Considering the multiple mechanisms by which DOX-induced mitochondrial impairment can lead to dysfunction or even cardiomyocyte death, many pharmacological and non-pharmacological interventions have been studied targeting mitochondria in order to minimize DOX cardiac alterations, including physical exercise.

Mitochondrial adaptations are likely to be crucial in exercise-induced cardioprotection, as the increased demand in contractility results in a consequent rise in oxygen consumption and in the rate of mitochondrial ATP synthesis (Ascensao et al., 2007; Ascensao et al., 2011c). Those are important metabolic adaptations on the mitochondrial phosphorylative system that can result in improved ability to oxidize substrates (Ascensao et al., 2005b; Ascensao et al., 2006a; Ascensao et al., 2006b; Ascensao et al., 2007; Burelle et al., 2004; Magalhaes et al., 2013) and could afford an

increased tolerance of these organelles to harmful physiopathological conditions associated with mitochondrial dysfunction, including DOX treatment.

The heart is particularly susceptible to DOX-induced oxidative damage because of the large density/volume of mitochondria. Therefore, since chronic physical activity is known to interfere with function and plasticity of these organelles, it is expected that exercise positively modulate some important cardiac defense systems to antagonize the toxic effects caused by DOX treatment (Ascensao et al., 2011c; Ascensao et al., 2012).

Chronic physical exercise is widely documented to improve the antioxidant machinery and consequent decreased of oxidative stress markers (Ascensao et al., 2005b, 2006c; Ascensao et al., 2006a; Ascensao et al., 2007; Kavazis et al., 2008; Kavazis et al., 2009; Starnes and Taylor, 2007). This is particularly important, as heart has lower antioxidant defenses compared with other tissues (Ascensao et al., 2005b; Kaiserova et al., 2007) and a common response to DOX treatment is the decrease in the content/activity of antioxidant enzymes (Carvalho et al., 2014; Pereira et al., 2011; Takemura and Fujiwara, 2007). Additionally, exercise as been associated to an augmented capacity of mitochondria to tolerate calcium, with further consequences on the control of mitochondrial-driven apoptotic cell death (Ascensao et al., 2005b; Kavazis et al., 2008; Siu et al., 2004). Again, these evidences provide a reasonable hypothesis for mitochondrial protection afforded by exercise training, as increased levels of mitochondrial-mediated apoptotic cell death markers have also been observed following DOX treatment (Carvalho et al., 2014; Pereira et al., 2011). Although the effects of exercise on calcium induced mPTP susceptibility are not completely understood (for refs see Ascensao et al., 2011c), it has been reported that acute and chronic exercise prevented increased mPTP susceptibility in cardiac mitochondria of DOX treated animals (Ascensao et al., 2005b; Ascensao et al., 2005a; Ascensao et al.,

2011b; Marques-Aleixo et al., b), shifting heart mitochondria from exercised animals to a more resistant phenotype against DOX-induced apoptosis.

During stressful conditions, cardiac myocytes early develop a defense mechanism against aberrant mitochondria that can cause harm to the cell, involving selective sequestration and subsequent degradation of the dysfunctional mitochondria before it causes activation of cell death (Kubli and Gustafsson, 2012a). Besides energy production, a critical role of mitochondria to ensure proper cardiac muscle contraction involves the regulation and adaptations in mitochondrial network structure (Rimbaud et al., 2009). The mitochondrial plastic proprieties are driven from the mitochondrial dynamic interaction of fusion, fission, auto(mito)phagy and mitochondrial biogenesis, which ensures proper organization of the mitochondrial network (Chen and Chan, 2004). Currently, DOX-induce cardiotoxicity has been suggested to be associated with disorganization of the mitochondrial network into a fragmented profile (Parra et al., 2008; Sardao et al., 2009) and the inhibition of mitochondrial fission protects the heart against DOX-induced cardiac injury (Gharanei et al., 2013). Moreover, the loss of mitochondrial connectivity can predisposes cardiomyocytes to apoptosis (Chen and Knowlton, 2010; Martinou and Youle, 2006), and/or mitochondrial division can designate dysfunctional organelles with low membrane potential for mitophagy (Youle and Narendra, 2011).

The precise mechanism linking DOX cardiomyopathy to mitochondrial dynamics, apoptosis and auto(mito)phagy is still lacking. Recent divergent results regarding the effects of DOX treatment on auto(mito)phagic flux suggest both adaptive and maladaptive consequences as follows, probably dependent on the severity of the stimulus: (i) DOX treatment attenuate Parkin-mediated mitophagy (Hoshino et al., 2013), (ii) cause a shift from autophagy to apoptosis (Dickey et al., 2013), and (iii) inducing autophagy could be an adjuvant therapy to mitigate DOX-induced myocardial

damage (Dickey et al., 2013; Kawaguchi et al., 2012; Sishi et al., 2013); others associate DOX treatment with elevated autophagic signaling, suggesting that increased autophagy mediates DOX-induced cardiotoxicity (Dimitrakis et al., 2012; Kobayashi et al., 2010; Lu et al., 2009; Marques-Aleixo et al., b; Smuder et al., 2013; Xu et al., 2012; Zhang et al., 2009)

Recent studies suggested that chronic exercise-induced cardioprotection could be associated with an improvement of mitochondrial quality control (Campos et al., 2012; Sun et al., 2013). Exercise increases cardiac mitochondria renewal and remolding by promoting mitochondrial biogenesis, fusion, and auto(mito)phagy, to eliminate damaged mitochondria (Sun et al., 2013). Together with redox-related and anti-apoptotic protective adaptations, the mitochondrial network modulation provided by chronic physical exercise can be an important step in the mitigation of DOX-induced cardiac and mitochondrial toxicity (Marques-Aleixo et al., a; Marques-Aleixo et al., b; Smuder et al., 2013).

The current knowledge concerning mitochondrial-mediated mechanisms by which exercise preconditioning and exercise during therapy can counteract DOX cardiotoxicity are detailed bellow.

**The exercise preconditioning-like effect**

Physical exercise preconditioning (performed before acute single dose of DOX treatment) has been reported to be an effective strategy to counteract DOX-induced ROS production thought the enhancement of antioxidant signaling up regulation along with the improvement of mitochondrial bioenergetics, apoptotic signaling and calcium loading capacity, important mechanisms commonly disarranged and considered

markers of DOX cardiotoxicity (Ascensao et al., 2011c; Ascensao et al., 2012; Scott et al., 2011).

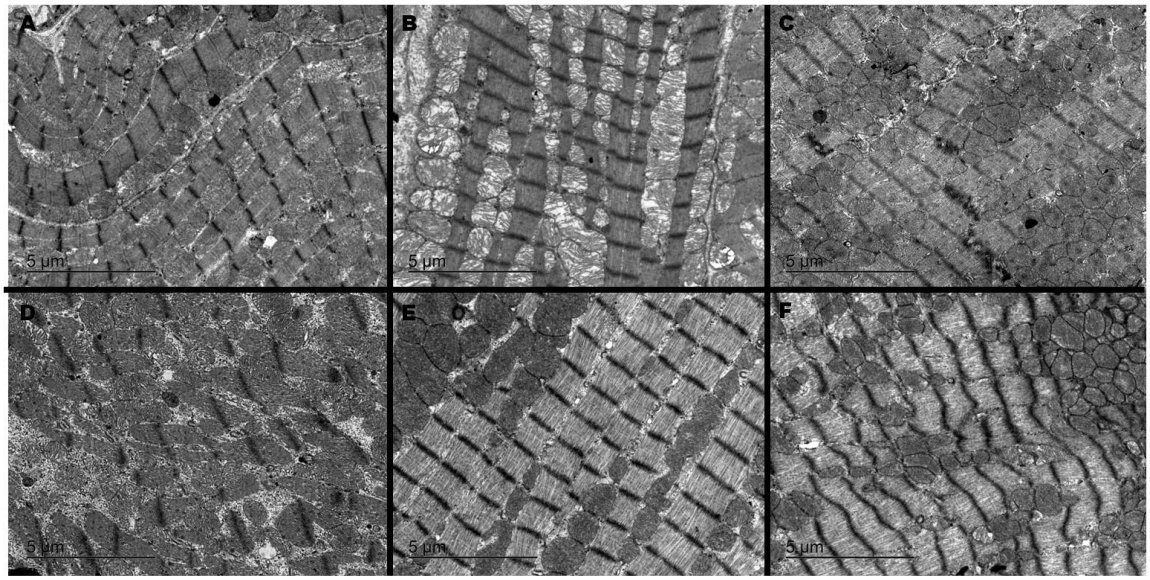
Several authors reported that exercise preconditioning (short and long-term exercise training - 5 days to 14 weeks) is an effective strategy to counteract acute single dose of DOX (10 to 20 mg/kg)-induced cardiac oxidative damage (Ascensao et al., 2005a; Ascensao et al., 2005b; Ascensao et al., 2006a; Ashraf and Roshan, 2012; Chicco et al., 2006b; Kavazis et al., 2010; Shirinbayan and Roshan, 2012), mitochondrial bioenergetics (Ascensao et al., 2005b; Ascensao et al., 2006a; Kavazis et al., 2010) and apoptotic signaling (Ascensao et al., 2005b; Kavazis et al., 2010). Generally, these results suggested that an active life style prior to DOX treatment could prevent DOX cardiotoxicity, targeting mitochondrial bioenergetics collapse and pro-apoptotic and oxidant unbalance characterizing DOX treatment. Table 1 detailed the effects of chronic exercise preconditioning against the alterations in markers of mitochondrial dysfunction induced by DOX.

**Exercise during the course of DOX treatment**

Despite exercise-based rehabilitation has been considered as an integral component of clinical cardiac disease management, some of the cellular and molecular mechanisms underlying the effects of exercise on target organs remains incompletely understood. The effect of chronic exercise performed during DOX therapy were first examined by Kanter and co-workers (1985), reporting that 21 weeks of swimming increased blood antioxidant levels, mitigating DOX-induced cardiac structural damage. Short-term (2 weeks) and low intensity treadmill exercise performed during the course of sub-chronic DOX treatment enhanced antioxidant protection and inhibit apoptotic signaling (Chicco et al., 2006a). Accordingly, concurrent moderate aerobic training and DOX treatment

increased MnSOD, decreased oxidative damage and attenuated the decreased activity of mitochondrial ETC complexes I and II in DOX-treated mice (Dolinsky et al., 2013).

Regardless exercise intensity, recent studies support the protective effect of exercise against DOX by exploring some molecular mechanisms and signaling pathways related to mitochondrial disruption. Voluntary exercise and treadmill running performed during sub-chronic DOX treatment counteracts the impaired mitochondrial morphology (Figure 2), bioenergetics, complex I activity and content, increased oxidative damage and the worsted antioxidant machinery, increased apoptotic signaling and mPTP susceptibility characterizing DOX (Marques-Aleixo et al., a; Marques-Aleixo et al., b).




**Chapter 2.1 - Figure 2.** Representative electron micrographs of cardiac tissue from (A) saline+sedentary group, (B) saline+TM (12-weeks treadmill) group, (C) saline+FW (12-weeks voluntary free-wheel) group, (D) DOX (7-weeks of sub-chronic DOX treatment 2mg.kg-1 *per* week)+sedentary group (E) DOX+TM group and (F) DOX+FW group (magnification: 12000 x). Note the protection afforded by chronic exercise (E and F) against impaired mitochondrial density and morphological alterations characterizing heart tissue from sedentary DOX-treated group (D). From Marques-Aleixo et al.(a) with permission.

**Exercise modulates mitochondrial dynamics and auto(mito)phagy in the context of DOX treatment**

As mitochondria are organelles with multiple interconnected cell functions, recent works have explored the effects of chronic exercise on mitochondrial network responses including auto(mito)phagy and dynamics as potential subcellular consequences of the attenuated cardiac stress of DOX-treated animals and/or mechanistic targets aiming at counteracting DOX-related cardiac side-effects (Dolinsky et al., 2013; Marques-Aleixo et al., b; Smuder et al., 2013).

The effect of exercise on the regulation of mitochondrial dynamics is scarcely explored, as we only identify two studies examining the modulation of primary regulators of mitochondrial dynamics machinery, including Mfn1/2 (Dolinsky et al., 2013), OPA1 and DRP1 (Marques-Aleixo et al., b) by chronic exercise during the course of DOX treatment. Both studies suggested that exercise might have contributed to a proper regulation of mitochondrial dynamics essential for cellular survival.

Additionally, auto(mito)phagy are also tightly associated with mitochondrial network regulation and renewal. It has been suggested that DOX effect on cell death pathways is variable, probably depending on the therapy time course and drug doses. Considering these conditions, a physiological stress scale is suggested, ranging from i) mild stress with autophagy induction removing aggregates and damaged organelles, ii) intermediate stress with apoptosis and mitochondrial involvement to iii) intense stress with necrosis following ATP depletion (Zhang et al., 2009). However, studies have shown the coexistence of increased apoptosis and auto(mito)phagy in hearts of DOX-treated rats, both attenuated by voluntary and forced endurance exercise during DOX treatment (Marques-Aleixo et al., b). Similarly, exercise preconditioning might have a role a against DOX-induced activation of cardiac autophagy/lysosomal system pathway (Smuder et al., 2013). From the data obtained, it is likely that the used acute and sub-

chronic DOX dosages in animal studies are above the referred mild stress or adaptive threshold, in which auto(mito)phagy activation contributes to increase cellular turnover by eliminating aggregates and damaged organelles. In this context of cardioprotection, physical exercise seems to appear as a strategy to attenuate unnecessary high levels of auto(mito)phagy activation by DOX. However, it is possible that a delicate balance between life and death in the myocytes during stress may occur, being the final outcome dependent on the complex cross-talk between mitophagy and apoptotic cell death (Kubli and Gustafsson, 2012a). These authors argue that under tolerable and mild stressful conditions, mitophagy functions as an early cardioprotective response, favoring adaptation to stress by removing few damaged or aberrant mitochondria, which are rapidly sequestered by autophagosomes. In contrast, in response to severe stress, there is an overwhelming mitochondrial damaged that autophagosomes are unable to efficiently clear. These structurally and functionally damaged mitochondria increase free radical production with consequent oxidative stress, release of prodeath proteins from intermembrane space, and increase apoptotic protease activity, resulting in attenuated mitophagic flux and allowing for the execution of cell death.

The combination of different DOX schedules and therapies and exercise models as cardioprotective interventions against DOX effects on myocardial death pathways needs to be further investigated. Table 1 summarizes the effects of exercise preconditioning and exercise performed during DOX treatment against DOX-induced deregulation of mitochondrial dynamics and auto(mito)phagy.

**Chapter 2.1 - Table** 1. Summary of some described mitochondrial-related alterations associated with DOX-induced cardiotoxicity and the modulation effect afforded by chronic exercise preconditioning and exercise during DOX treatment course.

| | DOX effect | | Chronic exercise preconditioning | | Exercise during DOX treatment | |
|---|---|---|---|---|---|---|
| | | References | | References | | References |
| **Mitochondrial morphology** | | | | | | |
| Abnormal mitochondria | ↑ | Ascensão et al. (2005b; 2006b); Berthiaume et al. (2005); Marques-Aleixo et al. (a); Oliveira et al. (2006); Santos et al. (2002); Ikeda et al. (2010) | ↓ | Ascensão et al. (2005b; 2006b) | ↓ | Marques-Aleixo et al. (a) |
| Density | ↓or↑ | Marechal et al. (2011); Marques-Aleixo et al. (a) | | | ↑ | Marques-Aleixo et al. (a) |
| **Oxidative stress** | | | | | | |
| Antioxidants | ↓or= | Ascensão et al. (2005b; 2006a); Chicco et al. (2006a); Dolinsky et al. (2013); Kavazis et al. (2010); Marques-Aleixo et al. (a) | ↑ | Ascensão et al. (2005b; 2005a; 2006a) | ↑or= | Chicco et al. (2006a); Dolinsky et al. (2013); Marques-Aleixo et al. (a) |
| Oxidative damage | ↑or= | Ascensao et al. (2006a); Berthiaume et al. (2005); Childs et al. (2002); Dolinsky et al. (2013); Kavazis et al. | ↓ | Ascensão et al. (2005b; 2005a; 2006a); Kavazis et al. (2010) | ↓or= | Chicco et al. (2006a); Dolinsky et al. (2013); Marques-Aleixo et al. (a) |

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|---|---|---|---|---|---|---|
| | | (2010); Marques-Aleixo et al. (a); Ikeda et al. (2010) | | | | |
| HSPs | ↑or= | Ascensão et al. (2005a; 2006a); Chicco et al. (2006a) | ↓ | Ascensão et al. (2005a; 2006a); Kavazis et al. (2010) | = | Chicco et al. (2006a) |
| Sirt3 | ↓ | Marques-Aleixo et al. (a) | | | ↑ | Marques-Aleixo et al. (a) |
| p66Shc(pSer$^{36}$)/p66Shc | ↑ | Marques-Aleixo et al. (a) | | | ↓ | Marques-Aleixo et al. (a) |
| **Mitochondrial bioenergetics** | | | | | | |
| State 3 | ↓ | Ascensão et al. (2005b; 2006a); Childs et al. (2002); Kavazis et al. (2010); Marechal et al. (2011); Marques-Aleixo et al. (a); Santos et al. (2002); Yen et al. (1999) | ↑or= | Ascensão et al. (2005b; 2006a) | ↑ | Marques-Aleixo et al. (a) |
| RCR | ↓or= | Ascensão et al. (2005b; 2006a); Kavazis et al. (2010); Marechal et al. (2011); Marques-Aleixo et al. (a); Pereira et al. (2012); Santos et al. (2002); Yen et al. (1999) | ↑or= | Ascensão et al. (2005b; 2006a); Kavazis et al. (2010) | ↑ | Marques-Aleixo et al. (a) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Maximal $\Delta\psi$ | ↓or= | Marechal et al. (2011); Marques-Aleixo et al. (a); Pereira et al. (2012) | | | ↑ | Marques-Aleixo et al. (a) |
| Lagphase | ↑or= | Marques-Aleixo et al. (a); Pereira et al. (2012) | | | ↓ | Marques-Aleixo et al. (a) |
| Complex I | ↓ | Dolinsky et al. (2013); Marques-Aleixo et al. (a); Santos et al. (2002); Yen et al. (1999) | | | ↑ | Dolinsky et al. (2013); Marques-Aleixo et al. (a) |
| Complex II | ↓or= | Dolinsky et al. (2013); Marques-Aleixo et al. (a); Yen et al. (1999) | | | ↑or= | Dolinsky et al. (2013); Marques-Aleixo et al. (a) |
| Complex V | ↓or= | Marques-Aleixo et al. (a) | | | ↑ | Marques-Aleixo et al. (a) |
| **Apoptosis** | | | | | | |
| mPTP susceptibility | ↑ | Marechal et al. (2011); Marques-Aleixo et al. (b); Santos et al. (2002) | ↓ | Ascensão et al. (2005b) | ↓ | Marques-Aleixo et al. (b) |
| Bax/Bcl2 | ↑ | Ascensao et al. (2005b); Childs et al. (2002); Ikeda et al. (2010); Marques-Aleixo et al. (b) | ↓ | Ascensão et al. (2005b) | ↓ | Marques-Aleixo et al. (b) |
| Caspase 3 | ↑ | Chicco et al. (2006a); Childs et al. (2002); Dickey et al. (2013); Marques-Aleixo et al. (b) | ↓ | | ↓ | Marques-Aleixo et al. (b) Chicco et al. (2006a) |

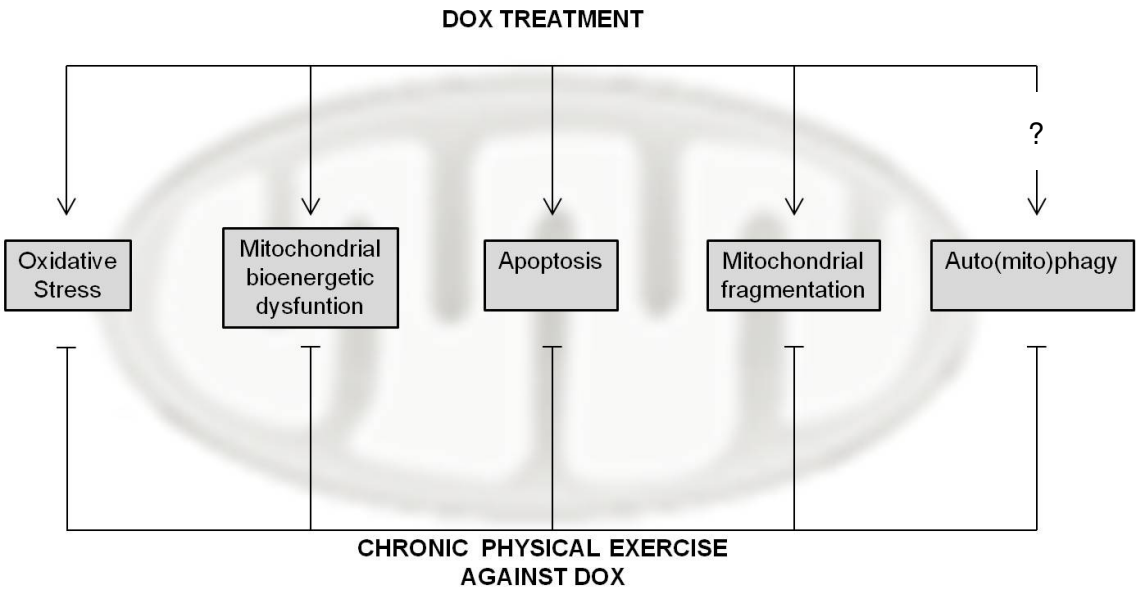
| | | | | | | |
|---|---|---|---|---|---|---|
| Caspase 8 | ↑ | Marques-Aleixo et al. (b) | | | ↓ | Marques-Aleixo et al. (b) |
| Caspase 9 | ↑ | Ascensão et al. (2005b) | ↓ | Ascensão et al. (2005b) | ↓ | Marques-Aleixo et al. (b) |
| **Mitochondrial biogenesis** | | | | | | |
| PGC1α | ↑or= | Marechal et al. (2011); Marques-Aleixo et al. (a) | | | = | Marques-Aleixo et al. (a) |
| TFAM | ↓ | Marques-Aleixo et al. (a); Ikeda et al. (2010) | | | ↑ | Marques-Aleixo et al. (a) |
| **Mitochondrial dynamics** | | | | | | |
| Mfn1/2 | ↓,↑or= | Marques-Aleixo et al. (b);Dolinsky et al. (2013); Marechal et al. (2011) | | | ↑ | Marques-Aleixo et al. (b); Dolinsky et al. (2013) |
| Opa1 | ↓or↑ | Marques-Aleixo et al. (b) Marechal et al. (2011) | | | ↑ | Marques-Aleixo et al. (b) |
| DRP1 | ↑or= | Marques-Aleixo et al. (b); Marechal et al. (2011) | | | ↓ | Marques-Aleixo et al. (b) |
| **Auto(mito)phagy** | | | | | | |
| Beclin | ↑ | Marques-Aleixo et al. (b); Smuder et al. (2013) | ↓or= | Smuder et al. (2013) | ↓ | Marques-Aleixo et al. (b) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Beclin/Bcl2 | ↑ | Marques-Aleixo et al. (b); Smuder et al. (2013) | ↓ | Smuder et al. (2013) | ↓ | Marques-Aleixo et al. (b) |
| Atg12 | ↑or= | Smuder et al. (2013) | ↓ | Smuder et al. (2013) | | |
| Atg12-Atg5 | ↑ | Smuder et al. (2013) | ↓ | Smuder et al. (2013) | | |
| Atg4 | ↑or= | Smuder et al. (2013) | ↓or= | Smuder et al. (2013) | | |
| Atg7 | ↑or= | Smuder et al. (2013) | ↓or= | Smuder et al. (2013) | | |
| LC3II/LC3I | ↑ | Marques-Aleixo et al. (b); Smuder et al.(2013) | = | Smuder et al. (2013) | ↓ | Marques-Aleixo et al. (b) |
| Pink | ↑ | Marques-Aleixo et al. (b) | | | ↓ | Marques-Aleixo et al. (b) |
| Parking | = | Marques-Aleixo et al. (b) | | | = | Marques-Aleixo et al. (b) |
| p62 | ↑ | Marques-Aleixo et al. (b) | | | = | Marques-Aleixo et al. (b) |

HSPs- heat shock proteins; Sirt3- silent mating type information regulation 2, homolog 3; p66(pSer$^{36}$)/p66Shc- ratio between p66Shc phosphorylated at serine in position-36 and p66Shc; RCR – respiratory control ratio; ∆ψ- transmembrane potential; mPTP- mitochondrial permeability transition pore; Bax/Bcl2- ratio between pro-apoptotic (Bax) and anti-apoptotic (Bcl2); PGC1α - Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; TFAM - mitochondrial transcription factor A; Mfn1/2- mitofusins 1 and 2; Opa1 – optic atrophy 1; DRP1 - dynamin-related protein 1; Atg- autophagy-related proteins; LC3 - Microtubule-associated protein 1A/1B-light chain 3; Pink - PTEN-induced putative kinase 1; ↑increase; ↓decrease; = no alterations.

## Concluding Remarks

To mitigate DOX cardiac tissue and mitochondrial toxicity remains an actual challenge. The data here discussed suggests that chronic physical exercise is a promising strategy to prevent (preconditioning) or to act as an adjuvant therapy (during the course of DOX treatment) against DOX cardiac side effects. Exercise-induced beneficial adaptations are associated, at least in part, with the modulation of mitochondrial-related mechanisms, including up-regulation of antioxidant machinery, the regulation of apoptotic and auto(mito)phagy cell death pathways and quality control as well as with efforts to reestablish cardiac mitochondrial network (Figure 2). Cardiac mitochondrial plasticity and ability to adapt could be central to explain the protective phenotype induced by chronic exercise before and during DOX exposure. Further studies are required to better elucidate mitochondrial molecular mechanisms underlying the cardio and mitochondrial protective proprieties of exercise. Finally, exercise intensity, volume, timing and duration should be further investigated in order to fully explore the benefits of exercise in the setting of DOX therapy.




**Chapter 2.1 - Figure 3.** Scheme summarizing the described adaptations induced by chronic physical exercise against heart mitochondrial dysfunction induced by DOX treatment.

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# 2.2

# Physical exercise as a possible strategy for brain protection: Evidence from mitochondrial-mediated mechanisms

Marques-Aleixo I[1], Paulo J Oliveira[2], Paula I. Moreira[3] José Magalhães[1], António Ascensão[1]

[1]CIAFEL - Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Portugal

[2]CNC - Centre for Neuroscience and Cell Biology, Department of Life Sciences, University of Coimbra, Portugal

[3]Institute of Physiology, Faculty of Medicine, University of Coimbra, Portugal

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**ABSTRACT**

Aging and neurodegenerative conditions such as Alzheimer and Parkinson diseases are characterized by tissue and mitochondrial changes that compromise brain function. Alterations can include increased reactive oxygen species production and impaired antioxidant capacity with a consequent increase in oxidative damage, mitochondrial dysfunction that compromises brain ATP production, and ultimately increased apoptotic signaling and neuronal death. Among several non-pharmacological strategies to prevent brain degeneration, physical exercise is a surprisingly effective strategy which antagonizes brain tissue and mitochondrial dysfunction. The present review aims to discuss the role of physical exercise in the modulation of the mechanisms involved in neuroprotection including the activation of signaling pathways underlying brain protection.

**Keywords:** exercise, brain, bioenergetics, neuroprotection

## Introduction

Aging is accompanied by structural and neurophysiological alterations in the brain, leading to variable degrees of cognitive decline associated with neurodegenerative diseases. In fact, with increasing life expectancy, age-related neurodegenerative disorders are dramatically becoming more prevalent and represent one of the major health problems in our society. However, the etiology of most neurodegenerative diseases, such as Alzheimer (AD) and Parkinson (PD) diseases, is highly complex and multifactorial. These diseases are not only consequence of genetic predisposition but also a result from environmental and endogenous factors (Correia et al., 2010; Migliore and Coppede, 2009b, 2009a). Hypertension, hypercholesteromia, obesity, diabetes and chronic inflammation can significantly influence the onset and the progression of neurodegenerative diseases (for refs see Kern and Behl, 2009).

Viable mitochondria are vital to the homeostasis of mammalian systems. The decline of mitochondrial function can be a primary contributor to the aging process (Aliev et al., 2009a; Bishop et al., 2010; Boveris and Navarro, 2008a) and to the development of several neuropathological conditions (Aliev et al., 2009b). In most mammalian studies, the decline of mitochondrial function is associated with health impairment and shorter lifespan and may contribute to brain aging and increased neuronal susceptibility to age-related pathologies (Bishop et al., 2010; Haigis and Yankner, 2010; Lin and Beal, 2006; Moreira et al., 2010b). Among other factors, the overproduction of mitochondrial reactive oxygen species (ROS) could be a crucial contributor to brain senescence and neurodegeneration (Boveris and Navarro, 2008b; Gilmer et al., 2010; Meng et al., 2007; Navarro and Boveris, 2007b, 2010). The gradual and chronic accumulation of oxidation products can also compromise brain cell structure and its constituents, particularly mitochondrial structure and function, and trigger apoptotic pathways that ultimately result in neuronal death (Andersen, 2004; Boveris and Navarro, 2008a).

Considering the importance of mitochondrial machinery in neuronal function, mitochondria are a potential target for pharmacological and non-pharmacological approaches to counteract neurodegenerative disorders. Physical exercise has been proposed as one of the best non-pharmacological strategies that can be used to antagonize brain dysfunction associated with age-related neurodegenerative diseases (Radak et al., 2010). Endurance training involves a series of adaptations usually leading to the upregulation of tissue protective mechanisms (Ascensao et al., 2007; Goto et al., 2007; Somani and Husain, 1996). These adaptations include increased mitochondrial biogenesis and function, and improvement in antioxidant networks, leading to a more effective control of free radical production. These same responses have also been reported in brain tissue, suggesting that physical exercise is an important therapeutic and/or protective mediator of neuroprotection through mitochondrial-mediated mechanisms (Navarro et al., 2004). Indeed, mitochondria are essential organelles involved in appropriate bioenergetic adaptation of neurons, increased neuronal activity and synaptic plasticity in response to exercise (Dietrich et al., 2008). Additionally, moderate exercise triggers regulatory responses that delay some age-dependent brain mitochondrial decline such as increased oxidative stress and decreased mitochondrial enzymatic activities (Navarro et al., 2004).

The present review discusses neurodegenerative mechanisms mediated by mitochondrial dysfunction and describes the potentiality of physical exercise as a mediator of neuroprotection. The role of mitochondria as critical organelles responsible for adaptive responses with potential beneficial effects in prevention and/or attenuation of neurodegenerative diseases is also described.

**Mitochondrial dysfunction in aging and neurodegeneration**

Functional mitochondria are crucial for ATP production, intracellular calcium ($Ca^{2+}$) regulation as well as for redox and apoptotic signaling. In particular, neurons require large amounts of

energy and need to conduct this energy waves across long distances. Mitochondria supply most of the energy used in neurons through oxidative phosphorylation. ATP-dependent processes such as ion transport, receptors function, vesicle release and recycling of neurotransmitters are critically dependent on mitochondrial bioenergetics (Chan, 2006; Hoppins et al., 2007; Knott and Bossy-Wetzel, 2008). Importantly and as will be described below, the ability of mitochondria to fuse, divide and migrate throughout the extended neuronal processes explain their plasticity in terms of ATP supply where it is most needed (Lovas and Wang, 2012). Mitochondria also play a vital role in synaptic maintenance through their ability to buffer cytosolic $Ca^{2+}$ (Knott and Bossy-Wetzel, 2008).

**Aging and neurodegeneration: role of mitochondrial dysfunction and oxidative stress**

The mechanistic distinctions between normal aging and neurodegenerative diseases are difficult to define. Throughout normal brain aging, gradual alterations are expected to occur in memory and cognition processes, as well as in physical or motor skills, although in a much less severe rate than in neurodegenerative diseases. Numerous theories have attempted to explain the aging process from molecular to systemic level, reflecting the complexity of the whole process. One of the most highlighted, “the free-radical theory of aging”, was first postulated by Harman (1956), and has been subject of an intense debate in the scientific community. Briefly, this theory sustains that critical cellular components are under constant injury by free radicals, resulting in structural damage and altered function in many of these components. A few years later, Harman (1972) modified his own theory into the new “mitochondrial theory of ageing”, based on the dependency of mammalian cells and systems on healthy mitochondria and their inherent production of free radicals. In this revised theory, a progressive cellular aging occurs due to accumulating oxidative damage to mitochondria and failure of cellular bioenergetic processes.

Mitochondrial dysfunction is associated with the pathogenesis of several neurodegenerative diseases, including AD (for refs see Moreira et al., 2010a) and PD (for refs see Arduino et al.,

2010). Mitochondrial dysfunction plays a critical role in the pathologic mechanisms of neurologic disorders or diseases associated with the aging process (Beal, 2005; Morais and De Strooper, 2010; Moreira et al., 2010b; Nicholls, 2009; Soane et al., 2007). Dysfunctional mitochondrial machinery induces the disruption of energy metabolism that culminates with a decrease in ATP production, $Ca^{2+}$ buffering impairment and exacerbated generation of ROS (Beal, 2005). Whereas brain mitochondrial dysfunction is an important component of neurodegeneration, age-related decline in mitochondrial function is controversial (table 1), with data suggesting either no alterations (Davies et al., 2001; Gilmer et al., 2010; Meng et al., 2007) or significant disruption of mitochondrial respiration (Moreira et al., 2003; Petrosillo et al., 2008), and in the activity of individual electron transport chain (ETC) complexes (Boveris and Navarro, 2008b; Kwong and Sohal, 2000; Long et al., 2009; Navarro and Boveris, 2010; Petrosillo et al., 2008; Sandhu and Kaur, 2003). In this regard, Gilmer et al. (2010) reported age-related decline in mitochondrial ATP producing ability, together with increased oxidative damage in specific brain regions of Fischer 344 rats. Similar results showing age-related decline in mitochondrial enzymatic activity were found in whole brains of mice (Navarro et al., 2002), rats (Navarro and Boveris, 2004) and primates (Bowling et al., 1993).

**Chapter 2.2 - Table 1.** Aging and brain mitochondrial alterations

| Animal Model | Aging-related main findings | Reference |
|---|---|---|
| **A** | | |
| Female Wistar rats:<br>Young (2-3 mo)<br>Old (24 mo) | ~ Oxidative phosphorylation complexes enzymatic activity<br>↑ Protein carbonylation | Davies et al. (2001) |
| Fischer 344 rats:<br>Young (12–13 mo)<br>Old (26–28 mo) | ~ ATP production or content | Drew and Leeuwenburgh (2003) |
| Fischer 344 rats:<br>Young (8 mo)<br>Old (26 mo) | ~ RCR<br>~ $\Delta\psi m$<br>~ Mn-SOD activity and content | Meng et al. (2007) |
| Fischer 344 rats:<br>Young (3-5 mo)<br>Middle age (12-14 mo)<br>Aged (22-24 mo) | ~ Respiratory endpoints (synaptic and extra-synaptic mitochondria)<br>↑ Protein carbonyl<br>↑ 4-hydroxynonenal<br>↑ Nitrotyrosine | Gilmer et al. (2010) |
| **B** | | |
| Male C57BL/6 mice:<br>Young (3.5 mo)<br>Adult (12-14 mo)<br>Old (28-30 mo) | ↓ Complex II activity (adult to old )<br>↓ Complex III activity | Kwong and Sohal (2000) |
| Fischer 344 male rats<br>Young (4.5 mo)<br>Old (24.5 mo) | ↑ Mitochondrial structural abnormalities<br>↑ Oxidative damage to nucleic acids | Liu et al. (2002) |
| Male Wistar rats:<br>1.5 mo<br>12 mo<br>24 mo | ↓ RCR<br>↓ ADP/O<br>~$\Delta\psi m$ | Moreira et al. (2003) |

| | | |
|---|---|---|
| Male Wistar strain albino rats:<br>1 mo<br>3–4 mo<br>12 mo<br>24 mo | ↓ Oxidative phosphorylation complex activity | Sandhu and Kaur (2003) |
| C57BL6 mice<br>2 mo<br>12 mo<br>18 mo<br>24 mo | ↓ Gene expressions of mitochondrial complexes (adult to old)<br>↑ 8-OHdG (young to adult)<br>↑ Cytochrome c (young to adult) | Manczak et al. (2005) |
| Male Wistar rats<br>Young (5 mo)<br>Old (24 mo) | ↓ Complex I activity<br>↓ State 3<br>↑ Content of peroxidized cardiolipin<br>↑ $H_2O_2$ | Petrosillo et al., (2008) |
| Male Fischer 344 rats<br>Young (4.7 mo)<br>Old (22 mo) | ↓ Activities of complex I, IV and V<br>~ Mitochondrial complex protein levels<br>↑ Lipid Peroxidation<br>↑ Protein carbonyl<br>↓ SOD activity and GSH content | Long et al. (2009) |
| Male Fisher 344 rats<br>Young (4 mo)<br>Old (22 mo) | ↑ Mitochondrial morphological changes | Aliev et al. (2009a) |
| Male and Female Beagles<br>Young (4.1 yr)<br>Old (10.69 yr) | ↓ RCR (Complex I)<br>↑ Mitochondrial ROS production | Head et al. (2009) |

**A.** Studies reporting no age-related changes in mitochondrial function; **B.** Studies reporting age-related changes in mitochondrial function; CAT- catalase; Cu/Zn-SOD - Copper/zinc superoxide dismutase; GPx - Glutathione peroxidase; GSH - Glutathione; Mn-SOD - Manganese superoxide dismutase; RCR - Respiratory control ratio; ROS - Reactive oxygen species; SOD - Superoxide dismutase; $\Delta\psi m$ - Transmembrane electric potential; 8-OHdG - 8-hydroxydeoxyguanosine; yr – years; mo – months; wk(s) – week(s);↑ significant increase; ↓ significant decrease; ~ no significant changes.

Incomprehensibly, some studies that did not find age-related alterations in brain mitochondrial function reported increased levels of oxidative damage (Davies et al., 2001; Gilmer et al., 2010; Meng et al., 2007). On the other hand, higher activities of superoxide dismutase (SOD, both Mn and Cu-Zn forms) and catalase (CAT), but not glutathione peroxidase (GPx) were observed in older vs. younger animals, when both groups were fed a virgin olive oil diet (Ochoa et al., 2011). A decrease in total SOD activity, but not in GPx or CAT was also described (Meng et al., 2007). Other studies showed that glutathione (GSH) content and total SOD activity were decreased in older rats, whereas CAT activity did not differ between young and old animals (Long et al., 2009). In contrast, there are also reports that total SOD, GPx and CAT activities remained unchanged in aged brain mitochondria (Alabarse et al., 2011). With such disparate results regarding the modulation of mitochondrial antioxidant enzyme activity, it is possible that compensatory mechanisms of the redox systems in brain mitochondria occur in order to counteract the increased oxidative stress associated with the aging process (Ochoa et al., 2011). Indeed, it is still not clear how aging affects brain mitochondrial antioxidative defense systems. Under some conditions, the increased generation of ROS results in a compensatory regulation of antioxidant enzymes activity. However, in most studies the increase in oxidative damage parallels the decline in antioxidant systems capacity. When the excessive production of ROS is sustained, the endogenous reserves of antioxidants become insufficient, which increases the vulnerability of the brain to the deleterious effects of global oxidative stress (Finkel and Holbrook, 2000).

The brain is extremely susceptible to oxidative stress due to its limited glycolytic function and high dependence on aerobic oxidative phosphorylation (Moreira et al., 2009; 2010b). The mitochondrial respiratory chain is one of the primary sources of cellular ROS production, having an important impact in brain (dys)function due to the lower activity of antioxidant enzymes and high levels of polyunsaturated fatty acids, which are extremely susceptible to oxidation (Picklo and Montine, 2007; Santos et al., 2001). The brain tissue is also rich in

transition metals, such as iron that can act as a powerful catalyst for ROS formation (Jomova et al., 2010; Kann and Kovacs, 2007; Migliore and Coppede, 2009a; Nunomura et al., 2006).

Almost all neurodegenerative disorders converge in an overproduction of ROS by mitochondria, either directly or as a secondary consequence of other malfunctions (Boll et al., 2008; Migliore and Coppede, 2009a; Moreira et al., 2010b). Therefore, the overproduction of ROS in neuronal impairment can result in oxidative damage to lipids, proteins and nucleic acids (Migliore et al., 2005; Migliore and Coppede, 2009a). The result of continuous oxidative damage throughout life causes modifications of mitochondrial components that when not repaired accumulate, leading to cellular alterations and cell death. In fact, all known markers of global oxidative damage have been shown to be increased in neurodegenerative disorders and are generally accompanied by reduced levels of antioxidants (Andersen, 2004; Garcia-Mesa et al., 2011; Ischiropoulos and Beckman, 2003; Nunomura et al., 2006). Even in the absence of global oxidative stress causing macromolecular damage, alterations in the redox environment in different cellular compartments can also result in the disruption of several critical redox signaling processes. Alterations in GSH/GSSG, or cysteine/cystine redox pairs by localized ROS production can disrupt redox organization and thus disturb gene expression and bioenergetics during aging (Jones and Go, 2010).

**Mitochondrial DNA during brain aging and degeneration**

Particularly, mitochondrial DNA (mtDNA) is extremely susceptible to high levels of oxidative stress mainly due to its proximity to the mitochondrial respiratory chain, and to the lack of protective histones (Ames et al., 1993; Yang et al., 2008). Mitochondrial DNA oxidative damage in the brain has been described to be inversely correlated with maximal life span of mammals (Barja and Herrero, 2000), suggesting that the accumulation of mtDNA mutations might play an important role in the aging process and neurodegeneration. Other results have shown that other signaling pathways can determine life span in the absence of alterations in

oxidative damage (Caballero et al., 2011). Additionally, the repairing mechanisms of mtDNA could be compromised in aging and may also contribute to neurodegenerative disorders (Ledoux et al., 2007; Weissman et al., 2007).

As a result of accumulated oxidative damage in mtDNA, mitochondrial function can be markedly altered due to the perpetuating production of aberrant and dysfunctional oxidative phosphorylation components, which are partly encoded by mtDNA (Gilmer et al., 2010; Kukat and Trifunovic, 2009; Ochoa et al., 2011). Overall, disrupted ETC can result in a partial impairment of electron flow as well as mitochondrial bioenergetic deficiency resulting in higher levels of ROS production. This creates a vicious cycle, causing energy depletion and increasing ROS production (Petrozzi et al., 2007). In addition, mitochondrial oxidative stress and mtDNA damage contribute to neuronal cell degradation and death through the increased susceptibility to the mitochondrial permeability transition pore (mPTP) opening and activation of apoptosis (Correia et al., 2010; Moreira et al., 2007; Toman and Fiskum, 2011). The opening of mPTP can result in the release of cytochrome c and apoptosis inducing factor (AIF), among other pro-apoptotic factors, to the cytosol. Cytochrome c forms a complex with Apaf-1 and pro-caspase-9, which results in the activation of the effector caspase-3 and consequently cell death. Mitochondrial-released AIF translocates to the nucleus where it induces chromatin condensation and fragmentation (Niizuma et al., 2010). Moreover, mtDNA damage can also trigger apoptosis through p53 and proapoptotic members of Bcl-2 family, a cell death mechanism that has been implicated in many age-related neurological and neurodegenerative disorders (Antonsson, 2004; Mattson, 2006). The increased vulnerability to mPTP opening is also favored by abnormal levels of intramitochondrial $Ca^{2+}$ (Kowaltowski et al., 2001; Toman and Fiskum, 2011). Calcium overload and oxidative stress can interact synergistically, since much lower levels of $Ca^{2+}$ are required to activate the mPTP when mitochondria are undergoing oxidative stress (Fiskum, 2000).

**Mitochondrial dynamics in aging and neurodegenerative diseases**

As previously reported, neurons are also particularly sensitive to changes in mitochondrial movement and distribution. Mitochondrial dynamics is regulated by fission and fusion mechanisms by which smaller or elongated organelles and tubular structures are generated, respectively. Fission-related proteins include dynamin-related protein 1 (Drp1) and fission 1 (Fis1), whereas mitofusins (Mfn1/2) and optic atrophy type 1 (Opa1) operate as fusion proteins. These fission/fusion-associated proteins are present in neurons, suggesting rapid alterations in mitochondrial dynamics that are important in the context of mitochondrial metabolism (Nakamura et al., 2010). These processes are associated with the redistribution of mitochondria to distinct subcellular localizations, thus responding to high-energy requirements, and leading to biogenesis and mtDNA mixing that is critical for the repair of defective mtDNA (Chen and Chan, 2006; Knott et al., 2008). In fact, the impairment of mitochondrial fission/fusion can induce alterations in mitochondrial number and morphology and compromise mtDNA mixing, having a deleterious impact in mitochondria functionality and contributing to neurodegenerative disorders (Bossy-Wetzel et al., 2003; Chan, 2006; Cho et al., 2010a; Knott and Bossy-Wetzel, 2008; Su et al., 2010a). Additionally, the unbalance of mitochondrial dynamics in neurons can also contribute to the disruption of $Ca^{2+}$ homeostasis (Frieden et al., 2004; Knott and Bossy-Wetzel, 2008; Szabadkai et al., 2004), mitochondria depolarization, translocation of proapoptotic mediators to mitochondria and the consequent release of cytochrome c prompting apoptosis (Yuan et al., 2007). Abnormal and dysfunctional brain mitochondria frequently result from dysfunction in the fission/fusion machinery in neurodegenerative disorders, (Chan, 2006; Quintanilla et al., 2011; Su et al., 2010b). Extensive mitochondrial fragmentation is implicated in the pathogenesis of several neurodegenerative diseases including AD (Rui et al., 2006; Wang et al., 2008) and PD (Jendrach et al., 2009; Lutz et al., 2009), due to the inhibition of mitochondrial fusion, or overexpression of mitochondrial fission proteins. Excessively fragmented mitochondria have been associated with cell growth inhibition, a reduction in the respiratory rates and loss of

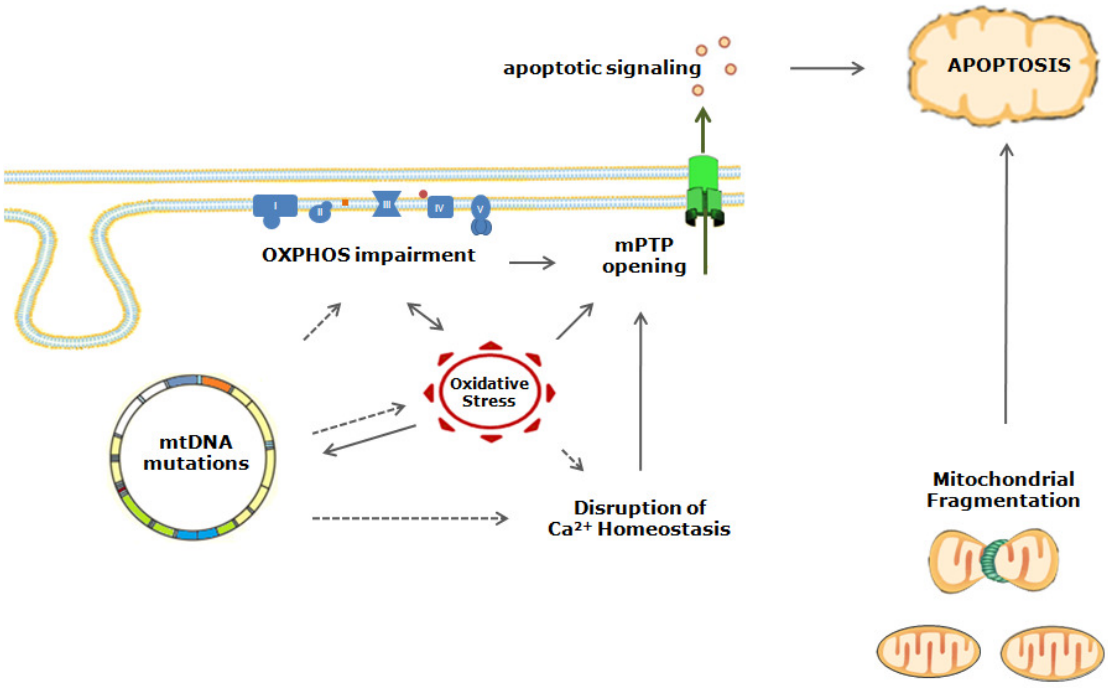
mitochondrial membrane potential (Chen et al., 2005). Similarly, defective mitochondrial fusion in Purkinje cells resulted in aberrant mitochondrial distribution and ultrastructure and compromised ETC activity (Chen et al., 2007). Therefore, alterations in mitochondrial dynamics are, most probably, a common pathway underlying mitochondrial dysfunction with consequent neuronal degeneration. Additionally, oxidative and nitrosative stress appear to be important inducers of mitochondrial fission (Knott and Bossy-Wetzel, 2008), resulting in abnormal mitochondrial distribution and inherent dysfunction that characterize neurodegenerative disorders (Cho et al., 2010a; Su et al., 2010b).

Subsequent cycles of mitochondrial fission/fusion are also associated with segregation and elimination of dysfunctional or less active mitochondria from the networking population (Gottlieb and Carreira, 2010; Santos et al., 2010). In this sense, Twig et al (2008) reported that decreased levels of fission or increased fusion inhibit mitophagy, therefore establishing a link between mitochondrial dynamics and mitophagy. The upregulation of fission with consequent mitochondrial fragmentation observed in neurodegenerative diseases are probably an attempt of neuronal cells to segregate damaged or inactive mitochondria through mitophagy (Santos et al., 2010). Briefly, mitochondrial fission originates metabolically different daughters structures, characterized by biochemical, morphological and functional features usually found in autophagocyted mitochondria. These include a decrease in Opa1, reduced size and reduced membrane potential. Furthermore, mitochondrial sub-populations with decreased membrane potential are less likely to re-fuse with the more functional mitochondria and are more likely to be targeted by mitophagy-related mechanisms (Twig et al., 2008).

The term mitophagy was proposed by Lemasters et al. (2005) to described a selective autophagy of mitochondria (Lemasters, 2005). This pathway of mitochondrial degradation can be triggered by the alteration of $K^+/H^+$ exchanger activity and loss of cation homeostasis, the impairment of oxidative phosphorylation system, and increased ROS levels (Santos et al., 2010). Mitochondrial elimination by mitophagy could be an effective cytoprotective

response because, as previously discussed, dysfunctional mitochondria overproduce ROS and release proapoptotic mediators, promoting damage to neighboring mitochondria and the entire cell (Gottlieb and Carreira, 2010). Therefore, similarly to other cellular death pathways, mitophagy can exert a protective effect by preserving the integrity of mitochondrial population and cellular homeostasis. Whether or not mitophagy plays a definitive role in mitochondrial remodeling in neurodegenerative diseases is still a matter of debate and further studies are needed to better elucidate this connection (Santos et al., 2010).

In summary, global or localized oxidative stress, the disruption of $Ca^{2+}$ homeostasis, mtDNA mutations, and altered mitochondrial dynamics seem to be tightly interconnected (Figure 1) in aging and neurodegenerative diseases. In an attempt to antagonize the harmful brain effects of the aging process or neurodegenerative diseases, several pharmacological and non-pharmacological preventive and therapeutic strategies have been developed and studied. These include physical exercise, a topic discussed in the next sections.




**Chapter 2.2 - Figure 1.** Relationship between some mitochondrial-mediated mechanisms in neurodegeneration. Mitochondrial dysfunction has been implicated in the etiology of neurodegenerative diseases. The decline in respiratory chain complexes leads to insufficient ATP generation, unbalanced ROS production and oxidative damage. Moreover, mitochondrial DNA (mtDNA) mutations have also been implicated in neurodegeneration, which is aggravated by the interaction with ROS, leading to further ROS production, oxidative damage and ATP depletion, in a vicious cycle. mtDNA is also associated with disruption of calcium ($Ca^{2+}$) homeostasis. Depletion in energy production, mtDNA mutations and increased ROS production, associated with $Ca^{2+}$ overload, could increase mitochondrial susceptibility to undergo permeability transition pore (mPTP) opening with subsequent activation of apoptotic signaling. All together, these mechanisms and mitochondrial dynamics are reciprocally regulated and may result in mitochondrial fragmentation, an early event during apoptosis.

## Physical exercise-induced neuroprotection

### Protection of brain function by physical exercise

Considering the multiple mechanisms by which mitochondrial impairment can lead to dysfunction or even death of brain cells, many neuroprotective interventions have targeted mitochondria, including antioxidant supplementation, caloric restriction, short-term mild hypoxia and also physical exercise. However, the development of effective strategies for

therapy or prevention of many neuropathophysiological conditions has been faltering, mainly due to the uncertainty of the mechanisms behind the histopathological and clinical changes that characterize the different neurodegenerative pathologies.

Physical activity can improve the quality of life and prevent or delay the onset of dementia and cognitive decline (Geda et al., 2010; Middleton et al., 2008; Miller et al., 2011; Rockwood and Middleton, 2007). Moreover, physical exercise has been associated with health benefits against several chronic diseases (Booth et al., 2008), including age-related neurodegenerative disorders such as AD (Arcoverde et al., 2008; Lautenschlager et al., 2008; Rolland et al., 2007; Rolland et al., 2008; Wolf et al., 2006) and PD (Dibble et al., 2009b; Dibble et al., 2009a; Goodwin et al., 2008; Weintraub and Morgan, 2011). Accordingly, physical inactivity and a sedentary lifestyle are considered significant risk factors to develop dementia and neurodegeneration (Radak et al., 2010). Appendixes A and B detail human studies analyzing the influence of physically active lifestyle as a non-pharmacological preventive and/or therapeutic strategy against numerous signs of cognitive impairment and neurologic diseases as well as a counteracting behavior that can potentially minimize risk factors associated with neurodegenerative diseases.

Evidences from animal studies confirm that endurance training induces molecular brain alterations that enhance learning and cognitive function (Cotman and Berchtold, 2002; Cotman et al., 2007; Ding et al., 2006a; Gomes Da Silva et al., 2010; Gomez-Pinilla et al., 2008; Radak et al., 2001a). Also, endurance training delays some brain alterations associated with the aging process (Boveris and Navarro, 2008a; Cotman et al., 2007; Dishman et al., 2006; Radak et al., 2008) and facilitates functional recovery from brain injury (Chytrova et al., 2008; Griesbach et al., 2004; Griesbach et al., 2009; Szabo et al., 2010). Importantly, exercise also protects against the onset of chronic diseases including neurodegenerative disorders (Van Praag, 2008). Evidence suggests that voluntary physical exercise reduces both cortical and hippocampal β-amyloid peptide levels in a mouse model of AD (Adlard et al., 2005b; Um et al., 2008). Moreover, lesions on *Substantia Nigra pars*

*compacta* in a mouse model of PD were markedly reduced when these animals were submitted to a chronic treadmill exercise program (Lau et al., 2011), suggesting that physical exercise may have a role in preventing or delaying neuropathologies.

Although the brain is a non-contractile tissue, an increase in energy metabolism seems to indirectly influence neuronal function (Dishman et al., 2006). Brain-skeletal muscle cross-talk may explain how physical exercise modulates brain function. Changes in the content and activity of oxidative enzymes in skeletal muscle induced by exercise could limit the use of glucose and have indirect effects on brain metabolism, protecting the increased metabolic needs of brain during and after exercise (Pilegaard et al., 2000). Moreover, during high intensity exercise, lactate released from skeletal muscle is likely metabolized by the brain (Dalsgaard et al., 2004), which explains intercellular lactate shuttles between muscle and brain (Brooks, 2007).

Chronic physical activity stimulates growth and development of new brain cells, which is a neuroregenerative and neuroprotective effect (Dishman et al., 2006; Rhodes et al., 2003; Van Praag et al., 1999; 2005). Indeed, physical exercise seems to regulate neurogenesis, providing not only quantitative but also qualitative benefits to the brain tissue (Van Praag et al., 1999; 2005; 2008). Exercise is associated with the up-regulation of proteins involved in neuronal and synaptic plasticity including the cytoskeletal protein α-internexin and some molecular chaperones in the hippocampus, a brain region responsible for learning and memory (Ding et al., 2006b). Regular physical activity also increases the proliferation of brain endothelial cells (Cotman et al., 2007), and therefore influences brain vasculature by improving metabolic capacity through the increase in nutrients and oxygen supply (Lopez-Lopez et al., 2004). Indeed, as neurons have high metabolic requirements, their viability is regulated by the bioavailability of energy substrates (Stranahan and Mattson, 2011) and oxygen supply (Lopez-Lopez et al., 2004). Alterations related to energy metabolism induced by physical exercise comprise among others, the up-regulation of multiple proteins involved in glycolytic and oxidative neurometabolism, including fructose-bisphosphate aldolase C,

phosphoglycerate kinase 1, ATP synthase and mitochondrial creatine kinase (Ding et al., 2006b). Increases in the content of isocitrate dehydrogenase [NAD] subunit α, malate dehydrogenase, ubiquinol-cytochrome-c reductase complex core protein 1, and ubiquitin carboxyl-terminal hydrolase isozyme L1 were also observed after chronic exercise (Kirchner et al., 2008). Therefore, exercise may contribute to increase cell number and improved cell function in conditions where the neuronal cell loss such as aging and neurodegenerative diseases (Vaynman and Gomez-Pinilla, 2006).

**Exercise-induced modulation of brain mitochondrial function**

Considering the different pathways, brain mitochondrial metabolism seems to be highly modulated by physical exercise. Indeed, mitochondria have been implicated in the cross-tolerance phenomena by which physical exercise confers neuroprotection. The study of metabolic brain alterations induced by physical exercise has been based on the analysis of the content and/or activity of several enzymes, mainly those involved in aerobic energy production. Ding et al. (2006b) reported that free wheel voluntary exercise increased the content of some hippocampus enzymes involved in the breakdown of glutamate to α-ketoglutarate. The same authors also described an increase in ATP synthase complex protein content and hypothesized that this effect may be a mechanism by which exercise increases energy production. Another proteomic study conducted by Kirchner et al. (2008) revealed that different exercise types such as free wheel voluntary running and treadmill forced running have different outcomes regarding hippocampal mitochondrial proteins in old rats. Despite this, increased levels of mitochondrial ubiquinol-cytochrome-c reductase complex core protein 1, a component of ETC complex III, were observed after both voluntary and forced training (Kirchner et al., 2008). The increase in hippocampus isocitrate and malate dehydrogenases protein content after forced but not after voluntary activity reinforce the idea that brain mitochondrial alterations mediated by exercise are intensity dependent.

Nevertheless, other factors including differential stress levels may also play a role in the observed differences between exercise types (Arida et al., 2004). However, forced or voluntary exercise in Sprague-Dawley rats affected differently brain metabolism. Forced exercise resulted in an increased glycolytic rate when compared with voluntary exercise, as observed by increased expression of glycolytic proteins (Kinni et al., 2011), suggesting that exercise type and intensity remodel mitochondrial function in the context of a broader cellular metabolic remodeling.

Contrarily to what was expected, Tong et al. (2001) reported a decrease in a large number of transcripts related to mitochondrial metabolic enzymes induced by free wheel running training in adult rat hippocampus. Similarly, Stranahan et al. (2010) observed that a number of redox-related transcripts associated with mitochondrial function were down-regulated in aged runners compared to sedentary counterparts. However, the authors reported that mice exposed to lifelong running following learning (water maze) or swimming stress exhibited an upregulation in genes involved in mitochondrial function (Stranahan et al., 2010). In general, both studies suggest that increased physical activity induces changes in translational efficiency or protein stability that may compensate upstream transcriptional responses (Stranahan et al., 2010; Tong et al., 2001).

In an attempt to uncover novel mitochondrial mechanisms by which exercise induces brain plasticity, Dietrich et al. (2008) reported that mitochondrial density and function increased after 4 weeks of free wheel running in adult male and female mice. In the same study, voluntary exercise induced elevations in mitochondrial respiration states. Particularly, increased respiration during ATP synthesis may be indicative of increased energy utilization and improvement in respiratory chain function. This suggests that similarly to other cell types, exercise induces important adaptations in the mitochondrial activity of hippocampal neurons in order to sustain increased metabolic demands. An increase in $O_2$ consumption during basal respiration could be related to augmented proton leak, with concomitant decrease in membrane potential. Increased proton leak may contribute to the phenomenon of mild

uncoupling, which has been described to decrease mitochondrial ROS production by the respiratory chain (Skulachev, 1996). Interestingly, an increase in uncoupling protein 2 (UCP2) gene expression in hippocampus was associated with mitochondrial bioenergetic adaptations to voluntary exercise (Dietrich et al., 2008). UCP2 can represent a possible new target for therapy in several brain pathologies associated with energy deficits, including neurodegenerative disorders (Andrews et al., 2005; Dietrich et al., 2008). Additionally, some studies suggest that mild uncoupling is an effective strategy in the regulation of mitochondrial biogenesis by decreasing ROS overproduction, increasing ATP generation and improving calcium homeostasis (Kowaltowski et al., 2009). Mild uncoupling induced by exercise might also contribute to the ability of mitochondria to modulate synaptic release and gene expression (Andrews et al., 2005; Vaynman and Gomez-Pinilla, 2006). Nevertheless, the positive impact of mild uncoupling is not consensual. Johnson-Cadwell et al. (2007) presented evidence that FCCP-induced mild uncoupling in cultured rat cerebellar granule neurons did not decrease mitochondrial superoxide production. Furthermore, mild uncoupling using that model contributed to decrease spare respiratory capacity, which could explain a decreased resistance to insults including menadione-induced oxidative stress and N-methyl-d-aspartate receptor-induced delayed calcium deregulation (Johnson-Cadwell et al., 2007). This result may have two explanations: a) mild uncoupling is not a savior adaptation in all pathological events and cell systems or b) FCCP, the chemical uncoupler used, may interact with other mitochondrial targets which counteract any positive effect from mild uncoupling. Nevertheless, more evidence indicate that decrease in ROS production due to mild uncoupling under physiological conditions may not be as effective as demonstrated under several systems (Shabalina and Nedergaard, 2011).

Moderate running also delayed some age-related impairments in mitochondrial function (Boveris and Navarro, 2008b; Navarro et al., 2004; Navarro and Boveris, 2007a). Indeed, 24 weeks of moderate exercise ameliorated electron transfer in brain mitochondrial respiratory chain, by increasing the activity of complexes I, III and IV, and preventing the age-dependent

decline reported in sedentary rodents (Navarro et al., 2004). Likewise, the progressive decrease in enzymatic activities of brain mitochondrial complexes I and IV observed in aged animals were reverted with moderate exercise (Navarro and Boveris, 2007a). Interestingly and as previously described, mitochondrial damage during AD (Atamna and Kumar, 2010; De La Monte and Wands, 2006) and PD (Navarro and Boveris, 2009; Schapira et al., 1990) is characterized by decreases in the respiratory chain complexes activities. To our knowledge, there are few studies analyzing brain mitochondrial complexes activity or function in mouse models of neurodegenerative disorders following endurance training. However, since AD and PD are associated with impairments in mitochondrial complexes IV and I, respectively (Navarro and Boveris, 2010), and as exercise modulates both mitochondrial respiratory chain components in aged rodents, exercise may be a valuable neuroprotective strategy by targeting these particular mitochondrial components. A recent study developed by Lau et al. (2011) showed that endurance training protected against mitochondrial dysfunction in a chronic mouse model of PD, as assessed through respiratory control ratio and ATP content.

Exercise also increases the expression of brain-derived neurotrophic factor (BDNF) (Adlard et al., 2005a; Gomez-Pinilla et al., 2002), a neurotrophin that has been related to learning and memory (Fahnestock et al., 2010; Radak et al., 2006) and also with neuronal activity, plasticity and survival (Kovalchuk et al., 2002; Martin and Finsterwald, 2011). Markham et al. (2004) reported that brain mitochondrial function was increased by BDNF in a concentration-dependent manner, the effect being specific to complex I of the mitochondrial respiratory chain and dependent on the presence of a synaptosomal component. BDNF also counteracted the deleterious consequence of mitochondrial $Ca^{2+}$ overload. It was also observed that BDNF was ineffective in liver mitochondria preparations, suggesting that the effect of BDNF is tissue specific. Since aging (Hayashi et al., 2001) and neurodegenerative diseases (Murer et al., 2001) are accompanied by a decrease in BDNF levels, these findings sustain that the elevation in the levels of BDNF induced by exercise can possibly alter the

mitochondrial oxidative efficiency and apoptotic signaling with important preventive and/or therapeutic implications against these pathophysiological conditions.

**Exercise-induced brain mitochondrial biogenesis**

As mentioned, deleterious alterations in mitochondrial biogenesis and dynamics can be negatively associated with mitochondrial functionality, contributing to aging and neurodegeneration. Therefore, stimulation of mitochondrial biogenesis could be a compensatory mechanism against excessive fission and degradation that characterize most of neurodegenerative pathologies (Knott et al., 2008). Mitochondrial biogenesis can be promoted via an increase in the transcriptional coactivators of Silent Information Regulator T1 (SIRT1), which deacetylates and activates the peroxisome proliferator-activated receptor-γ coactivator 1-alpha (PGC-1α) increasing its transcriptional activity (Gerhart-Hines et al., 2007). PGC-1α is expressed in most tissues and responds to common signaling pathways involving calcium, cyclic AMP and ROS (St-Pierre et al., 2006; Wareski et al., 2009). PGC-1α has been recognized as a potent stimulator of mitochondrial biogenesis (Ji et al., 2010), antioxidant defense modulation and as a mediator of neuroprotection (Wareski et al., 2009). Elevations in PGC-1α content in rodent brains seem to be related to increased exercise tolerance (Davis et al., 2009; Steiner et al., 2011). In fact, Steiner et al. (2011) reported that brain adaptations to 8 weeks of endurance training included PGC-1α and SIRT1 mRNA overexpression, together with increased mtDNA content, suggesting increased mitochondrial biogenesis. Similarly, Bayod et al, (2011) also reported PGC-1α and SIRT1 upregulation, activation of AMPK, a decrease in p53 acetylation and concomitant increase in the content of mitochondrial respiratory complexes in the brains of rodents following endurance training. These adaptations suggest that exercise may induce mitochondrial biogenesis, being crucial in the prevention of the damage associated with defective mitochondrial function commonly

associated with aging and neurodegenerative diseases (Bayod et al., 2011; Steiner et al., 2011).

**Exercise-induced regulation of brain mitochondrial redox balance**

Aging and the pathogenesis of several neurodegenerative diseases are associated with increased local and global oxidative stress, which leads to mitochondrial and cellular dysfunction. Since exercise has been associated with a general improvement of brain antioxidant capacity and decreased oxidative stress-associated damage in different biological models (Table 3), it is reasonable to think that exercise may prevent the redox alterations associated with aging and neurodegenerative diseases. Either favorable brain redox adaptations (Somani and Husain, 1996) or an inability to revert the increased oxidative that characterizes the aging process were found (Jolitha et al., 2006). Navarro et al. (2004) reported that moderate exercise, when initiated at a young age, prevented the age-related increase in protein carbonyl content and the decrease in cytochrome c oxidase activity. Upregulation of antioxidant enzymes, the decrease in oxidative stress markers and the increased activity of mitochondrial enzymatic activity were thus associated with physical exercise (Navarro et al., 2004). The benefits of endurance training on brain mitochondrial function appear to be accompanied by the increase of antioxidant levels with consequent attenuation of oxidative damage markers which characterize aging (Navarro et al., 2004; Navarro and Boveris, 2007a) and PD (Lau et al., 2011). Exercise regimens with distinct intensity, duration and load lead to different physiological, biochemical and functional adaptations in brain (Ogonovszky et al., 2005). Moreover, age and animal characteristics also contribute to the experimental variability, and therefore explain the controversial results found regarding brain oxidative stress and physical exercise. For instance, Liu et al. (2000) reported that endurance training seemed to decrease lipid peroxidation, whereas acute exercise did not induce any significant alteration in brain mitochondria. These findings

suggest that mitochondrial adaptations may be influenced by the bioenergetic nature of exercise.

Moderate exercise combined with antioxidant supplementation reduced the age-dependent risk of oxidative modification of brain proteins and lipids and can substantially stimulate the endogenous antioxidant system (Devi and Kiran, 2004; Jolitha et al., 2006). In studies that use a mouse model for AD (Cho et al., 2010b; Garcia et al., 2011; Um et al., 2008) and PD (Lau et al., 2011) and in which brain oxidative stress levels are elevated, moderate exercise showed a beneficial effect in maintaining redox homeostasis. The proposed mechanism for exercise-induced improvement in mitochondrial and tissue antioxidant capacity is not exclusive of the brain. In fact, ROS generated during exercise may play a crucial role as regulatory mediators/signaling molecules in adaptive responses (Radak et al., 2001a; 2001c; 2006). ROS-mediated regulation can modulate not only antioxidant expression (Cadenas, 2004) but also uncoupling proteins (UCPs) (Andrews et al., 2005) and heat shock proteins (HSPs) (Calabrese et al., 2000). This joint upregulation mediated by feedback mechanisms prevent the extent of oxidative and apoptosis, ultimately leading to neuroprotection.

**Chapter 2.2 - Table 2.** Effects of physical exercise on antioxidant capacity and oxidative stress-induced damage

| Animal Model | Exercise training | Antioxidant capacity | Oxidative stress-induced biomolecule damage markers | Reference |
|---|---|---|---|---|
| Male Sprague-Dawley | 7.5 wk Treadmill running<br>30 min/5 days per wk<br>8.2 to 54.4 m/min<br>6 to 10º of inclination | ↑ SOD activity in brainstem and striatum<br>~ GPx activity<br>↑ GSH/GSSG in cortex and brainstem | | Somani et al. (1995) |
| Male Sprague-Dawley | Single swimming bout (30 min) | ~ GSH/GSSG | ~ MDA | Hara et al. (1996) |
| Male Fisher-344 | Single Treadmill running bout<br>(40 min)<br>0 to 12.5º inclination<br>2 to 30.3 m/min | ~ SOD or CAT activity<br>↑ GSH-Px activity in cortex<br>↓ GR activity in cortex | ↑ MDA in striatum | Somani et al. (1996) |

| | | | | |
|---|---|---|---|---|
| Male Fisher-344 | 6.5 weeks Treadmill running<br>30 min/5 days per wk<br>8.2 to 54.4 m/min<br>6 to 10º of inclination | ↑ SOD activity in striatum<br>↓ SOD activity in cerebellum and hypothalamus<br>↓ CAT activity in hypothalamus<br>↑ GPx activity in cortex and hypothalamus<br>↓ GPx activity in cerebellum<br>↑ GR activity in cortex and striatum<br>↓ GR activity in medulla<br>~ GSH levels | ↓ MDA | Somani and Husain (1997) |
| Female Sprague-Dawley<br>6 wk | 8 wk treadmill running<br>2 h/ 5days per wk<br>1.6 km/h.<br><br>2 wk treadmill running<br>10 min/3 days per wk 0.8 km/h<br>+ running to exhaustion at 1.6 km/h | ~ GSH<br>~ GSSG | ↓ MDA (chronic exercise)<br>~ Protein carbonyls<br>~ 8-OHdG | Liu et al. (2000) |
| Male Wistar<br>6 wk | Single swimming bout (2h) | | ~ TBARS<br>~ Proteins carbonyls<br>~ 8-OHdG | Radak et al. (2001b) |

| | | | | |
|---|---|---|---|---|
| Male Wistar<br>4 wk.<br>14 mo. | 9 wk Swimming<br>60 to 90 min/5 days per wk | | ↓ Proteins carbonyls<br>~ TBARS<br>~ 8-OHdG | Radak et al. (2001a) |
| Male Wistar<br>3 mo. | 8 wk Treadmill<br>10 to 60 min/day<br>0 to 6 % inclination | ~ Cu/Zn SOD activity<br>~ CAT activity<br>~ GPx activity | ~ TBARS | Ozkaya et al. (2002) |
| Male Wistar<br>4 mo. | 6 wk Swimming<br>90 min/5 days per wk | | ~ ROS measured by ESR<br>~ Proteins Carbonyls | Toldy et al. (2005) |
| male Wistar<br>24 wk. | 5 days Treadmill running<br>10 min/day<br>10 m/min<br>5º inclination<br>+ running to exhaustion<br>25 m/min | ~ SOD<br>~ GPx | ~ TBARS | Acikgoz et al. (2006) |
| Male Kunming albino<br>5wk | Swimming with 10, 13 and 15% of body weight during 2, 4 and 6 days respectably<br>8 x10 s<br>with 10 s rest interval<br>with 40 s rest interval | ~ SOD activity<br>↑ Total antioxidant capacity in short interval groups<br>↑ Total antioxidant capacity in long interval 6 days | ~ TBARS | Qiao et al. (2006) |

| | | | | |
|---|---|---|---|---|
| Male Wistar<br>13 mo | 8 wk Swimming<br>60min/5 days per wk- 1st 4wks<br>120min/5 days per wk- 2nd 4wks | ~ GOO1 mitochondrial activity | ↓ ROS measured in cerebellum by EPR | Radak et al. (2006) |
| Male CF1<br>6 wk. | 12 wk Treadmill running<br>10 to 60 min/5 days per week<br>10 to 16.5 m/min | ↑ CAT activity in striatum<br>↑ SOD activity in hippocampus and striatum | ↑ TBARS in hippocampus and striatum<br>↑ Protein carbonyls in hippocampus | Aguiar et al. (2008) |
| Male Sprague-Dawley<br>22 wk | 1 h Treadmill running<br>10,15 and 20 m/min<br><br>Treadmill running to exhaustion<br>25m/min<br>5º inclination<br><br>8 wk Treadmill running<br>1h/5 days per wk<br>10,15 and 20 m/min | ↑ SOD activity in striatum acute 20m/min<br>↓ SOD activity in hippocampus chronic 10 m/min 15 m/min<br>~ GPx | ~ TBARS | Aksu et al. (2009) |

CAT- Catalase; Cu/Zn-SOD; Copper/zinc superoxide dismutase; EPR– Electron paramagnetic resonance; ESR- Electron spin resonance; GOO1 – 8-oxoG-DNAglycosylase; GPx - Glutathione peroxidase; GSH - Glutathione; GSSG - Gluthatione disulfide; SOD - Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances; MDA - Malondialdehyde; ROS – Reactive oxygen species; 8-OHdG - 8-hydroxydeoxyguanosine; mo – months; wk(s) – week(s); h-hours; min- minutes; s-seconds; m-meters; ↑significant increase; ↓ significant decrease; ~ no significant change

**Effect of chronic exercise on apoptotic signaling in neurodegenerative diseases**

Excessive or inappropriate apoptotic responses are implicated in several neurodegenerative conditions. Evidence emerges that endurance training stimulates a variety of intracellular pathways involved in neuronal plasticity and survival (Ploughman, 2008).

Chronic physical exercise affords protection against deleterious stimuli and/or pathophysiological conditions including, trauma, ischemia-reperfusion injury, restrain-induced chronic stress and aging through the attenuation of increased apoptotic signaling that characterizes these harmful/senescent conditions (Chae and Kim, 2009; Haack et al., 2008; Itoh et al., 2011; Sim et al., 2004). Endurance training also increases the expression of chaperones, including HSPs in brain tissue (Chen et al., 2008; Cho et al., 2010b; Ding et al., 2006b), conferring cellular and mitochondrial protection by facilitating protein import, folding and assembly. This could be an important mechanism against the deleterious effect of neuropathologies as AD and PD, which are characterized by significant levels of oxidized and nitrated proteins in senile plaques and Lewy bodies, respectively (Calabrese et al., 2006). Indeed, HSP70 protein levels were found down regulated in the transgenic mouse model of AD, a process that was reverted following exercise (Cho et al., 2010b). Confirming the vital importance of cellular and mitochondrial chaperones, Veereshwarayya et al. (2006) reported that overexpression of HSP60 alone or in combination with HSP70 and HSP90 protected neurons by decreasing the cytotoxicity induced by intracellular β-amyloid protein. Moreover, elevated levels of mitochondrial HSPs decreased cytochrome c release, caspase-9 activation and protected mitochondrial enzymes and respiratory chain components from the deleterious effects triggered by β-amyloid protein. Similarly, Um et al. (2008) suggested that the increase of HSP70 with endurance training was associated with a decreased expression of apoptotic proteins in AD mice brain. Haack et al. (2008) showed that endurance training prevented the increase in Bax levels caused by chronic stress (21 consecutive days, 6 h/day restrained animals) on cortical mitochondria suggesting that

exercise suppressed Bax translocation to mitochondria with consequent decrease in its proapoptotic activity.

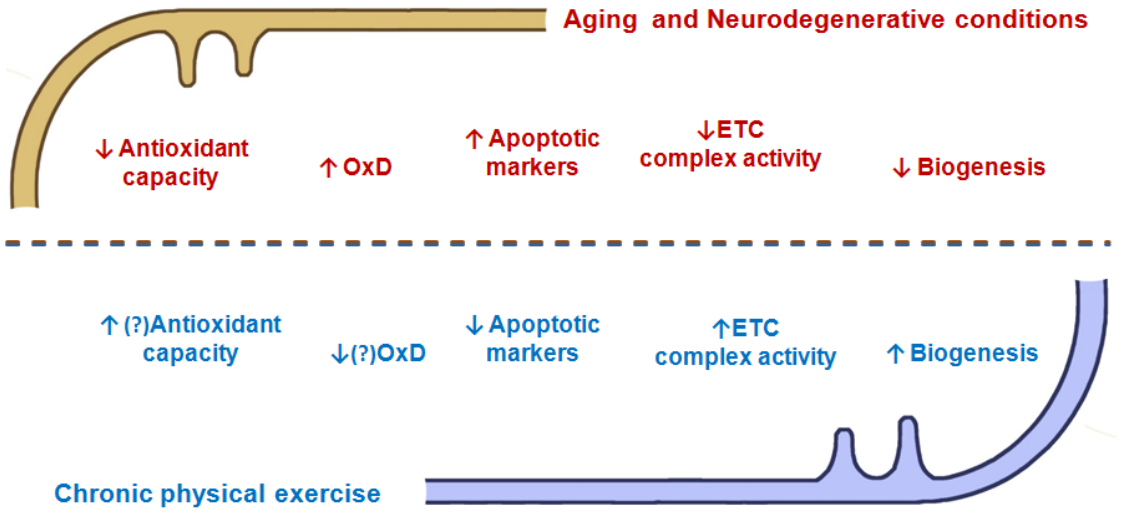
A decrease of several proapoptotic proteins, including caspases 9 and 3 as well as an increased Bcl-2 protein content was found following an exercise-training program (Um et al., 2011). The same research group also reported that NSE/APPsw animals, a transgenic mice model expressing AD phenotypes, including behavioral dysfunction, β-amyloid peptide-42 deposition and apoptosis activation at 12-13 months of age, exhibited reductions in brain cytochrome c, Bax, caspase 3 and 9 levels after the endurance training (Cho et al., 2010b; Um et al., 2008). These results suggest that endurance training attenuates neuronal cell apoptosis involved in the pathogenesis of AD. It is however of concern that the authors did not describe in detail the brain tissue fractions used for Western Blotting analysis, which can bias the significance of the results regarding Bax, Bcl-2 and cytochrome c as markers of apoptotic signaling. Regardless these methodological considerations, the reduction on apoptotic biomarkers (as far as we can understand, semiquantified in total brain tissue homogenate) were associated with improved antioxidant capacity, an elevation of HSP70 and BDNF levels mediated by an increase in the degradation or clearance of β-amyloid peptides (Cho et al., 2010b; Um et al., 2008; 2011).

**Conclusions**

In summary, physical exercise modulates several physiological mechanisms that may increase brain resistance to aging and neurodegeneration. Particularly, neuroprotective mechanisms triggered by physical exercise include beneficial adaptations to oxidative stress and apoptotic signaling. These, together with increased mitochondrial biogenesis and oxidative phosphorylation activity emphasize the role of mitochondria as plastic organelles central for adaptations resulting from physical exercise. Knowing that disturbances in mitochondrial machinery are important events to aging and neurodegeneration, the ability of

exercise to trigger neuroprotective mechanisms could represent an important strategy against many cerebral pathophysiological conditions. Figure 2 illustrates the reviewed mitochondrial alterations that contribute to neurodegeneration and the counteracting effects of physical exercise against those deleterious events.

Understanding the adaptations in brain mitochondrial machinery induced by physical exercise will provide important information necessary to develop new and more effective therapeutic strategies against neurodegeneration by targeting mitochondrial function. Therefore, approaches that counteract or attenuate mitochondrial dysfunction, such as physical exercise, may have preventive and/or therapeutic potential.




**Chapter 2.2 - Figure 2.** Exercise-induce brain mitochondrial improvement and neuroprotection. Scheme summarizing the described adaptations induced by chronic physical exercise against brain mitochondrial dysfunction characterizing aging and neurodegenerative conditions. OxD - Oxidative damage; ECT - Electron transport chain; ↑ increase; ↓decrease; ? no consensual information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**Chapter 2.2 - Figure 3.** Appendix A **(Supplementary data).** Association between physical activity and the risk of cognitive impairment and dementia-related diseases.

| Population | Follow-up period and assessment of PA | Major outcomes* | Reference |
|---|---|---|---|
| 1 090 individuals older than 60 years | 3-year Questionnaire. | Elderly people with limited PA had a higher risk for developing dementia | Li et al. (1991) |
| 828 individuals older than 65 years | 7-year Questionnaire | PA was preventive for AD | Yoshitake et al. (1995) |
| 1 192 individuals age 70 to 79 | 2 or 2.5-year Questionnaire | Strenuous but not moderate PA was associated with a reduced risk of cognitive decline | Albert et al. (1995) |
| 2 040 individuals older than 65 years | 1 and 3-years Interview | PA individuals had a lower risk of dementia | Fabrigoule et al. (1995) |
| 327 individuals older than 75 years | 3-year Questionnaire | No associations were found with dementia or AD | Broe et al. (1998) |
| 4 615 individuals older than 65 years. | 5-year Questionnaire | PA individuals had lower risks of cognitive impairment, AD, and dementia of any type | Laurin et al. (2001) |
| 347 men older than 65 years | 3-year Questionnaire | PA at older age may reduce the risk of cognitive decline | Schuit et al. (2001) |
| 2 030 individuals older than 70 years | 3 -year Interview | The lack of exercise was associated with an increased risk of cognitive impairment, particularly in women | Ho et al. (2001) |
| 5 925 women older than 65 years | 6 to 8 years self-reported | High levels of PA were associated with less susceptibility to develop cognitive decline | Yaffe et al. (2001) |
| 6 434 individuals older than 65 years. | 3 -year Interview | Regular PA was associated with a reduced risk of AD | Lindsay et al. (2002) |
| 364 individuals age 70 to 75 | 12 years Not detailed | Women with high-level PA at baseline were less likely to experience mental decline | Pignatti et al. (2002) |
| 732 individuals older than 75 years. | 4 and 7 years Interview | Unable to identify a significant beneficial effect of PA. Only a few elderly people were engaged in PA, leading to limited power to detect a moderate effect | Wang et al. (2002) |
| 469 individuals older than 75 years | 21 years Interview | There was no association between PA and the risk of dementia | Verghese et al. (2003) |
| 1 241 individuals age 62 to 85 | Reported PA between 15 and 25 years | Early life PA may delay late-life cognitive deficits | Dik et al. (2003) |
| 349 individuals older than 55 years | $VO_2$max as measure of cardiorespiratory fitness | Baseline measures of cardiorespiratory fitness were positively associated with cognitive performance evaluated 6 years later | Barnes et al. (2003) |
| 1 146 individuals older than 65 years | 2 years Self reported | Exercise was negatively associated with cognitive decline | Lytle et al. (2004) |
| 2 257 individuals age 71 to 93 | 7 years Questionnaire Physical tests | Fit and PA men are less likely to develop dementia | Abbott et al. (2004) |
| 16 466 women older than 70 years | 15 years Biennial questionnaire | Long-term PA was strongly associated with high cognitive function and less cognitive decline | Weuve et al. (2004) |

| | | | |
|---|---|---|---|
| 3 375 individuals older than 65 years | 8 years<br>Interview | Engaging in a number of different PA protects against subsequent risk of all-cause dementia, AD, and vascular dementia, although the potential benefits of exercise may be limited to APOE ε4 allele non-carriers | Podewils et al. (2005) |
| 1 449 individuals age 65 to 79 | 21 years<br>Questionnaire | Regular PA may reduce the risk or delay the onset of dementia and AD, especially among genetically susceptible individuals | Rovio et al. (2005) |
| 1 740 individuals older than age 65 years | 6.2 years<br>Questionnaire | Regular exercise was associated with a delayed onset of dementia and AD | Larson et al. (2006) |
| 10 714 men, mean age 67.6 years | Reported PA by questionnaire | The data do not strongly support the hypothesis that PA lowers the risk of PD | Logroscino et al. (2006) |
| 2288 individuals older than 65 years | 6 years<br>4 physical performance tests | Association of lower levels of physical function with an increased risk of future dementia and AD | Wang et al. (2006) |
| 1880 individuals older than 65 years | 14 years<br>Questionnaire every 1.5 years | Adherence and higher PA (dose-response manner) was associated with reduced risk for developing AD | Scarmeas et al. (2009) |
| 3 903 individuals older than 55 years. | 2-years follow-up<br>Questionnaire. | Moderate or high PA are associated with reduced incidence of cognitive impairment | Etgen et al. (2010) |
| 1 198 individuals with mild cognitive impairment and 1126 individuals with normal cognition aged 78 to 86 | Comparative study<br>Late life and midlife physical exercise questionnaire | Late life and midlife moderate physical exercise were associated with a reduced mild cognitive impairment | Geda et al. (2010) |
| 69 individuals, age 55 to 88 | Cerebrospinal fluid samples were collected<br>Retrospective questionnaire | Association between exercise engagement and AD-related biomarkers in cognitively normal older adults | Liang et al. (2010) |
| 213 701 individuals age 50 to71 | 10 years<br>Questionnaire for 4 past time points. | Higher levels of moderate to vigorous PA in mid or later life were associated with future lower risk of PD | Xu et al. (2010) |
| 197 individuals, mean age, 74.8 years | 5 or 8 years.<br>Total activity = activity energy expenditure - resting metabolic rate. | Energy expenditure is associated with a reduced incidence of cognitive impairment in older adults | Middleton et al.(2011) |
| 4 116 individuals older than 65 years | 14 years<br>Questionnaire every 1.5 years | Exercise may affect not only risk for AD but also subsequent disease duration: PA was associated with prolonged survival in AD | Scarmeas et al. (2011) |
| 109 subjects with mild cognitive impairment and 817 individuals with normal cognition, aged 70 to 93 | Comparative study<br>Questionnaire. | Moderate PA and computer use in late life was associated with decreased odds of having mild cognitive impairment. The synergistic interaction between the 2 activities occurred on an additive scale | Geda et al. (2012) |
| 59 889 individuals, age 20 to 88 | 17-year follow-up<br>Maximal treadmill exercise test. | Greater fitness was associated with lower risk of mortality from dementia in a large cohort of men and women | Liu et al. (2012) |

AD - Alzheimer disease, APOEε4 – apolipoprotein E genotype allele, PA - physical activity, PD - Parkinson disease, $VO_2$max - Maximal oxygen uptake; *Adjusted for confounders, if applicable.

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**Chapter 2.2 - Figure 4.** Appendix B **(Supplementary data).** Cognitive and behavioral-related effects of exercise programs in old individuals with cognitive impairment or dementia-related diseases.

| Population | Intervention/groups | Major Outcomes* | Reference |
|---|---|---|---|
| 30 old patients with moderate to severe AD | 10wk, 3days/wk, 30min/day<br>- Walking individually<br>- Control group | Walking program improved communication performance | Friedman and Tappen (1991) |
| 11 patients with AD<br>59 to 89 years | 1 year. At least 60m/wk of aerobic exercise + 2 days/wk of weight training | No clear benefits of exercise *per se* on cognitive, mood, and social outcomes observed by caregivers and corroborated by testing | Arkin (1999) |
| 16 patients with slightly to moderately PD | 14 wk, 2 days/wk<br>intensive exercise | Intensive exercise improved motor disability, mood and subjective well-being in early to medium stage PD patients | Reuter et al. (1999) |
| 153 patients with AD<br>55 to 93 years | 3 months, 30 min/day<br>- Moderate intensity exercise<br>- Control group | Exercise training combined with teaching caregivers behavioral management techniques improved physical health and depression in AD patients | Teri et al. (2003) |
| 25 old patients with dementia | 3 months, 30min/day<br>- Physical exercises supported by music<br>- Control group | Beneficial effect of a music-based exercise program on cognition in patients with moderate to severe dementia | Van De Winckel et al. (2004) |
| 75 old patients with mild cognitive impairment | 10wk, 3days/wk, 30min/day<br>- Frail aged appropriate, exercise program<br>- Social visit<br>- Control | Exercise may slowed the rate progression of cognitive symptoms related to dementia, slowed/reversed the disability in daily living activities | Stevens and Killeen (2006) |
| 6 men with PD<br>72.7 years | 8wk, 3days/ wk, 1h/day<br>-Individualized moderate intensity | Moderate exercise improved perceived cognition abilities in PD patients. | Baatile et al. (2000) |
| 134 patients<br>with mild to severe AD 62 to 103 years | 12mon, 2days/wk, 1h/day,<br>- walking, strength, balance, and flexibility<br>- control group | Exercise slowed disability in performance activities of daily living in AD patients, although no observed effect on behavioral disturbance or depression | Rolland et al. (2007) |
| 90 patients with moderate and severe AD | 16wks, 5days/wk, up to 30min/day<br>- walking<br>- walking, strength , balance, and flexibility<br>- social conversation | Long-term exercise resulted in better outcomes in mood and affect | Williams and Tappen (2007) |
| 36 patients with moderate to severe dementia,<br>85 years | 12wk, 3 days/wk, 30min/day<br>- moderate-intensity exercise<br>- before and after measurements | Moderate-intensity exercise reduced symptoms of depression | Edwards et al. (2008) |
| 50 years or older individuals who reported memory problems but did not meet criteria for dementia | 24-week PA intervention | PA program provided a modest improvement in cognition over a 18-month follow-up period in adults with subjective memory impairment | Lautenschlager et al. (2008) |

| | | | |
|---|---|---|---|
| | 16wk, 5days/ wk, 30 min/day | | |
| 45 patients with moderate to severe AD,<br>71 to 101 years | - strength, balance and flexibility + walking<br>- walking<br>- social conversation. | Depression was reduced in all three groups with some evidence of higher benefit in exercise groups | Williams and Tappen (2008) |
| 52 patients with a broad range of mental and functional pathologies, older than 65 years | 24wk, 4days/wk, 30min/day<br>- Tai Chi<br>- cognition-action exercise program | Light to moderate intensity exercise decreases mood disturbances, increases self-efficacy and enhances quality of life | Deschamps et al. (2009) |
| 27 patients with AD mean 75 years | 12wk<br>- exercise program:<br>Aerobic 3.6 days/wk<br>Strength 2.9 days/wk<br>Balance 2.7 days/wk<br>- control group | No significant differences between groups were noted in any of the cognitive outcome measures | Steinberg et al. (2009) |
| 20 patients with mild to moderate PD,<br>65.4 years | 6 months, 3days/wk, 60min/day<br>- Aerobic exercise<br>- control group | Exercise improved executive functions in PD patients. | Tanaka et al. (2009) |
| 230 patients with AD | 24wk, 150 min/wk<br>- moderate PA<br>- control group | PA in combination with usual care alleviated AD symptoms and improved their management and quality of life | Cyarto et al. (2010) |
| 9 patients with PD,<br>65.8 years | 6wk, 3days/wk, 50min/day<br>- treadmill walking with additional body load<br>- control group | Exercise had positive effects on cognition | Filippin et al. (2010) |
| 28 patients with PD | 12wk, 2days/wk, 60min/day<br>- aerobic and strength exercise<br>- control group | Exercise exerted a selective benefit for frontal lobe-based executive function | Cruise et al. (2011) |
| 132 patients with AD<br>81 years | 6 months, 7days/wk<br>- 30min/day walking,<br>-1 h/day light exercise<br>- combination walking+light<br>- control group | Walking, light exposure, and their combination improved sleep | Mccurry et al. (2011) |
| 24 patients with advanced AD,<br>older than 65 years | 6 months, 4days/wk, 30min/day<br>- aerobic walking<br>- control group | Walking program stabilized progressive cognitive dysfunctions and improve performance of activities of daily living | Venturelli et al. (2011) |
| 27 patients with AD<br>70.5 and 75.7 | 6 wks, 2h/wk<br>- non-aerobic exercise<br>- control group | Short course of non-aerobic exercise was effective at least in some aspects of cognitive functioning | Yaguez et al. (2011) |

AD - Alzheimer disease, PA - physical activity, PD - Parkinson disease, *Adjusted for confounders, if applicable.

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# 3.

# EXPERIMENTAL STUDIES

# 3.1

## Materials and Methods

**Reagents**

Deionized water (18.7 MΩ) from an arium®611VF system (Sartorius, Göttingen, Deutschland) was used in the preparation of all solutions. Doxorubicin hydrochloride, commercial/clinic use, was obtained from *Ferrer Farma* (Barcelona, Spain), prepared in a sterile saline solution, NaCl 0.9% (pH 3.0, HCl) and stored at 4 ºC for no longer than five days upon rehydration. Commercial RANSOD kit from Randox Labs (Antrim, UK), chemiluminescent reagent ECL-Plus™ 104 (RPN2236) from GE Healthcare (Amersham BioSciences UK Ltd., Buckinghamshire, UK) and PVDF membranes (#IPVH00010) from Millipore (Billerica, MA, USA). Primary antibodies were purchased as follows: anti-OXPHOS (ab110413) and anti-PGC1-α (ab106814), anti-cyclophilin D (ab110324), anti-p62 (ab56416), anti-OPA1 (ab119685), anti-PINK1 (ab23707) and anti-Parkin (ab15954) from *Abcam* (Cambrige, UK); anti-Bax (#2772), anti-Bcl-2 (#2870), anti-DRP1 (#8570), anti-cofilin (#5175) anti-SIRT3 (#2627), anti-p66sch (#2432) anti-beclin1 (#3495) and anti-acetylated-lysine (#9441) from *Cell Signaling Technology* (Danvers, MA, USA); anti-LC3 (PD014) from *MBL Medical & Biological Labs* (Nagano, Japan); anti-DNP (D9656) and anti-UCP2 (SAB2501087) from *Sigma Aldrich* (Sintra, Portugal); anti-shc/p66(pSer$^{36}$) (6E10) from *Calbiochem* (Merck Millipore Darmstadt, Germany); anti-ANT1/2 (sc-9299), anti-Mfn1 (sc-50330), anti-Mfn2 (sc-50331), anti-TFAM (sc-23588), anti-TOM 20 (sc-11415) and anti-β-actin (sc-1616) from *Santa Cruz Biotechnology Inc.* (Santa Cruz, CA, USA). Secondary antibodies were purchased from *Santa Cruz Biotechnology Inc.* (Santa Cruz, CA, USA). All other chemicals were purchased from *Sigma Aldrich* (Sintra, Portugal).

**Animals**

All experimental procedures involving animals were conducted in accordance with the European Convention for the Protection of Vertebrate Animal Used for Experimental and

Other Scientific Purposes (CETS no. 123 of 18 march 1986 and 2005 revision) and the Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes [C (2007) 2525]. The authors are accredited by the Federation of Laboratory Animal Science Associations (FELASA) for animal experimentation (class c). The Ethics Committee of the Research Centre in Physical Activity, Heath and Leisure (Faculty of Sport, University of Porto) approved the experimental protocol.

Thirty-six male Sprague-Dawley rats (aged 21 days old) were obtained from Charles River Laboratories (L'Arbresle, France) and randomly divided into six groups (n=6 *per* group). Only male rats were used to avoid hormone-dependent alterations in drug-induced toxicity. During the experimental protocol, animals were housed in collective cages (two rats per cage) and maintained in a room at normal atmosphere (21–22 ºC; ~50–60% humidity) receiving food (Scientific Animal Food and Engineering, A04, Perotech, Toronto, Canada) and water *ad libitum* in 12 h light/dark cycles.

Specific set-ups related with animal groups used in the different studies are as follows:

Study 1 and 2 (Chapter 3.2 and 3.3): Saline sedentary (SAL+SED), saline treadmill endurance training (SAL+TM), saline free wheel voluntary physical activity (SAL+FW), doxorubicin sedentary (DOX+SED) doxorubicin treadmill endurance training (DOX+TM) and doxorubicin free wheel voluntary running (DOX+FW).

Study 3 (Chapter 3.4): SED, TM and FW.

Study 4 (Chapter 3.5): SAL+SED, DOX+SED,DOX+TM and DOX+FW.

**Endurance training protocol**

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

**Voluntary physical activity**

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

**Doxorubicin treatment** (studies 1, 2 and 4)

After the 5$^{th}$ week of endurance training or free wheel exercise, the animals were treated with sub-chronic intraperitoneal injection for seven weeks with DOX or sterile saline solution NaCl 0.9% (2 mg/Kg of body weight). The animals assigned to the TM groups received DOX or SAL in a day-off training.

**Y-maze behavioral test** (studies 3 and 4)

Data from behavioral tests were obtained from videotape recordings that were analyzed off-line.

To measure spontaneous alternation of behavior and exploratory activity, a Y-maze, with arms of 41 cm length by 12 cm width and 14 cm height was used. Each animal received one trial, in the course of which the animal were placed into one of the three alleys and allowed free exploration of the maze for 5 min. Alternations and total number of arm choices were recorded and movements were tracked using a digital camera (Sony® DCR-HC42E). % spontaneous of alterations = number of arm choices differing from the previous two choices / total entries minus 2 )* 100. (King and Arendash, 2002).

**Open field behavioral test** (studies 3 and 4)

For open-field testing of activity and exploratory behavior, an open black box (70 X 70 cm) with 28.5 cm height, was used. The box floor was painted with lines to demarcate 16 squares (17.5 X 17.5 cm each). For the single trial, each animal was admitted to the center of the enclosure and permitted to explore the interior for 5 min and movements were tracked using a digital camera (Sony® DCR-HC42E). Distance-covered, the total number of line crossings, rearings and grooming were analyzed (King and Arendash, 2002). Additionally, the activity time and the time spent into the center of the box were also recorded.

**Animal euthanasia, blood, heart, brain cortex, cerebellum extraction and soleus extraction**

Forty-eight hours after the last TM exercise session, non-fasted rats were euthanized by cervical dislocation between 9:00 and 10:00 AM to eliminate possible effects due to diurnal variation. Approximately 3~5 mL of blood were collected and immediately centrifuged (500 x*g*

for 5 min at 4 ºC), and an aliquot of plasma was obtained and stored at -80 ºC for later determination of cardiac troponin I (cTnI). After decapitation, the whole brain cortex minus the hippocampus and cerebellum were rapidly removed, washed and weighed. After quickly opening the chest cavity, the heart was rapidly excised, rinsed, carefully dried and weighed. The right *soleus* muscle was processed in the same way and was rapidly frozen at -80 ºC for later determination of citrate synthase (CS) activity.

Portions of approximately 25 mg of cardiac ventricle, brain cortex and cerebellum were separated, homogenized in RIPA buffer (#20-188) from Millipore (Merck, Darmstadt, Germany), contaning phosphatase and protease inhibitors in ratio of 100 mg/mL. the homogeneization was performed using a Teflon pestle on a motor driven Potter-Elvehjem glass homogenizer at 0–4 ºC three to five times for 5 s at speed low setting, with a final burst at a higher speed setting. Homogenates were centrifuged (2 min at 12000 x*g*, 4 ºC, in order to eliminate cellular debris) and the resulting supernatants were prepared and stored at -80 ºC for later semi-quantification of protein expression by Western Blotting. Protein content from homogenates were spectrophotometrically determined by the Bradford method using bovine serum albumin as standard (Bradford, 1976). Plasma cTnI was assayed in Abbott Architect System by chemiluminescent microparticle immunoassay.

**Isolation of heart mitochondria** (studies 1 and 2)

Heart mitochondria were isolated using conventional methods of differential centrifugation (Bhattacharya et al., 1991). Briefly, the heart was washed and minced in an ice-cold isolation medium containing 250 mM sucrose, 0.5 mM EGTA, 10 mM HEPES (pH 7.4) and 0.1% defatted bovine serum albumin (BSA, Sigma, cat. no. A7030). The minced blood-free tissue was then resuspended in 40 mL of isolation medium containing 0.75 mg/mL protease Subtilisin A Type III (Sigma P5380) and homogenized with a tightly fitted “Potter-Elvejhem” glass homogenizer. The suspension was incubated for 1 min (4 ºC) and re-homogenized. The homogenate was then centrifuged at 13000 x*g* for 10 min, the resulting supernatants were

decanted and the pellet, essentially free of protease, was gently re-suspended with a loose-fitting homogenizer. The suspension was centrifuged at 750 x*g* for 10 min and the resulting supernatant was centrifuged at 12000 x*g* for 10 min. The pellet was re-suspended in washing medium and centrifuged at 1200 xg for 10 min. The pellet was gently ressuspended to obtain the final mitochondrial suspension. EGTA and defatted BSA were omitted on the final washing medium. All the procedures were performed within at 4 ºC. Mitochondrial protein content was determined by the Biuret method method using bovine serum albumin as standard (Gornall et al., 1949).

**Brain cortex and cerebellum mitochondria isolation** (studies 3 and 4)

Briefly, after animal decapitation, the brain cortex and cerebellum were washed and carefully homogenized with a tightly fitted Potter–Elvehjem homogenizer and a Teflon pestle at 4 ºC in 30 mL of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA and 0.1% bovine serum albumin BSA, pH 7.4.) containing 5 mg of bacterial subtilisin protease type VIII and then centrifuged at 750 ×*g* for 5 min. The resulting supernatant was centrifuged at 12000 ×*g* for 10 min. The pellets, including the fluffy synaptosomal layer, were re-suspended in 10 mL of the isolation medium containing 0.02% digitonin and centrifuged at 12000 ×*g* for 10 min. The brown pellets minus the synaptosomal layer were re-suspended again in 10 mL of isolation medium and centrifuged at 12000 ×*g* for 5 min. The pellets were re-suspended in 10 mL of washing medium (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, pH 7.4) and centrifuged at 12000 ×*g* for 5 min. The final pellets were gently re-suspended in 150-200 µL of the washing medium to obtain the suspension mitochondrial. All procedures were performed at 4 ºC. Mitochondrial protein concentration was spectrophotometrically determined by using the Biuret method using bovine serum albumin as standard (Gornall et al., 1949).

Aliquots of heart, brain cortex and cerebellum mitochondrial suspension were separated and frozen at –80 ºC for later analysis. Other aliquots were separated and prepared for later semi-

quantification of protein expression by Western Blotting as detailed below. The remaining fresh mitochondrial suspensions were maintained on ice throughout this period and used within 2–3 h for *in vitro* assays of mitochondrial oxygen consumption and transmembrane electrical potential. There was no significant alteration of the mitochondrial respiratory control ratio (RCR) between the first and the last measurements.

**Mitochondrial respiratory activity** (studies 1, 3 and 4)

Mitochondrial respiratory function was measured polarographically at 30ºC using a Biological Oxygen Monitor System (Hansatech Instruments, Norfolk, UK) and a Clark type oxygen electrode (Hansatech DW1, Norfolk, UK). Reactions were conducted in a 0.75 mL closed, thermostatted and magnetically stirred glass chamber containing 0.5 mg/mL of heart mitochondrial protein in a respiration buffer (50 mM KCl, 130 mM sucrose, 2.5 mM $KH_2PO_4$, and 0.5 mM HEPES, pH 7.4) or 0.8 mg/mL of brain cortex and cerebellum mitochondrial protein in a respiration buffer (100 mM KCl, 100 mM sucrose, 10 µM EGTA, 2 mM $KH_2PO_4$, and 5 mM HEPES, pH 7.4).

After 1 min equilibration period, mitochondrial respiration was initiated by adding glutamate/malate (G/M) to a final concentration of 5 and 2.5 mM, respectively. State 3 respiration was determined after adding ADP (150 nmol); state 4 was measured as the rate of oxygen consumption after ADP phosphorylation. The RCR (state 3/state 4) and the ADP/O ratios, the number of nmol of ADP phosphorylated by natom of oxygen consumed, were calculated according to Estabrook (1967).

**Mitochondrial transmembrane electrical potential** (studies 1, 3 and 4)

Mitochondrial transmembrane electrical potential ($\Delta\psi$) was monitored indirectly based on the activity of the lipophilic cation tetraphenylphosphonium ($TPP^+$) using a $TPP^+$ selective electrode prepared in our laboratory as previously described by Ascensão et al. (2011a) and

according to Kamo et al (1979). No correction for the passive binding of $TPP^+$ to mitochondrial membranes was performed, since the purpose of this study was to show relative and not absolute Δψ values. As a consequence, a slight overestimation of the Δψ values is anticipated. The Δψ was estimated from the following equation (at 30°C): $\Delta\psi = 59 \times \log (v/V) - 59 \times \log (10^{\Delta E/59} - 1)$, where v, V, and ΔE stand for mitochondrial volume, incubation medium volume, and deflection of the electrode potential from the baseline, respectively.

Mitochondrial transmembrane electric potential were carried out at 30 ºC in 1 mL of heart reaction buffer (50 mM KCl, 130 mM sucrose, 2.5 mM $KH_2PO_4$, and 0.5 mM HEPESpH 7.4) or brain reaction buffer (100 mM KCl, 100 mM sucrose, 10 µM EGTA, 2 mM KH2PO4, and 5 mM Hepes pH 7.4), supplemented with 3 µM $TPP^+$ and 0.5 mg/mL (heart) or 0.8 mg/mL (brain cortex or cerebellum) of mitochondrial protein. For the measurements of ∆ψ with complex I-linked substrates, energization was carried out with G/M (5 mM and 2.5 mM, respectively) and ADP (150 nmol) was used to produce a phosphorylation cycle. The lag phase, which reflects the time needed to phosphorylate the added ADP, was also measured during the experiments.

**Brain cortex and cerebellum mitochondrial calcium accumulation and mPTP induction**

(studies 3 and 4)

Mitochondrial calcium accumulation capacity was determined by adding small pulses of calcium (45 nmol per pulse) until mPTP opening was observed as an irreversible fall in ∆ψ. The assays were performed in 1 mL of reaction medium (200 mM sucrose, 10 mM Tris, 10 µM EGTA and 5 mM $KH_2PO_4$, pH 7.4) supplemented with 3 µM rotenone and 8 mM succinate with 0.8 mg of protein/mL at 30 ºC continuously stirred. A negative control was performed with cyclosporine A (1µM) to inhibit mPTP (Broekemeier et al., 1989).

**Heart mitochondrial osmotic swelling** (study 2)

Previous reports on substrate-specific regulation of mPTP showed lower calcium retention capacity with complex I-linked substrates compared with substrates for complex II due to the fact that electron flow though complex I acts as a potent pore sensitizer independently of other regulators such as the redox state of pyridine nucleotides, ΔΨ, pH and ROS production (Fontaine et al., 1998). Hence, all studies involving mPTP induction were performed with succinate as the substrate. Heart mitochondrial osmotic volume changes were spectrophotometrically monitored by the decrease of absorbance at 540 nm (Jasco V-630 spectrophotometer, Italy). Swelling amplitude and rate of absorbance decrease upon calcium addition were considered as mPTP susceptibility indexes. The assays were performed in 1 mL of reaction medium containing 240 mM sucrose, 10 mM HEPES, 5 mM $KH_2PO_4$, 10 µM EGTA , pH 7.4, supplemented with 1.5 µM rotenone, 8 mM succinate and a single pulse of 80 nmol of calcium with 0.5 mg/mL protein (160 nmol/mg protein) at 30 ºC continuously stirred. Control trials were performed using 1 µM of cyclosporin-A, the selective mPTP sensitizer (Broekemeier et al., 1989).

**Blue-native PAGE separation of heart mitochondria OXPHOS complexes** (study 1)

Blue-native PAGE separation of mitochondria membrane complexes was performed using the method described by Schagger and von Jagow (1991) with minor modifications. Heart mitochondria (200 µg of protein) were centrifugated at 20000 x*g* for 10 min at 4 ºC and the pellet as resuspended in solubilization buffer (50 mM NaCl, 50 mM Imidazole, 2 mM ε-amino n-caproic acid, 1 mM EDTA pH 7.0) with 1% (w/v) digitonin. After 10 min on ice, insoluble material was removed by centrifugation at 20000 x*g* for 20 min at 4℃. Soluble components were combined with 0.5% (w/v) Coomassie Blue G250, 50 mM ε-amino n-caproic acid, 4% (w/v) glycerol and separated on a 4–13% gradient acrylamide gradient gel with 3.5% sample gel on top. The anode buffer contained 25 mM Imidazole pH 7.0. Cathode buffer (50 mM tricine and 7.5 mM Imidazole pH 7.0) containing 0.02% (w/v) Coomassie Blue G250 was

used during 1 h at 70 V, the time needed for the dye front reach approximately one third of the gel. Cathode buffer was then replaced with one containing only 0.002% (w/v) Coomassie Blue G250 and the native complexes were separated at 200 V for 3 h at 4 ºC. A native protein standard HMW-native marker (GE Healthcare, Buckinghamshire, UK) was used. The gels were stained with Coomassie Colloidal for protein visualization and scanned with Gel Doc XR System (Bio-Rad, Hercules, CA, USA). Band detection, quantification and matching were performed using QuantityOne Imaging software (v4.6.3, Bio-Rad).

**In-gel activity of heart mitochondrial complexes IV and V** (study 1)

The in-gel activity and histochemical staining assays of complexes IV and V were determined using the methods described by Zerbetto et al. (1997) with minor modifications. Complex IV-specific heme stain in BN-PAGE gels was determined using 10 µL horse heart cytochrome c (5 mM) and 0.5 mg diaminobenzidine (DAB) dissolved in 1 mL of 50 mM sodium-phosphate, pH 7.2. The reaction was stopped by 50% (v/v) methanol, 10% (v/v) acetic acid, and the gels were then transferred to water. ATP hydrolysis activity of complex V was analyzed by incubating the native gels with 35 mM Tris, 270 mM glycine buffer, pH 8.3 at 37 ºC, that had been supplemented with 14 mM $MgSO_4$, 0.2% (w/v) $Pb(NO_3)_2$, and 8 mM ATP. Lead phosphate precipitation proportional to the enzymatic ATP hydrolysis activity was stopped by 50% (v/v) methanol (30 min), and the gels were then transferred to water.

**Heart mitochondria respiratory chain complexes I and V activity** (study 1)

For spectrophotometric determination of respiratory chain complexes I and V activity, mitochondrial fractions were disrupted by a combination of freeze–thawing to allow free access to substrates for all assays. Complex I activity was measured by following the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm for 4 min. After the 4 min reduction, rotenone was added and the absorbance was measured again for 4 min (Janssen

et al., 2007). ATP synthase activity was measured according to Simon et al. (2003). The phosphate produced by hydrolysis of ATP reacts with ammonium molybdate in the presence of reducing agents to form a blue-colour complex, the intensity of which is proportional to the concentration of phosphate in solution. Oligomycin was used as a specific inhibitor of mitochondrial ATPase activity.

**Caspase-like activity** (studies 2, 3 and 4)

To measure caspase 3, 8 and 9 activities, heart, brain cortex and cerebellum tissue were homogenated in lysis buffer in ratio of ratio 1mg/mL containing 25 mM HEPES, 137Mm NaCl, 0,2mM EDTA, 0,5mM EGTA, pH 7.4 10 % glycerol, 1 % Triton X-100, 0.1 % CHAPS , 10 mM DL-Dithiothreitol (DTT) and centrifugated at 7000 x*g* for 5 min. The homogenates resulting were incubated with 100 µM of the caspase substrate Ac (N-acetyl)-LEHD-pNA (p-nitroaniline) (Calbiochem-Merk, Billerica, MA, USA)] for 2 h at 37ºC. Caspase-like activities were determined by following the detection of the chromophore pNA after cleavage from the labeled substracte Ac-LEHD-p-nitroanilide at 405nm, as previously described (Lumini-Oliveira et al., 2011). The method was calibrated with known concentrations of p-nitroanilide (Calbiochem,UK). Caspase-like activity was calculated by pNA released for equal µg protein loaded.

**Mitochondrial oxidative damage and antioxidants** (studies 1, 3 and 4)

Before analysis, heart, brain cortex and cerebellum mitochondrial membranes of isolated mitochondrial fractions were disrupted by a combination of freeze–thawing cycles to allow free access to substrates. The extent of lipid peroxidation in heart, bain cortex and cerebellum mitochondria was determined by measuring MDA contents through a colorimetric assay, according to a modified procedure described previously (Buege and Aust, 1978). Suspended mitochondria (2 mg/mL) were centrifuged at 12000 x*g* for 10 min and re-

suspended in 150 µL of a medium containing 175 mM KCl and 10 mM Tris-HCl, pH 7.4. Subsequently, mitochondria from the six groups were mixed with 2 volumes of trichloroacetic acid (10 %) and 2 volumes of thiobarbituric acid (1 %). The mixtures were heated at 90 ºC for 10 min, cooled in ice for 10 min before centrifugation (4000 x*g* for 10 min, 4 ºC). The supernatants were collected and the absorbance measured at 535 nm. The amount of MDA content formed was expressed as nanomoles of MDA per milligram of protein ($\varepsilon_{535}$=1.56 x $10^{-5}$ $M^{-1}$ $cm^{-1}$).

The mitochondrial content of oxidative modified -SH groups, including reduced glutathione and other SH-containing proteins, was quantified by a spectrophotometric measurement according to the method proposed by Hu (1990). Briefly, a mitochondrial suspension containing 5 mg/mL protein was mixed with 0.25 M Tris buffer pH 8.2 and 10 mM DTNB and the volume was adjusted to 1 mL with absolute methanol. Subsequently, the samples were incubated for 30 min in the dark at room temperature and centrifuged at 3000 x*g* for 10 min. The sample blank (without reagent) was prepared in a similar manner. The colorimetric assay of supernatant was performed at 414 nm against a blank test. Total -SH content was expressed in nanomoles per milligrams of mitochondrial protein ($\varepsilon_{414}$=13.6 $mM^{-1}$ $cm^{-1}$).

Aconitase activity was measured spectrophotometrically by monitoring the formation of cis-aconitate from isocitrate at 240 nm and 25 ºC as previously described (Ascensao et al., 2005b). Mitochondrial fractions were re-suspended in a buffer containing 50 mM Tris–HCl (pH 7.4) and 0.6 mM $MnCl_2$ to a final concentration of 2 mg/mL. Subsequently, 25 µL of each sample were collected, the volume was adjusted to 500 µL and isocitrate (200 mM) was added. One unit was defined as the amount of enzyme necessary to produce 1 µmol of cis-aconitate per minute ($\varepsilon_{240}$=3.6 $mM^{-1}$ $cm^{-1}$).

Manganese-dependent superoxide dismutase (Mn-SOD) activity was measured spectrophotometrically at 505 nm with a commercially available kit (RANSOD, Randox Labs, cidade, UK). In brief, heart, brain cortex and cerebellum mitochondria were mixed with a phosphate buffer (10 mM, pH 7.0) to a final concentration of 0.5 mg/mL. Subsequently, 1 mL

of the provided mixed substrate [0.05 mmol/L xanthine and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T)] and 30 µL of mitochondrial samples were added to a cuvette and the reaction was initiated by the addition of 150 µL xanthine oxidase (80 U/L) and maintained at 37 ºC for 3 min. Mn-SOD activity was determined against a blank measurement and measured as units per milligram of protein. One unit of SOD was defined as resulting in 50 % of inhibition of the rate of reduction of I.N.T under the conditions of the assay.

**Immunoblotting for detection of proteins** (studies 1, 2, 3 and 4)

Equivalent amounts of heart, brain cortex and cerebellum mitochondrial protein (1mg/mL) or tissues homogenates (3 mg/mL) were denatured in a sample loading buffer and separated by dodecyl sulphate-polyacrylamide gel electrophoresis SDS/PAGE (12 % gels) as described by (Laemmli, 1970), followed by blotting on PVDF membranes (Millipore, Massachusetts, USA) according to the method of Locke (1990). Heart, brain cortex and cerebellum mitochondrial content of the outer mitochondrial translocator TOM20 and tissue content in the β-actin were used as protein loading controls. In addition, membranes were stained with Ponceau-S to verify quality of transfer and equal protein loading. After blotting, non-specific binding was blocked overnight with 5 % (w/v) non-fat dry milk powder in Tris-base buffered saline with 0,1 % Tween-20 (TBS-T).

Proteins blotted into membranes were then incubated with PGC-1α (3 µg/mL, ab106814 goat polyclonal IgG); anti-OPA1 (1 µg/mL, ab119685 mouse monoclonal IgG); anti-PINK1 (1:500, ab23707 rabbit polyclonal IgG); anti-Parkin (1:500, ab15954 rabbit polyclonal IgG); anti-p62 (1:1000, ab56416 mouse monoclonal IgG); anti-cyclophilin D (1:1000, ab110324 mouse monoclonal IgG); anti-p66shc (1:500 #2432 rabbit polyclonal IgG); anti-beclin1 (1:1000, #3495 rabbit monoclonal IgG); anti-Bcl-2 (1:1000, #2870 rabbit monoclonal IgG); anti-Bax (1:1000, #2772 rabbit polyclonal IgG); anti-DRP1 (1:1000, #8570 rabbit monoclonal IgG); anti-cofilin (1:1000, #5175, rabbit monoclonal IgG); anti-SIRT3 (1:1000, #2627 rabbit

monoclonal IgG), anti-p66shc(pSer$^{36}$) (1:750, 6E10 mouse monoclonal IgG), anti-LC3 (1:1000, PD014 rabbit polyclonal IgG); anti-β-actin (1:500, sc-1616 goat polyclonal IgG); anti-ANT (1:750, sc-9299 goat polyclonal IgG); anti-TOM 20 (1:500, sc-11415 rabbit polyclonal IgG); anti-Mfn1 (1:1000, sc-50330 rabbit polyclonal IgG), anti-Mfn2 (1:1000, sc-50331 rabbit polyclonal IgG); anti-TFAM (1:1000, sc-23588 goat polyclonal IgG); anti-UCP2 (1:1000, SAB2501087 goat polyclonal IgG); and anti-acetylated-lysine (1:1000, #9441 rabbit polyclonal IgG) ). Additionally, mitochondrial oxidative phosphorylation proteins were semi-quantified by using the MitoProfile® Total OXPHOS Western blotting kit (1:1000, ab110413 mouse monoclonal IgG), containing a cocktail of monoclonal antibodies: NDUFB8-20 kDa of complex I (ab110242), SDHB-30 kDa of complex II (ab14714), Core protein 2-48 kDa of complex III (ab14745) subunit I-40kDa of complex IV (ab14705) and alpha subunit-55 kDa of ATP synthase (ab147487).

Carbonylated proteins were assayed according to Robinson et al. (1999) with some modifications. Briefly, 50 µL (1 V) of mitochondria containing 20 µg of protein was derivatized with 2,4-dinitrophenylhydrazine (DNPH). The sample was mixed with 1 V of 12 % SDS plus 2 V of 20 mM DNPH/10% TFA, followed by 30 min incubation in dark, after which 1.5 V of 2 M Tris–base/18% of β-mercaptoethanol was added for neutralization. Then, derivatized proteins were subjected to 12% SDS–PAGE gel and electrotransferred to PVDF membrane blots as described. Immunodetection of carbonylated proteins was performed using rabbit anti-DNP (1:500, D9656 rabbit polyclonal IgG) as primary antibody.

Primary antibodies were diluted in TBS-T containing 2 % of non-fat dried milk or BSA for 8 h at 4 ºC. Following antibody incubation membranes were washed and incubated with secondary horseradish-peroxidase-conjugated anti-mouse (sc-358922), anti-goat (sc-2354) or anti-rabbit (sc-4004) IgG antibodies (1:10000) for 2 h at room temperature, containing 2 % of non-fat dried milk or BSA. Protein bands were visualized by treating the immunoblots with ECL® PlusTM, visualized with the ChemiDoc XRS+ system (Bio-Rad Laboratories, Amadora, Portugal) and analyzed with the image analysis program Image Lab software (Bio-Rad

Laboratories, Amadora, Portugal). The densitometry analysis was carried out immediately before saturation of the immunosignal. Data were observed as band intensity of immunostaining values (arbitrary units), and the results were expressed as percentage of SED group.

**Soleus citrate synthase activity** (studies 1, 2, 3 and 4)

Portions of approximately 50 mg of one *soleus* muscle were separated and homogenized (100 mg/mL) in RIPA buffer (#20-188) from Millipore (Merck Millipore, Darmstadt, Germany) supplemented with protease (P8340 from Sigma) and phosphatase (P5726 from Sigma) inhibitor cocktail, using a Teflon pestle on a motor driven Potter-Elvehjem glass homogenizer at 4 ºC three to five times for 5 s at speed low setting, with a final burst at a higher speed setting. Homogenates were then centrifuged (2 min at 12000 *xg*, 4 ºC) and the supernatants collected. Protein content was spectrophotometrically determined by the Bradford protein assay method using bovine serum albumin as standard (Bradford, 1976).

*Soleus* CS activity was measured using the method proposed by Coore et al (1971). The principle of assay is based in the reaction of acetyl-CoA with oxaloacetate and measure the release of CoA-SH to 5,5-dithiobis (2-nitrobenzoate). The colorimetric assay of supernatant was performed at 30 ºC with 16 s intervals for 2 min, against a blank test. The repeated (16 s) absorbance values were then plotted against time. CS activity was expressed in nanomoles per milligrams of mitochondrial protein ($\varepsilon_{412}$=13.6 $mM^{-1}$ $cm^{-1}$).

**Transmission electron microscopy** (studies 1 and 2)

To analyze heart mitochondrial morphology, cardiac muscle samples were processed for transmission electron microscopy (TEM). Briefly, the heart tissue was cut into pieces smaller than 1 mm and fixed in 2.5 % glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 4 h. Samples were then post-fixed in 2 % osmium tetroxide in 0.2 M sodium cacodylate buffer

(pH 7.4) for 2 h, dehydrated through graded concentrations of ethanol and propylene oxide and subsequently embedded in Epon. Ultrathin sections (50–70 nm) were contrasted with uranyl acetate and lead citrate, and examined with a Hitachi 800-MT TEM (Hitachi Inc., Japan). The images obtained were collected at original magnifications of 12000 x, digitalized and stored in a tagged image file format in the microscope computer image analysis program (Quartz PCI, Scientific Image Management System, v 5.1, Quartz Image Corp., Canada). The percentage of abnormal mitochondria were analyzed according to the criteria previously established (Ascensao et al., 2006b) as follows: 1) no alterations; 2) mitochondria were considered as abnormal only if presented mild focal loss of cristae density and 3) mitochondria evidencing extensive degeneration or even loss of cristae, intramitochondrial vacuoles and notorious myelin figures that probably resulted in the formation of secondary lysosomes and mitochondria swelling. Mitochondrial area (12000 x) was estimated as the mitochondrial area ($\mu m^2$) per total area ($\mu m^2$) of the corresponding TEM image and expressed as percentage of SAL+SED group.

**Statistical analysis** (studies 1, 2, 3 and 4)

All data are expressed as the mean±SEM (Standard Error of the Mean). Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad software, San Diego, CA, USA) or Statistical Package for the Social Sciences version 18.0 (SPSS Inc., Chicago, Illinois). One-way or Two-way ANOVA followed by Bonferroni was used to examine possible effect of treatment and/or exercise for all variables. In all cases, the significance level was set at $p \leq 0.05$.

# 3.2

[Study 1]

## Physical exercise prior and during treatment reduces sub-chronic Doxorubicin-induced mitochondrial toxicity and oxidative stress

Marques-Aleixo I[1], Estela Santos-Alves[1], Diogo Mariani[1], David Rizo-Roca[2], Ana I Padrão[3], Sílvia Rocha-Rodrigues[1], Ginés Viscor[2], J Ramon Torrella[2], Rita Ferreira[3], Paulo J Oliveira[4], José Magalhães[1], António Ascensão[1]

[1]CIAFEL - Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Portugal

[2]Department of Physiology and Immunology, Faculty of Biology, Universitat de Barcelona

[3]QOPNA - Chemistry Department, University of Aveiro, Portugal

[4]CNC - Centre for Neuroscience and Cell Biology, Department of Life Sciences, University of Coimbra, Portugal

**ABSTRACT**

Doxorubicin (DOX) is an anti-cancer agent whose clinical usage results in a cumulative and dose-dependent cardiotoxicity. We have previously shown that exercise performed prior DOX treatment reduces the resulting cardiac(mito)toxicity. We sought to determine the effects on cardiac mitochondrial toxicity of two distinct chronic exercise models (endurance treadmill training–TM and voluntary free-wheel activity-FW) when used prior and during DOX treatment.

Male-young Sprague-Dawley rats were divided in six groups (n=6 per group): SAL+SED (saline sedentary), SAL+TM (12-weeks TM), SAL+FW (12-weeks FW), DOX+SED (7-weeks of chronic DOX treatment 2mg.kg-1 per week), DOX+TM and DOX+FW. Heart mitochondrial ultrastructural alterations, mitochondrial function (oxygen consumption and membrane potential), semi-quantification of oxidative phosphorylation (OXPHOS) proteins and their in gel activity, as well as proteins involved in mitochondrial oxidative stress (SIRT3, p66shc and UCP2), biogenesis (PGC1α and TFAM), acetylation and markers for oxidative damage (carbonyl groups, MDA, -SH, aconitase, Mn-SOD activity) were evaluated.

DOX treatment resulted in ultrastructural and functional alterations and decreased OXPHOS. Moreover, DOX decreased complex I activity and content, mitochondrial biogenesis (TFAM), increased acetylation and oxidative stress. TM and FW prevented DOX-induced alteration in OXPHOS, increase in oxidative stress, the decrease in complex V activity, and in complex I activity and content. DOX-induced decrease in TFAM and SIRT3 content were prevented by TM only.

Both chronic models of physical exercise performed before and during the course of sub-chronic DOX treatment translated into an improved mitochondrial bioenergetic fitness, which may result in part from the prevention of mitochondrial oxidative stress and damage.

**Key words:** cardioprotection; Adriamycin, exercise, bioenergetics, oxidative damage

## Introduction

Doxorubicin (DOX, or Adriamycin) is an effective antibiotic used to treat several malignancies. Unfortunately, its clinical use is limited by the development of a dose-dependent cardiac toxicity that results in life-threatening cardiomyopathy. DOX-induced cardiomyocyte dysfunction is associated with increased levels of oxidative damage with the involvement of mitochondrial bioenergetic collapse in the process (Wallace, 2007). In fact, sub-chronic DOX-treated rats show defects on heart mitochondrial function, accompanied by compromised mitochondrial electron transport chain activity and increased oxidative stress and damage (Abd El-Gawad and El-Sawalhi, 2004; Berthiaume et al., 2005; Santos et al., 2002).

Among the strategies proposed as effective counteracting the cardiac side effects associated with DOX treatment, physical exercise has been recommended as a non-pharmacological tool against myocardial injury (Ascensao et al., 2006c; Ascensao et al., 2007; Ascensao et al., 2011c; Powers et al., 2008). Previous work suggested that the advantage of both acute (Ascensao et al., 2011b; Wonders et al., 2008) and chronic exercise models (Ascensao et al., 2005a; Ascensao et al., 2005b; Ascensao et al., 2006a; Chicco et al., 2005, 2006b; Dolinsky et al., 2013) on triggering a preconditioning-like effect on DOX-treated rats with acute single doses include the protection of cardiac tissue and especially mitochondria against negative remodeling. Recent studies investigated the effects of exercise performed during and following late-onset DOX-induced cardiotoxicity showed improvements in hemodynamic parameters (Hayward et al., 2012b; Hydock et al., 2012b). However, the cellular and molecular mechanisms underlying this protective phenotype induced by exercise against sub-chronic DOX administration, particularly those targeting mitochondria, are unknown. Specifically, whether perturbations in heart mitochondrial oxidative phosphorylation capacity and oxidative modifications associated with sub-chronic cumulative DOX administration are mitigated by “forced” or “voluntary” long-term exercise models performed

prior and during the course of treatments have not been determined and represents the novelty of the present study. As patients undergoing chemotherapy experience severe fatigue and display severe exercise intolerance, the intensity and duration of tolerable exercise is likely to be severely limited (Emter and Bowles, 2008). Facing this, we aimed at analyzing the effects of two types of long-term exercise with distinct characteristics regarding volume and intensity on cardiac mitochondrial bioenergetics and oxidative stress markers in rats sub-chronically treated with DOX.

**Results**

Body and heart weights, mitochondrial protein isolation yield as well as the activity of *soleus* citrate synthase and cTnI are shown in Table 1. As expected, final body and heart weights decreased with DOX treatment (SAL+SED *vs.* DOX+SED) and TM and FW-induced heart hypertrophy (SAL+TM and SAL+FW *vs.* SAL+SED and DOX+TM and DOX+FW *vs.* DOX+SED). TM increased *soleus* citrate synthase activity in both SAL and DOX-treated animals (SAL+SED *vs.* SAL+TM and DOX+SED *vs.* DOX+TM).

**Chapter 3.2 - Table 1.** Animal data and yield of mitochondrial protein isolation

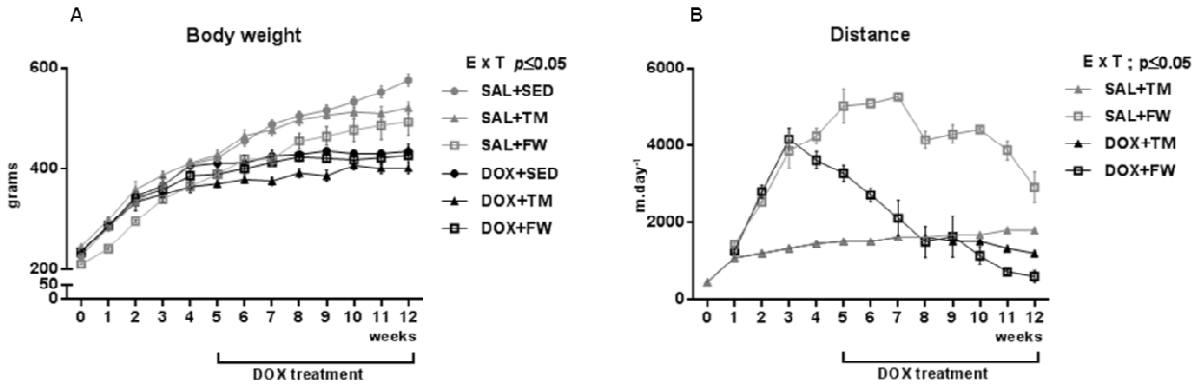
| | SAL+SED | SAL+TM | SAL+FW | DOX+SED | DOX+TM | DOX+FW | $p^{*}$ |
|---|---|---|---|---|---|---|---|
| Heart weight (g) | 1.44±0.03 | 1.92±0.08* | 1.85±0.10* | 1.10±0.03$^{\#}$ | 1.45±0.05* | 1.55±0.11* | E, T |
| Mitochondrial isolation yield (mg protein/g tissue) | 18.58±0.62 | 15.15±1.22 | 15.01±1.50 | 16.96±0.83 | 18.60±4.68 | 17.84±1.57 | NS |
| *Soleus* citrate synthase activity (mM. min$^{-1}$.mg$^{-1}$) | 10.94±2.07 | 23.34±1.79*$^{\ddagger}$ | 11.22±2.65 | 8.69±1.15 | 22.22±1.97*$^{\ddagger}$ | 10.27±1.32 | E |
| cTnI (pg.mL$^{-1}$) | 0.009±0.001 | 0.013±0.003 | 0.014±0.003 | 0.020±0.003 | 0.011±0.006 | 0.010±0.004 | NS |

Values are mean±SEM. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 per group,*SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED ($p \leq 0.05$); $^{\#}$SAL+SED vs. DOX+SED ($p \leq 0.05$); $^{\ddagger}$SAL+TM vs. SAL+FW and DOX+TM vs. DOX+FW ($p \leq 0.05$). $^{*}$ Significant ($p \leq 0.05$) effects of Exercise (E), Exercise and treatment (E, T) are shown; Non Significant (NS, $p > 0.05$).

Body weight alterations and distances covered by the animals during the entire protocol are shown in Figure 1. No significant differences were found in the mean body weight of the animals from the beginning of the protocol until the 5$^{th}$ week, when the sub-chronic DOX treatment was initiated. However, body weights of DOX-treated animals were lower than SAL counterparts at the end of the protocol (SAL+SED *vs.* DOX+SED; SAL+TM *vs.* DOX+TM; SAL+FW *vs.* DOX+FW). No differences in body weight between exercised groups were found (SAL+TM *vs.* SAL+FW and DOX+TM *vs.* DOX+FW). Both TM and FW decreased body weight at 12$^{th}$ and 9$^{th}$ week, respectively (SAL+TM and SAL+FW *vs.* SAL+SED). TM in DOX treated animals decreased body weight from the 5$^{th}$ week (DOX+SED *vs.* DOX+TM), whereas no significant differences were found between FW and SED-treated groups (DOX+SED *vs.* DOX+FW) (Figure 1A).

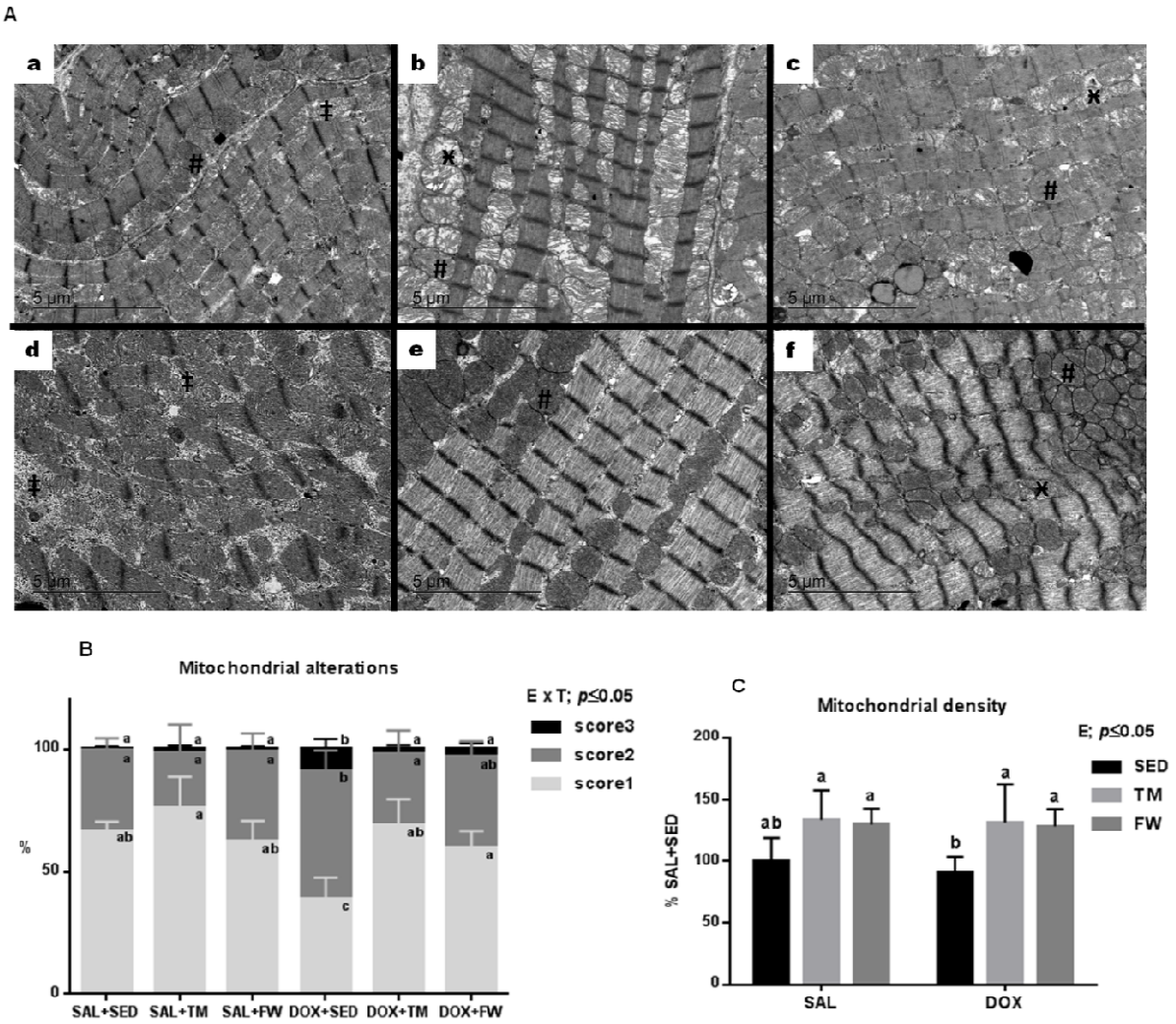
As can be seen in Figure 1B, voluntary running distance decreased significantly in DOX+FW after the 5$^{th}$ week and remained lower until the end of the protocol ($p \leq 0.05$). Animals from TM

group ran at the same velocity throughout the 8 weeks of the protocol and running velocity and distance significantly decreased in DOX animals, at the 11th and 12th weeks.



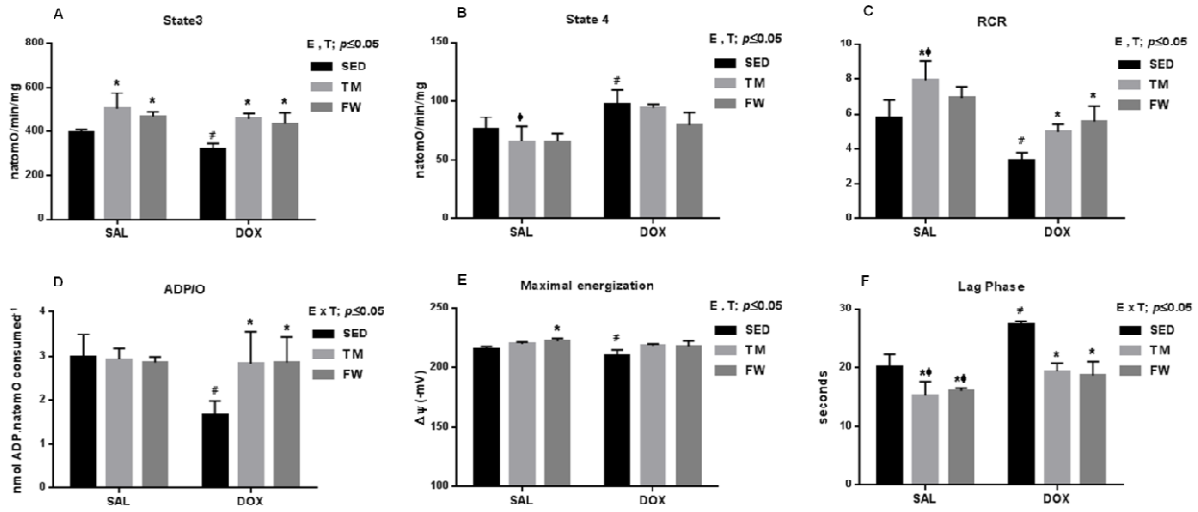

**Chapter 3.2 - Figure 1.** Effect of exercise and sub-chronic DOX treatment on (A) body weight alterations over time and (B) distance covered per day by animals of the TM and FW group during the 12 weeks of protocol. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 per group. Significant differences ($p \leq 0.05$) are mentioned in the text. Significant ($p \leq 0.05$) interaction effect of Exercise and Treatment (E x T) is indicated.

The effects of the two exercise models on DOX-induced toxicity regarding cardiac ultra-structure alterations, including mitochondria were analyzed by TEM (Figure 2). As shown, and despite no differences in mitochondrial density between sedentary-treated and saline groups, sub-chronic DOX treatment increased the percentage of abnormal mitochondria regardless the severity of the alterations (score 2 and 3, DOX+SED *vs.* SAL+SED). Both exercise models increased the percentage of normal mitochondria and prevented the increase in abnormal mitochondria induced by DOX treatment (DOX+TM and DOX+FW *vs.* DOX+SED). Mitochondrial density increased in exercised groups, when compared with the sedentary group treated with DOX (DOX+TM and DOX+FW *vs.* DOX+SED).




**Chapter 3.2 - Figure 2.** Effect of exercise and sub-chronic DOX treatment on (A) Representative electron micrographs of cardiac tissue from (a) SAL+SED, (b) SAL+TM, (c) SAL+FW, (d) DOX+SED, (e) DOX+TM and (f) DOX+FW groups (magnification: 12000 x); (B) the percentage of heart mitochondrial alterations and on (C) mitochondrial density. The percentage of abnormal mitochondria and mitochondrial density were analyzed according to the score-based criteria previously established: 1) no alterations; 2) mitochondria were considered as abnormal only if presenting mild focal loss of cristae density and 3) mitochondria evidencing extensive degeneration or even loss of cristae, intramitochondrial vacuoles and notorious myelin figures that probably resulted in the formation of secondary lysosomes and mitochondria swelling. Mitochondria density is expressed in percentage of $\mu m^2$ of mitochondria per $\mu m^2$ total TEM image. Values are mean±SEM. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=3 per group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED ($p \leq 0.05$); $^{\#}$SAL+SED vs. DOX+SED ($p \leq 0.05$). Significant effects of Exercise (E) or interaction effect of Exercise and Treatment (E x T) are indicated.

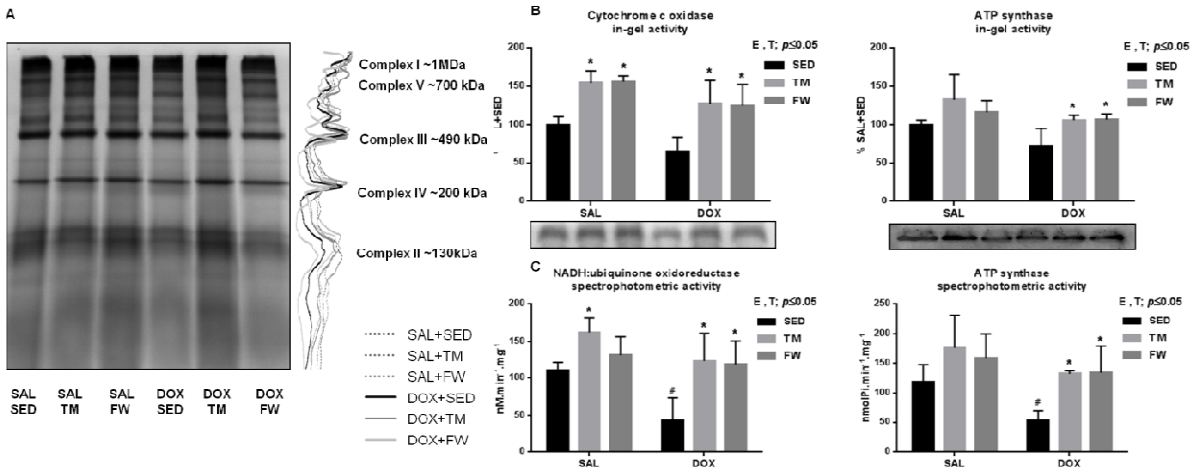
Mitochondrial oxygen consumption and Δψ associated with ADP phosphorylation were measured to identify exercise- and DOX-dependent effects (Figure 3). DOX treatment decreased heart mitochondrial respiratory rate during state 3, increased state 4 respiration, affected the coupling between oxygen consumption and ADP phosphorylation (RCR) and the phosphorylation efficiency, as measured by the ADP/O ratio, as well as the maximal Δψ and ADP lag-phase (DOX+SED *vs.* SAL+SED). Importantly, TM and FW increased state 3 respiration and decreased the ADP phosphorylation lag phase in both SAL and DOX groups (SAL+TM and SAL+FW *vs.* SAL+SED and DOX+TM and DOX+FW *vs.* DOX+SED). TM and FW were able to restore DOX-induced decreased RCR, ADP/O ratio and maximal Δψ caused (DOX+TM and DOX+FW *vs.* DOX+SED).




**Chapter 3.2 - Figure 3.** Effect of exercise and sub-chronic DOX treatment on heart mitochondria (A) state 3 of mitochondrial respiration, (B) state 4 of mitochondria respiration, (C) RCR, (D) ADP/O and on heart mitochondrial Δψ fluctuations (E) maximal energization and (F) ADP phosphorylation lag phase. Data are means±SEM obtained from different mitochondrial preparations (0.5 mg/mL protein) for each group. Respiration and Δψ developed were measured with G/M as substrates. RCR, respiratory control ratio (state 3/state 4); ADP/O, number of nmol ADP phosphorylated by natom of oxygen consumed; lag phase, time elapsed in the depolarization/repolarization cycle during ADP phosphorylation. Values are mean±SEM. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 per group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED (p≤0.05); $^{\#}$SAL+SED vs. DOX+SED (p≤0.05); $^{\Phi}$SAL+TM vs.

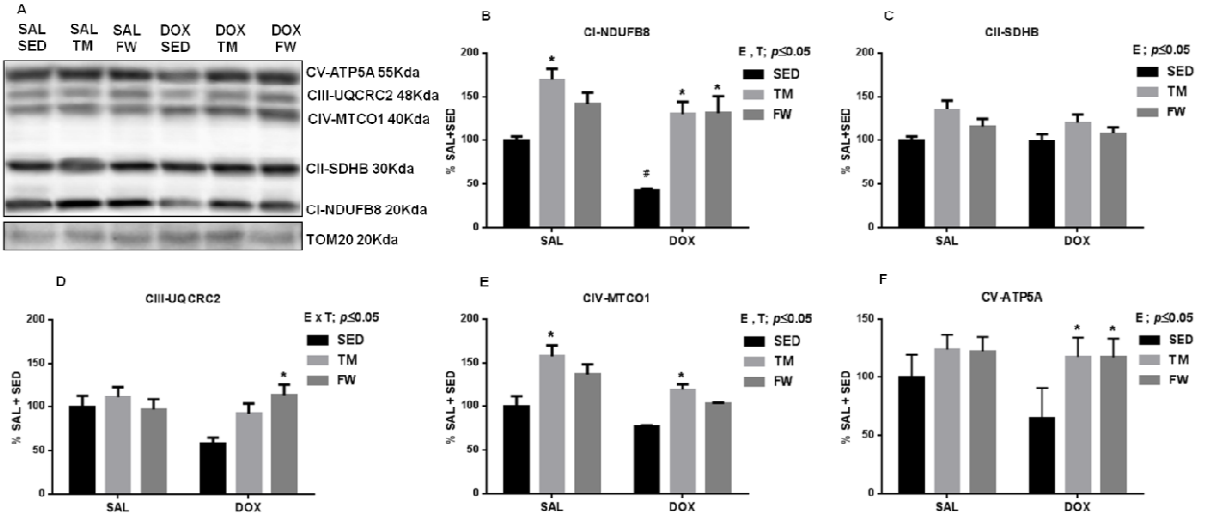
DOX+TM and SAL+FW vs. DOX+FW . Significant (p≤0.05) effects of Exercise and Treatment (E, T) and interaction effect of Exercise and Treatment (E x T) are indicated.

The effect of both exercise models on sub-chronic DOX treatment regarding mitochondrial respiratory chain complex BN-profile are presented in Figure 4A. The pattern of the five respiratory chain complexes was consistent with the molecular weight described (Schagger and Von Jagow, 1991) and no qualitative differences between groups were observed. The determined specific activity and/or expression of the complexes for DOX and exercised groups generally agreed with the fitness-related oxygen consumption and $\Delta\psi$ data above described. By using an in-gel activity analysis for complexes IV and V, the data showed that DOX treatment *per se* did not alter the activity levels of those complexes (SAL+SED *vs.* DOX+SED). Importantly, both exercise models increased complex IV and V activities in SAL and DOX groups (SAL+TM or SAL+FW *vs.* SAL+SED; DOX+TM or DOX+FW *vs.* DOX+SED) (Figure 4B). Spectrophotometric assays showed that DOX decreased, while TM increased NADH:ubiquinone oxidoreductase complex activity (DOX+SED *vs.* SAL+SED and SAL+TM *vs.* SAL+SED, respectively). Also, DOX decreased ATP synthase activity in the sedentary group (DOX+SED *vs.* SAL+SED). Increased activity levels of respiratory chain complexes I and V were observed in exercised groups treated with DOX compared to their sedentary counterparts (DOX+TM or DOX+FW *vs.* DOX+SED) (Figure 4C).



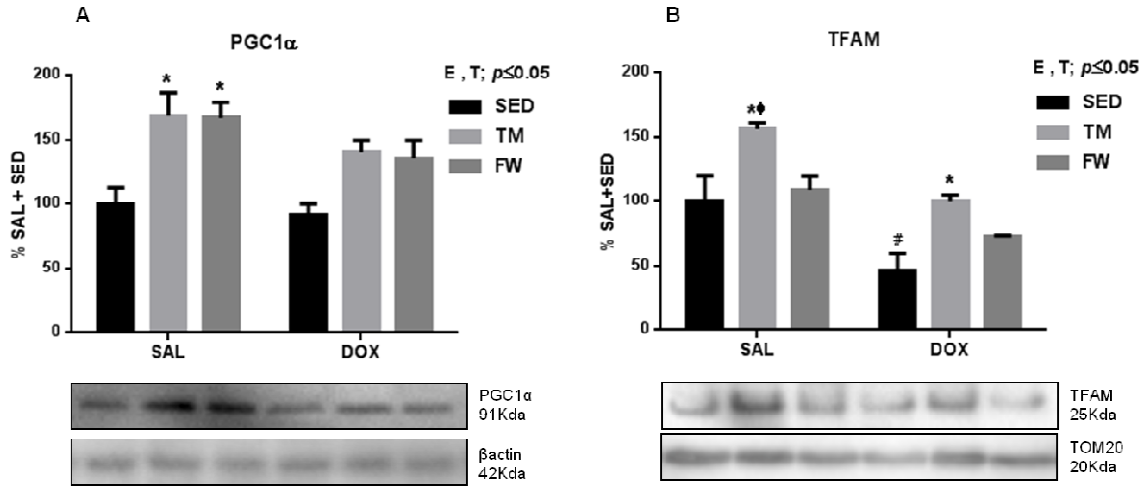

**Chapter 3.2 - Figure 4.** Effect of exercise and sub-chronic DOX treatment on heart mitochondria on: (A) BN-PAGE profile of mitochondrial respiratory complexes. An overlap of the density variation for all lanes is presented on the right side; (B) semi-quantitative analysis of in-gel activity of complexes cytochrome c oxidase and ATP synthase. The representative image of histochemical staining of in-gel activity of both complexes cytochrome c oxidase and ATP synthase are presented below the histograms and the results were expressed as percentage of SAL+SED group; and (C) Respiratory chain complexes NADH:ubiquinone oxidoreductase and ATP synthase activity in heart mitochondria, as measured spectrophotometrically. Values are mean±SEM. Values are mean±SEM. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 per group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED ($p \leq 0.05$); $^{\#}$SAL+SED vs. DOX+SED ($p \leq 0.05$). Significant ($p \leq 0.05$) effects of Exercise and Treatment (E, T) are indicated.

The analysis of heart mitochondria OXPHOS subunits (Figure 5) showed that DOX treatment decreased complex I subunit content (DOX+SED *vs.* SAL+SED). On the other hand, TM *per se* increased complex I and IV subunits content (SAL+TM *vs.* SAL+SED). In DOX-treated groups, TM increased complex IV subunit content, whereas FW increased complex III subunit (DOX+TM *vs.* DOX+SED and DOX+FW *vs.* DOX+SED, respectively). Both exercise models increased complex V subunit content in DOX-treated groups and were able to counteract DOX-induced decreased complex I (DOX+TM and DOX+FW *vs.* DOX+SED).



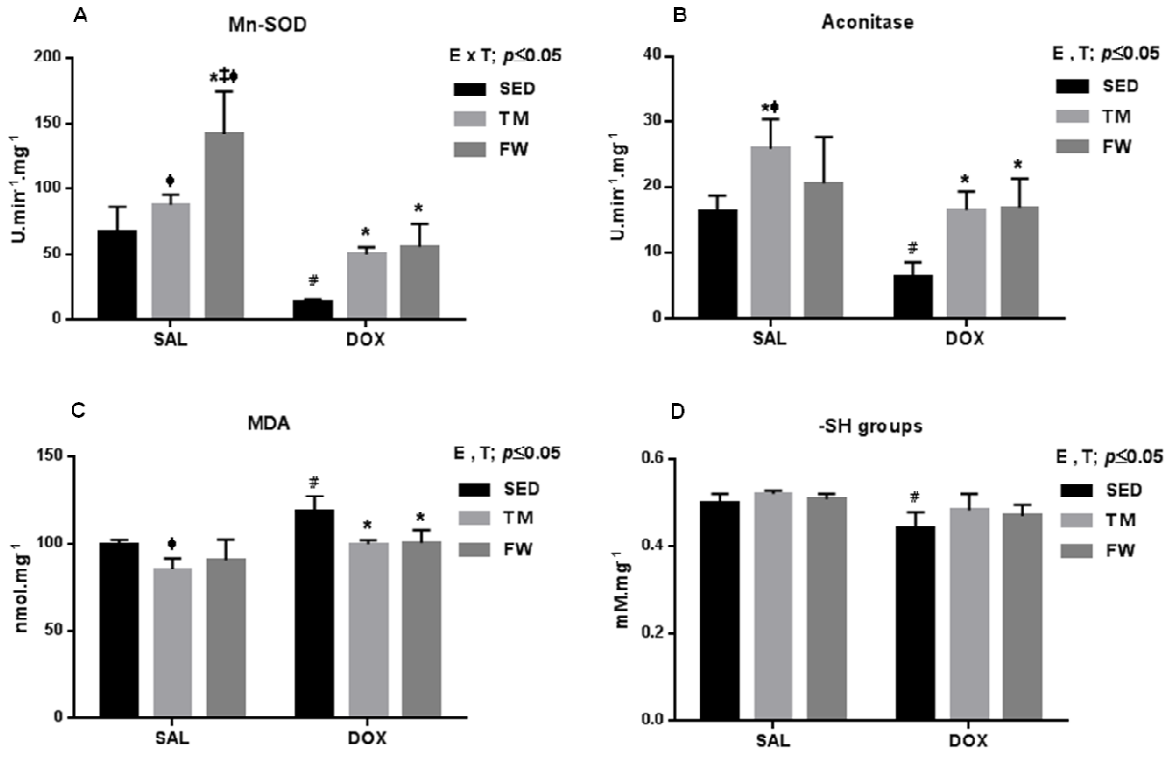

**Chapter 3.2 - Figure 5.** Effect of exercise and sub-chronic DOX treatment on heart mitochondrial OXPHOS subunits: (A) typical immunoblots (B); complex I, CI-NDUFB8 (C); complex II, CII-SDHB (D); complex III, CIII-UQCRC2 (E); complex IV, CIV-MTCO1 and (F) complex V, CV-ATP5A. Data are means±SEM obtained from different mitochondrial preparations for each group. The results were corrected for TOM20 and expressed as percentage of SAL+SED group. Values are mean±SEM. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=4 per group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED ($p \leq 0.05$); #SAL+SED vs. DOX+SED ($p \leq 0.05$). Significant ($p \leq 0.05$) effects of Exercise (E), Exercise and Treatment (E, T) and interaction effect of Exercise and Treatment (E x T) are indicated.

Immunoblotting was performed to semi-quantify heart mitochondrial proteins involved in mitochondrial biogenesis, namely PGC1α and TFAM (Figure 6). As shown, sub-chronic DOX treatment decreased TFAM expression (DOX+SED *vs.* SAL+SED). Both exercise models *per se* increased PGC1α levels (SAL+TM and SAL+FW *vs.* SAL+SED). Although not reaching statistical significance, increased levels of PGC1α were observed in exercised groups treated with DOX (DOX+TM or DOX+FW *vs.* DOX+SED). Only mitochondria from TM animals exhibited significantly higher levels of TFAM compared to sedentary (SAL+TM *vs.* SAL+SED and DOX+TM *vs.* DOX+SED).




**Chapter 3.2 - Figure 6.** Effect of exercise and sub-chronic DOX treatment on (A) whole tissue PGC1α and (B) mitochondrial TFAM. Typical immunoblots are presented below the histograms. Data are means±SEM for heart tissue or mitochondria obtained from different preparations for each group. The results were corrected for TOM20 or β-actin and expressed as percentage of SAL+SED group. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=4 per group,*SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED ($p \leq 0.05$); $^{\#}$SAL+SED vs. DOX+SED ($p \leq 0.05$); $^{\Phi}$SAL+TM vs. DOX+TM and SAL+FW vs. DOX+FW . Significant ($p \leq 0.05$) effects of Exercise and Treatment (E, T) is indicated.

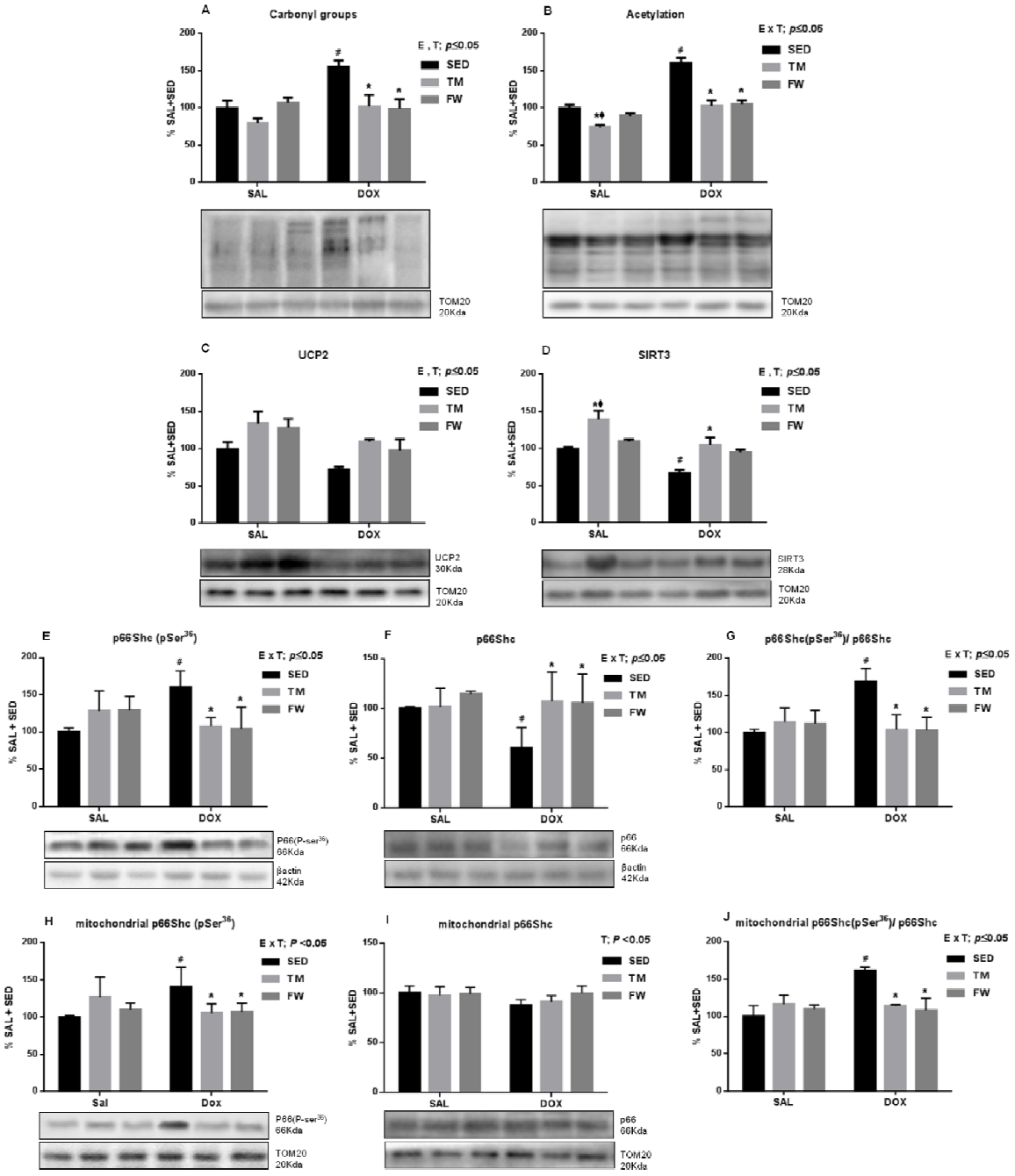
We next evaluated whether exercise altered sub-chronic DOX treatment-induced mitochondrial oxidative stress (Figure 7). DOX treatment decreased Mn-SOD and aconitase activities and –SH content, while increasing MDA content (SAL+SED *vs.* DOX+SED). TM *per se* decreased mitochondrial MDA levels and increased aconitase activity (SAL+TM *vs.* SAL+SED), whereas FW increased Mn-SOD activity (SAL+FW *vs.* SAL+SED). Both models of exercise were able to prevent DOX-induced alterations in oxidative stress and damage as seen by Mn-SOD, aconitase and MDA (DOX+SAL *vs.* DOX+TM and DOX+FW).



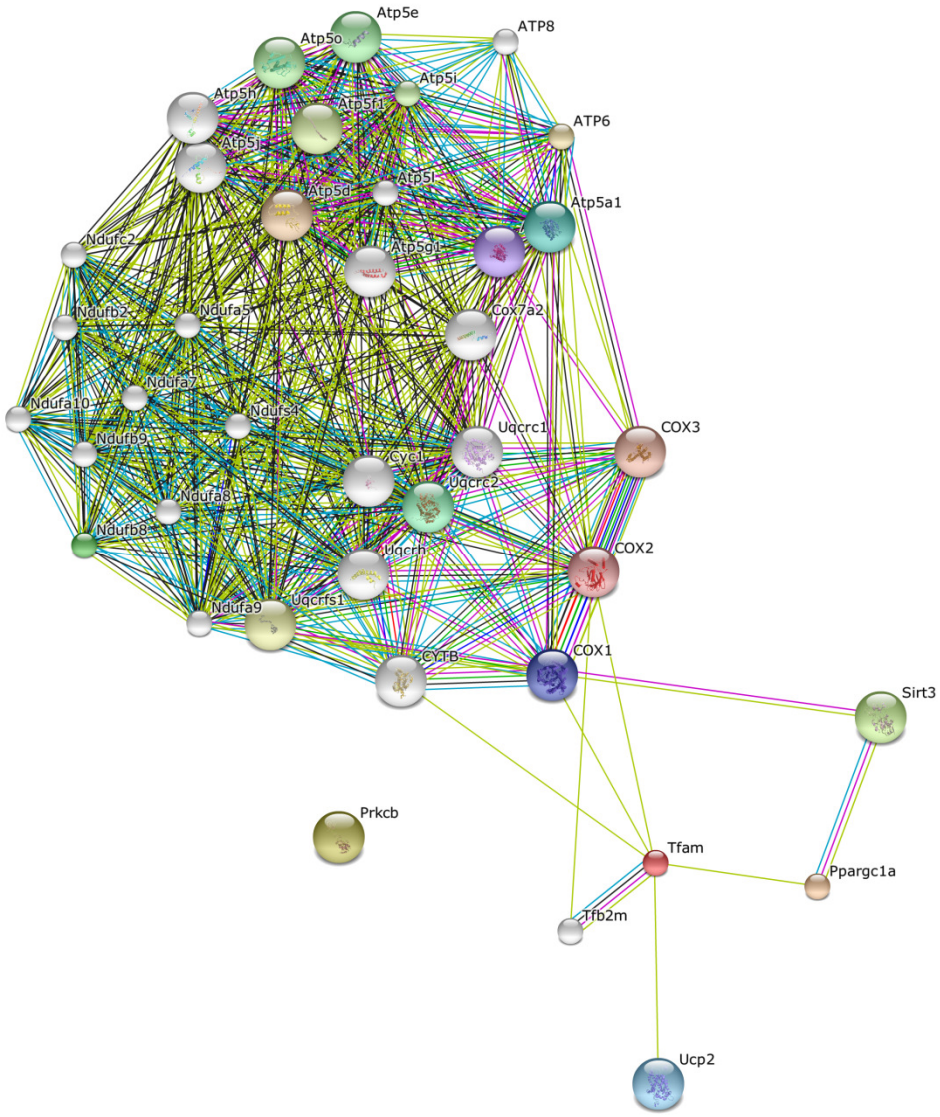

**Chapter 3.2 - Figure 7.** Effects of exercise and sub-chronic DOX treatment on heart mitochondrial (A) Mn-SOD activity (B) aconitase activity (C) MDA content and (D) reduced sulfhydryl contents. Data are means±SEM obtained from different mitochondrial preparations for each group. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 per group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED (p≤0.05); $^{\#}$SAL+SED vs. DOX+SED (p≤0.05); $^{\ddagger}$ SAL+TM vs. SAL+FW and DOX+TM vs. DOX+FW (p≤0.05); $^{\Phi}$SAL+TM vs. DOX+TM and SAL+FW vs. DOX+FW Significant (p≤0.05) effects of Exercise and Treatment (E, T) and interaction effect of Exercise and Treatment (E x T) are indicated.

Alterations in heart mitochondrial carbonylated proteins content as well as in SIRT3, UCP2 and p66shc can be seen in Figure 8. As shown, DOX treatment significantly increased protein carbonylation levels and p66shc(Ser$^{36}$)/p66shc ratio in total heart tissue and in isolated mitochondria, and decreased SIRT3 content (DOX+SED *vs.* SAL+SED). TM increased SIRT3 levels in both SAL and DOX groups and decreased acetylated proteins in SAL group (SAL+TM vs. SAL+SED and DOX+TM *vs.* DOX+SED). Protein content of SIRT3, PGC1$\alpha$ and complex IV subunit 1 (COX1) were similar and in accordance with protein-

protein interactions with SIRT3 (according to String analysis shown in Figure 9). We also measured the mitochondrial expression of acetylated proteins, which increased after DOX treatment. Both TM and FW exercise models were able to counteract DOX-induced increased mitochondrial carbonylated and acetylated proteins as well as p66shc($Ser^{36}$)/p66shc ratio in both heart homogenate and isolated mitochondria (DOX+TM and DOX+FW *vs.* DOX+SED). No differences between groups were observed regarding the content of mitochondrial UCP2.




**Chapter 3.2 - Figure 8.** Effect of exercise and sub-chronic DOX treatment on semiquantification by western blotting of heart (A) mitochondrial carbonyl groups (B) acetylation (C) UCP2 (D) SIRT3 (D) p66shc(pSer$^{36}$) (E) p66shc (F) p66shc(pSer$^{36}$)/p66shc ratio (G) mitochondrial p66shc(pSer$^{36}$) (H) mitochondrial p66shc and (I) mitochondrial p66shc(pSer$^{36}$)/p66shc ratio. Typical immunoblots are presented below the histograms. Data are means±SEM for heart mitochondria and cardiac tissue obtained from different preparations for each group. The results were corrected for TOM20 or β-actin and expressed as percentage of SAL+SED group. SAL+SED-saline sedentary, SAL+TM-saline treadmill, SAL+FW-saline free wheel, DOX+SED-doxorubicin sedentary, DOX+TM-doxorubicin treadmill, DOX+FW-doxorubicin free wheel, n=4 per group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED (p≤0.05); $^{\#}$SAL+SED vs. DOX+SED (p≤0.05); $^{\Phi}$SAL+TM vs. DOX+TM and SAL+FW vs. DOX+FW. Significant (p≤0.05) effects of Exercise and Treatment (E, T) and interaction effect of Exercise and Treatment (E x T) are indicated.




**Chapter 3.2 - Figure 9.** Protein-protein interaction with SIRT3 (String v9.05). String analysis evidenced a direct interaction between SIRT3 and PGC1α (Ppargc1a) and OXPHOS complex IV subunit (COX1). No direct interaction was observed for TFAM, UCP2 and the remaining analyzed OXPHOS complex subunits.

## Discussion

The main finding of the present study was that TM and FW, performed before and during the course of sub-chronic DOX treatment, prevented mitochondrial dysfunction and regulate oxidative stress. Our objective was to find out whether any of these exercise models was effective in counteracting cardiac mitochondrial structural alterations, compromised

mitochondrial biogenesis, oxidative damage and bioenergetic disruption caused by cumulative sub-chronic DOX treatment. The present study demonstrated for the first time that both models of chronic exercise normalize the referred cardiac mitochondrial abnormalities characterizing sub-chronic DOX treatment under such conditions.

In the attempt to ascertain the relevance of mitochondrial bioenergetics and oxidative stress, the major reasons to justify the present novel set were related to the timing- and the possible intensity-dependent effects of exercise-induced cardioprotection against DOX. In our study, the initial DOX injection was administrated 5wks after the beginning of exercise with subsequent weekly injections until the end of the protocols. Additionally, patients undergoing chemotherapy experience severe fatigue or display severe exercise intolerance (Lucia et al., 2003) that may severely limit the ability to follow some exercise regimens, particularly those comprising elevated intensities around 75% $VO_2$max (Emter and Bowles, 2008). As a result, the duration and intensity patients are able to tolerate under such programs are likely to be severely limited. Therefore, two distinct chronic exercise models regarding intensity and duration (TM, which imposes a forced pace running and FW, that depends on animal motivation and which is self-paced) were implemented.

Electron microscopy imaging of hearts from sedentary sub-chronic DOX treated animals revealed the presence of morphological evidences of mitochondrial injury, including swollen mitochondria with extensive degeneration or even loss of cristae (Ashour et al., 2012; Zhou et al., 2001b), which were prevented by both exercise models (Figure 2). The observed mitochondrial morphologic alterations induced by sub-chronic DOX treatment were accompanied by impairments on heart mitochondrial bioenergetics. In fact, the decreased state 3 respiration, a functional marker of DOX-induced mitochondrionopathy (Oliveira et al., 2006; Oliveira and Wallace, 2006; Santos et al., 2002), was accompanied by an increase in state 4 respiration, which results in a decreased coupling between respiration and ADP phosphorylation (i.e., lower RCR) and ADP/O. Moreover, a decrease in maximal mitochondrial $\Delta\Psi$ and an increase in the phosphorylation cycle lag phase confirmed that sub-

chronic DOX treatment was associated with defective function of the phosphorylative system. However, 12 weeks of endurance TM training and voluntary FW running improved mitochondrial fitness endpoints when animals were treated with DOX, suggesting that exercise increased mitochondrial respiratory activity and improved the ADP phospholylation capacity. Interestingly, FW was more effective at counteracting DOX-induced impairments in RCR than TM, which may be a consequence of a slight decreased in state 4 observed in DOX+FW group.

One possible explanation for the observed protective effect of exercise in DOX treated animals could be related to improvements on complex I and V (Ascensao et al., 2011b). Alterations in mitochondrial oxidative phosphorylation resulting from DOX treatment could be due to several factors including the decreased activity, content and/or BN profile of OXPHOS complexes or other proteins of the phosphorylation system such as ATP synthase, which may result in part from increased oxidative stress (Pereira et al., 2011). Accordingly, our results showed that DOX treatment decreased complex I activity and the content of one of its subunit, which partially justifies diminished electron transport through the ETC in the SAL+DOX group. In opposition, both exercise models prevented DOX-induced inactivation of complex I and V (Figures 4, 5), which again may result from oxidative damage (Figures 7 and 8), as discussed below. Possible explanations for the bioenergetics preservation after sub-chronic DOX treatment in exercised groups may include the up-regulation of oxido-reductase activity and/or increased availability of reduced equivalents (Nulton-Persson and Szweda, 2001), increased capability of the phosphorylative system due the possible up-regulation of Krebs cycle enzymes (Holloszy et al., 1970) and in ATP synthase activity (Bianchi et al., 1987).

As mitochondrial complexes include enzymes susceptible to inactivation resulting from increased reactive oxygen species (ROS), further accumulation of products of protein and lipid oxidation is expected. It is suggested that DOX generates ROS in a futile redox cycling on cardiac mitochondrial complex I (Davies and Doroshow, 1986; Doroshow and Davies,

1986). The increased generation of ROS by DOX may directly damage mitochondria or alter the synthesis of proteins associated with the mitochondrial ETC with subsequent inhibition of oxidative phosphorylation and decrease in cardiac energy supply (Berthiaume and Wallace, 2007; Zhou et al., 2001a). In the present study, exercise was able to prevent the altered mitochondrial redox homeostasis imposed by DOX, namely the oxidative damage measured as MDA and -SH content, aconitase activity and carbonylated proteins, consistent with alterations in mitochondrial bioenergetic function and complex I activity/content (Figure 7). Importantly, increased Mn-SOD activity suggests that exercise induced up-regulation of mitochondrial antioxidant defenses contributes to the observed exercise-induced mitochondrial tolerance against DOX effects.

SIRT3 and p66shc, two important proteins in the control of mitochondrial physiology and in the modulation of ROS production, were analyzed in the context of exercise-induced cardioprotection against DOX toxicity. SIRT3 is a mitochondrial deacetylase that modulates the activity of several other mitochondrial proteins involved in metabolism. Mitochondrial SIRT3 increases the activity of enzymes supplying intermediates for the tricarboxylic acid cycle as well as of multiple protein subunits in the ETC, namely complex I, IV and V. Also, SIRT3 shows antioxidant functions by multiple mechanisms. For instance, SIRT3 deacetylates and directly activates Mn-SOD and increases NADPH levels necessary to increase the pool of available reduced glutathione (GSH) through the activation of glutamate dehydrogenase (GDH) and isocitrate dehydrogenase 2 (IDH2) (Bell and Guarente, 2011; Sack, 2011). According to the results of the present study, DOX treatment decreased the content in mitochondrial SIRT3 and increased acetylation levels, which is in agreement with decreased complex I activity and content, increased oxidative damage and decreased Mn-SOD activity. Interestingly, the elevation of SIRT3 content in TM group could have contributed to exercise modulation of cardiac mitochondrial susceptibility to DOX-induced oxidative stress (Figures 7 and 8). Moreover, SIRT3 is suggested to be involved in mitochondrial biogenesis mediating the effects of PGC1α on mitochondrial metabolism and

*vice versa*, also supported by String analysis (Figure 9). Although TFAM is involved in mitochondrial DNA replication and transcription (Brenmoehl and Hoeflich, 2013; Kong et al., 2010), the interaction between SIRT3 and TFAM appears to be of second level. Despite no alterations were found in PGC1α content, TM prevented the observed decrease in TFAM expression caused by sub-chronic DOX treatment (Figure 6).

p66shc is suggested serve as a thiol-based redox sensor that signals mitochondria to overproduce ROS when ROS levels in cytoplasm become elevated leading to a vicious cycle (Li et al., 2013; Pinton and Rizzuto, 2008) of cell damage and elimination. Despite an unexpected decrease in total p66shc expression in sedentary DOX group, the present results suggest that DOX-induced mitochondrial oxidative stress increased the relative expression p66shc($Ser^{36}$) and/or *vice versa*. Both exercise models decreased mitochondrial oxidative stress and damage and increased Mn-SOD activity, which agrees with the observed decrease in p66shc phosphorylation in the exercised groups, thus possibly contributing to mitigate DOX cardiotoxicity (Figures 7 and 8). It is however important to consider that the role of p66shc in DOX-treated cardiomyocytes may still be elusive. Guo et al. (Guo et al., 2009) suggest that p66shc downregulation is not cytoprotective, which goes against the current trend that states that pharmacological therapies that inhibit p66shc expression or action act as panaceas for clinical disorders characterized by increased oxidative stress. Also, the authors claim for other predicted cell specific role of p66shc, including an anti-apoptotic effect. Further studies are needed in order to better determine the relevance of p66shc in the modulation of cardiac redox state by DOX and exercise.

Given that the observed changes in some variables in response to exercise appear to occur in a similar manner in both saline and DOX-treated rats, one can believe that exercise is inducing physiological adaptations that would be beneficial to cardiac function and mitochondrial bioenergetics regardless the remodeling stimulus. In this regard, the analysis of the interaction effects of Exercise and Treatment evidenced that the measured variables showing this interaction include body weight, distance covered, mitochondrial histological

alterations, ADP/O ratio and ADP lag phase, complex III subunit, Mn-SOD, p66shc, p66shc(Ser$^{36}$) and p66shc(Ser$^{36}$)/p66shc. Moreover, the variation in most of all other variables/markers had the independent effect of both exercise and DOX treatment, which probably mean that the effects of exercise may translate into a cardiac mitochondrial phenotype more resistant to stress caused by other deleterious stimuli besides DOX.

One of the limitations of the present study is the lack of functional data to see how changes in mitochondrial fitness and biochemical parameters correlate with heart function. According with previous cross-tolerance studies on exercise against DOX (Dolinsky et al., 2013; Hayward et al., 2012a; Hydock et al., 2012a) and considering several reports evidencing that compromised mitochondrial functionality results in deterioration of cardiac function (Hoshino et al., 2013; Lee et al., 2012), it would be expected that chronic exercise translate into a phenotype more resistance to DOX-induced cardiac dysfunction.

In summary, the novel findings of the present study were that the protected role of both exercise modalities performed prior and during the course of sub-chronic DOX treatment was associated to an increased mitochondrial function and reduced oxidative damage. After the published data on exercise pre-conditioning against acute DOX dosages, this study represents a further step in the study of the cardioprotection afforded by exercise against DOX-side effects. It suggests that the modulation of SIRT3 and p66shc may also contribute to the protective mitochondrial phenotype associated to exercise. Interestingly, no major differences were found between exercise models, which support that further investigation regarding exercise intensities and modalities should be addressed for better understanding exercise strategy in the context of cardioprotection to mitigate sub-chronic DOX side-effects. In addition, and based on the present observations that no major differences were found between TM and FW, both chronic exercise interventions might be used as possible effective co-adjuvant tools in clinical practice for counteracting DOX-induced cardiac mitochondrial toxicity.

**Acknowledgments**

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# 3.3

[Study 2]

## Exercise prior and during sub-chronic Doxorubicin treatment modulates cardiac mitochondrial permeability transition, apoptotic signaling, mitochondrial dynamic and auto(mito)phagy

Marques-Aleixo I[1], Santos-Alves E[1], Rizo DR[2], Torrella JR[2], Viscor G[2], Oliveira PJ[3], Magalhães J[1], Ascensão A[1]

[1]CIAFEL - Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Portugal

[2]Department of Physiology and Immunology, Faculty of Biology, Universitat de Barcelona

[3]CNC - Centre for Neuroscience and Cell Biology, Department of Life Sciences, University of Coimbra, Portugal

**ABSTRACT**

The cross-tolerance effect of exercise against heart mitochondrial-mediated remodeling and death-related mechanisms associated with sub-chronic Doxorubicin (DOX) treatment is yet unknown. Therefore, the effects of two distinct chronic exercise models (endurance treadmill training – TM and voluntary free-wheel activity - FW) performed during the course of the sub-chronic DOX treatment on mitochondrial susceptibility to permeability transition pore (mPTP), apoptotic and autophagic signaling and mitochondrial dynamic were analyzed.

Male Sprague-Dawley rats were divided into six groups (n=6 *per* group): saline sedentary (SAL+SED), SAL + TM (12-wks treadmill), SAL+FW (12-wks voluntary free-wheel), DOX+SED [7-wks sub-chronic DOX treatment ($2mg.kg^{-1}.wk^{-1}$)], DOX+TM and DOX+FW. Apoptotic signaling and mPTP regulation were followed by measuring caspase 3, 8 and 9 activities, Bax, Bcl2, CypD, ANT, and cophilin expression. Mitochondrial dynamics (Mfn1, Mfn2, OPA1 and DRP1) and auto(mito)phagy (LC3, Beclin1, Pink1, Parkin and p62)-related proteins were semi-quantified.

DOX treatment results in augmented mPTP susceptibility and apoptotic signaling (caspases 3, 8 and 9 and Bax/Bcl2 ratio). Moreover, DOX decreased the expression of fusion-related proteins (Mfn1, Mfn2, OPA1), increased DRP1 and the activation of auto(mito)phagy signaling. TM and FW prevented DOX-increased mPTP susceptibility and apoptotic signaling, alterations in mitochondrial dynamics and inhibits DOX-induced increases in auto(mito)phagy signaling.

Collectively, our results demonstrate that both chronic models of exercise performed before and during the course of sub-chronic DOX treatment limit cardiac mitochondrial-driven apoptotic signaling and regulate alterations in mitochondrial dynamics and auto(mito)phagy in DOX treated animals.

**Keywords:** Exercise, Adriamycin, mitochondria, cardiac cell death, quality control

## Introduction

The anthracycline quinone Doxorubicin (DOX, or adriamycin) is widely prescribed in the treatment of several tumors and leukemias. Despite its effectiveness, the clinical use of DOX is limited by a dose-dependent and cumulative cardiotoxicity that results in life-threatening cardiomyopathy (Wallace, 2003). During the last decades, several mechanism have been proposed to explain DOX toxicity, including increased free radical generation as a result of a mitochondrial complex I-mediated redox cycle, with consequent increased oxidative stress and damage to mitochondrial membranes, proteins and nucleic acids (Carvalho et al., 2014). One particular consequence of DOX toxicity is the triggering of the calcium-dependent mitochondrial permeability transition pore (mPTP) (Wallace, 2007). In addition to the cessation of mitochondrial ATP synthesis, mPTP opening may signal the triggering of cell death cascades due to the release of pro-apoptotic factors from dysfunctional mitochondria (Carvalho et al., 2014). On the other hand, damaged and/or less functional mitochondria are selectively engulfed by autophagossomes and delivered to lysosomes, a process called mitophagy. This way, mitochondria are selectively removed following its inability to produce a transmembrane electric potential, suggesting that mitophagy mediates the renewal of defective mitochondria, which constitutes an adaptive mechanism ultimately ensuring mitochondrial quality control (Youle and Narendra, 2011). Several reports showed the modulation of autophagic signaling in cardiac tissue after DOX treatment (Dimitrakis et al., 2012; Sishi et al., 2013) and recently, Hoshino et al. (2013) provide evidence that mitophagy activation has an important role in DOX-induced cardiomyopathy.

Physical exercise has been used as one important non-pharmacological strategies to mitigate DOX side effects in cardiac tissue, including by preserving mitochondrial function (Ascensao et al., 2007; Ascensao et al., 2011c; Ascensao et al., 2012). In our previous works, we reported the advantage of both acute (Ascensao et al., 2011b) and chronic exercise models (Ascensao et al., 2005b; Ascensao et al., 2005a; Ascensao et al., 2006a)

regarding cardiac preconditioning of DOX-treated rats against the deleterious side-effects associated with DOX treatment. Moreover, a five-days running program before acute DOX attenuated DOX-induced autophagy (Smuder et al., 2013). We recently reported that heart mitochondrial oxidative phosphorylation capacity and pro-oxidant redox modifications associated with sub-chronic DOX administration were decreased by long-term physical exercise performed before and during treatment (Marques-Aleixo et al., a). However, a further step into the understanding of the molecular mechanisms related to exercise-induced cardioprotection before and during DOX treatment regards whether the cross-tolerance effect is also associated with alterations in mPTP susceptibility, apoptotic signaling, as well as with mitochondrial dynamics and quality control. We here hypothesize that both models of long-term exercise, with different intensity-related characteristics and performed before and during the overall DOX administration, decrease mPTP opening susceptibility, apoptotic signaling and mitigates alterations in auto(mito)phagy signaling markers and mitochondrial dynamics induced by drug treatment.

## Results

*Characterization of animals and exercise protocols*

Body and heart weights, distance covered by exercised animals, mitochondrial protein isolation yield as well as the activity of *soleus* citrate synthase and cTnI are shown in Table 1. The distance covered by FW was higher than TM groups (SAL+TM *vs.* SAL+FW and DOX+TM *vs.* DOX+FW). As expected, final body and heart weights decreased with DOX treatment (SAL+SED *vs.* DOX+SED) and TM and FW induced heart hypertrophy (SAL+TM and SAL+FW *vs.* SAL+SED and DOX+TM and DOX+FW *vs.* DOX+SED). TM increased of *soleus* citrate synthase activity in both SAL and DOX-treated animals (SAL+SED *vs.* SAL+TM and DOX+SED *vs.* DOX+TM).

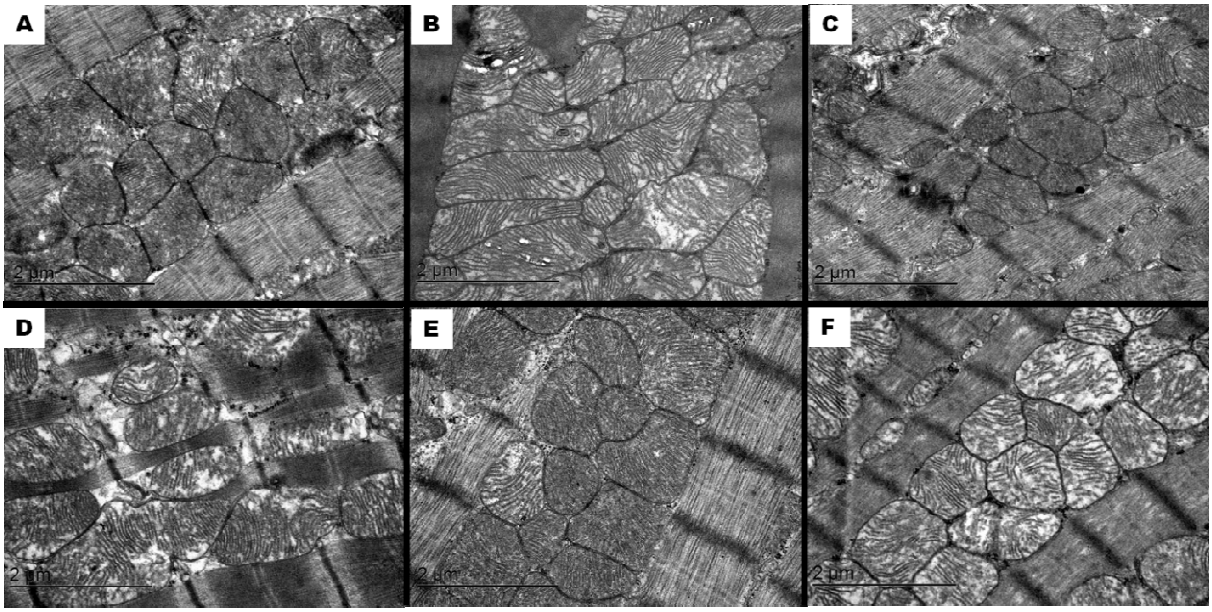
**Chapter 3.3 - Table 1.** Animal data, distance covered and yield of mitochondrial protein isolation

| | SAL+SED | SAL+TM | SAL+FW | DOX+SED | DOX+TM | DOX+FW | $p^*$ |
|---|---|---|---|---|---|---|---|
| Initial body weight (g) | 207.4±3.9 | 214.0±3.8 | 211.5±1.6 | 208.9±4.7 | 207.0±4.6 | 208.9±2.6 | NS |
| Final body weight (g) | 598.5±10.6 | 522.3±9.9* | 498.3±6.0* | 438.0±6.2$^{\#}$ | 426.0±10.8 | 428.5±16.9 | ExT |
| Distance (m/day) | - | 1437±65$^{\ddagger}$ | 3920±163 | - | 1326±50$^{\ddagger}$ | 2122±169 | - |
| Heart weight (g) | 1.44±0.03 | 1.92±0.08* | 1.85±0.10* | 1.10±0.03$^{\#}$ | 1.45±0.05* | 1.55±0.11* | E, T |
| Mitochondrial isolation yield (mg protein/g tissue) | 18.6±0.6 | 15.2±1.2 | 15.0±1.5 | 16.9±0.8 | 18.6±4.7 | 17.8±1.6 | NS |
| *Soleus* CS activity (mM. min$^{-1}$.mg$^{-1}$) | 10.9±2.1 | 23.3±1.8*$^{\ddagger}$ | 11.2±2.6 | 8.7±1.2 | 22.2±1.9*$^{\ddagger}$ | 10.3±1.3 | E |

Values are mean±SEM. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 *per* group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED ($p \leq 0.05$); $^{\#}$SAL+SED vs. DOX+SED ($p \leq 0.05$);$^{\ddagger}$ SAL+TM vs. SAL+FW and DOX+TM vs. DOX+FW ($p \leq 0.05$). $^{*}$ Significant ($p \leq 0.05$) effects of Exercise (E), Exercise and Treatment (E, T) or their interaction (E x T) are shown; Non Significant (NS, $p > 0.05$).

*Transmission electron microscopy*

Qualitative and semi-quantitative analysis of cardiac ultra-structure, including heart mitochondria, was performed by electron microscopy (Figure 1 and Table 2). As shown in Table II, DOX treatment did not induce significant differences in mitochondrial area (SAL+SED *vs.* DOX+SED), however exercise increased mitochondrial area when compared with the sedentary group treated with DOX (DOX+TM and DOX+FW *vs.* DOX+SED).




**Chapter 3.3 - Figure 1.** Representative electron micrographs of cardiac tissue from (A) SAL+SED – saline sedentary, (B) SAL+TM – saline treadmill, (C) SAL+FW – saline free, (D) DOX+SED – doxorubicin sedentary, (E) DOX+TM – doxorubicin treadmill and (F) DOX+FW – doxorubicin free wheel group. (magnification: 30,000 x).

**Chapter 3.3 - Table 2.** Effect of exercise and sub-chronic DOX treatment on mitochondrial area

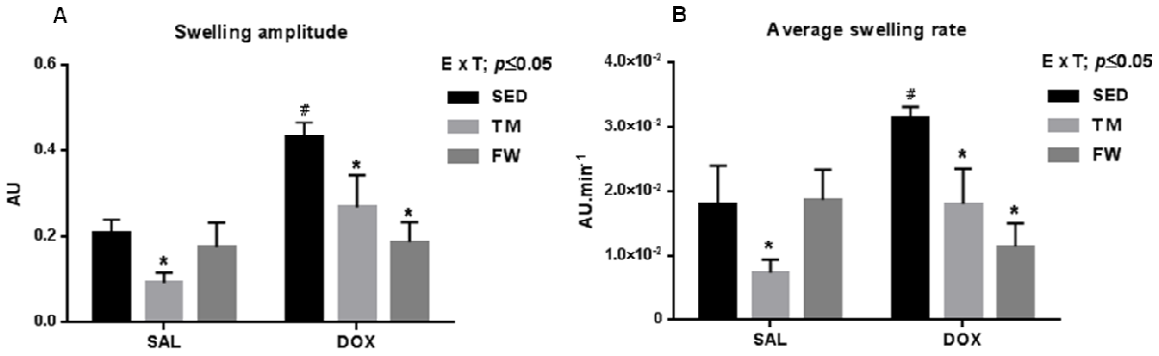
| | SAL+SED | SAL+TM | SAL+FW | DOX+SED | DOX+TM | DOX+FW | p* |
|---|---|---|---|---|---|---|---|
| Mitochondrial area | 100.00±7.68 | 133.44±9.77 | 129.79±5.16 | 90.07±5.43 | 131.40±12.68* | 128.48±5.45* | E |

Values (mean±SEM) and expressed as percentage of SAL+SED group. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 *per* group. Mitochondria area is expressed in $\mu m^2$ of mitochondria per $\mu m^2$ total TEM image (12000 x). *SAL+TM or SAL+FW *vs.* SAL+SED and DOX+TM or DOX+FW vs. DOX+SED ($p \leq 0.05$). *Significant ($p \leq 0.05$) effect of Exercise (E) is shown.

*Mitochondrial osmotic swelling*

The effects of both exercise models and DOX treatment on *in vitro* susceptibility to calcium-induced mPTP opening were analyzed. Incubation of mitochondrial suspension with cyclosporine-A, a specific mPTP sensitizer (Broekemeier et al., 1989), limits the absorbance decrease after calcium addition, which demonstrate the association with mPTP opening.

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

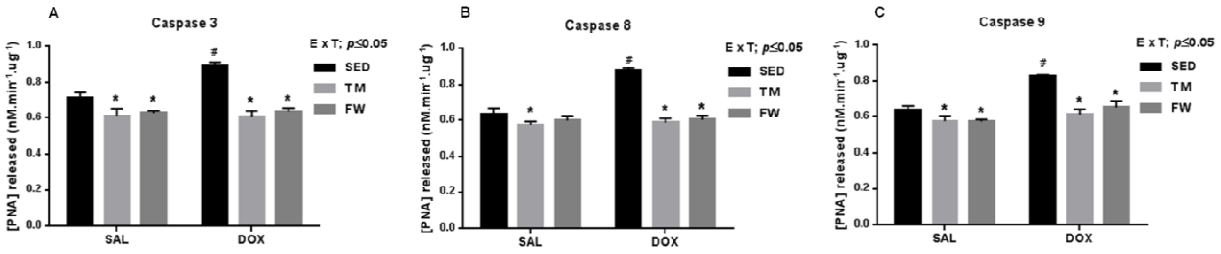



**Chapter 3.3 - Figure 2.** Effect of exercise and sub-chronic DOX treatment on cardiac calcium-induced mPTP (A) Swelling amplitude and (B) Average swelling rate. Values are mean±SEM. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 *per* group. The absorbance of mitochondrial suspension was followed at 540 nm. Mitochondria were incubated as described in methods. A 80 nmol of calcium pulse (160 nmol/mg protein) was added to 0.5 mg of mitochondrial protein in order to attain the cyclosporin A-sensitive swelling, indicating that the decreased optical density corresponding to the increased swelling was due to mPTP opening. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 per group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED (p≤0.05); #SAL+SED vs. DOX+SED (p≤0.05). Significant (p≤0.05) interaction effect of Exercise and Treatment (E x T) is indicated.

*Apoptotic signaling*

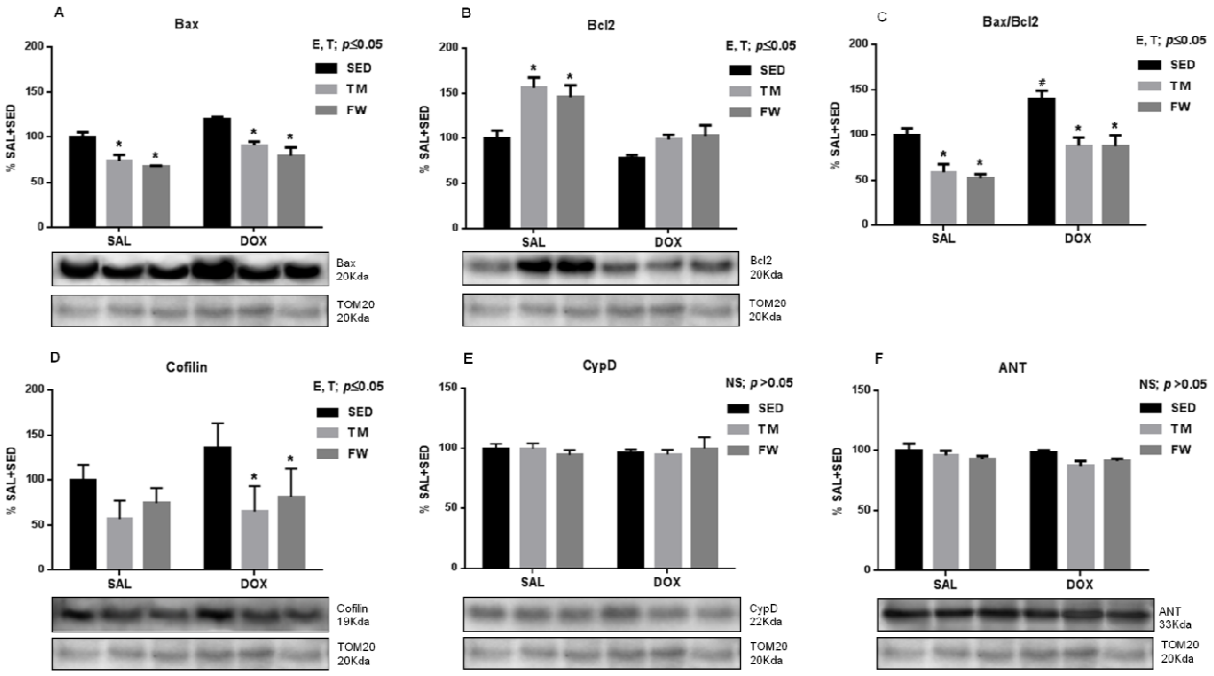
Caspase 3, 8 and 9-like activities were determined as tissue markers of apoptotic signaling activation. As seen in Figure 3, DOX treatment significantly increased caspase 3, 8 and 9-

like activity (DOX+SED vs. SAL+SED). Both models of exercise decreased caspase 3, 8 and 9-like activity in saline groups (SAL+TM and SAL+FW *vs.* SAL+SED), with the exception of caspase 8-like activity in SAL+FW group. Both types of exercise were able to counteract DOX-induced increased apoptotic signaling (DOX+TM and DOX+FW *vs*. DOX+SED).




**Chapter 3.3 - Figure 3.** Effect of exercise and sub-chronic DOX treatment on heart caspase 3, 8 and 9-like activities. Data are means±SEM for heart tissues. Caspase-like activity was measured colorimetrically by pNA release from specific caspase substrates, as detailed in methods. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=6 *per* group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED; #SAL+SED vs. DOX+SED (p≤0.05). Significant (p≤0.05) interaction effect of Exercise and Treatment (E x T) is indicated.

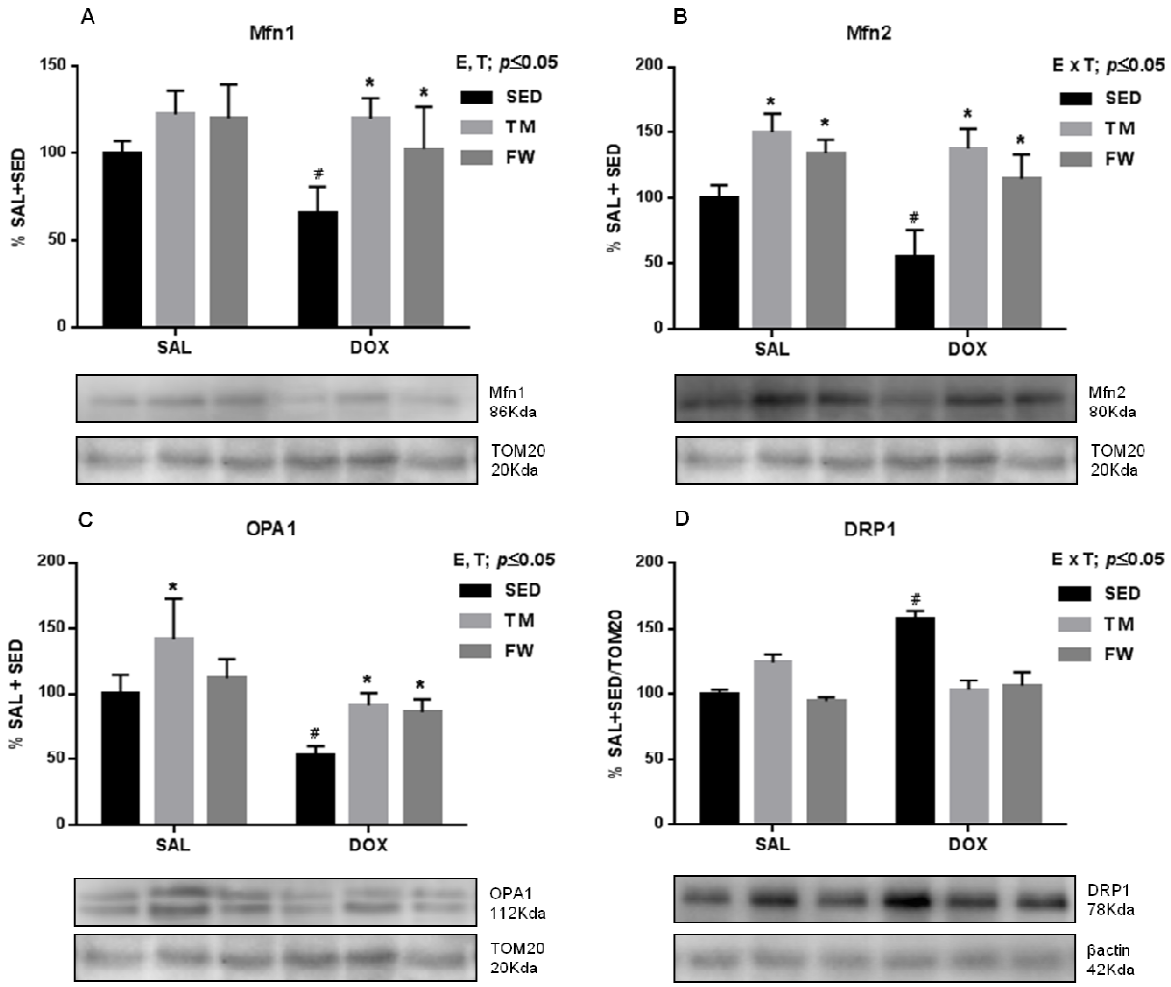
Immunoblotting was also performed to semi-quantify mitochondrial proteins involved in apoptotic signaling, including the pro-and anti-apoptotic Bcl-2 family proteins, Bax and Bcl-2, respectively, as well as Cyp D, a chaperone which has been involved in apoptosis and autophagy (Carreira et al., 2010). ANT and cofilin, also associated with mPTP regulation and induction, were also semi-quantified. As shown in Figure 4, DOX treatment significantly increased Bax-to-Bcl-2 ratio (DOX+SED *vs.* SAL+SED). Exercise *per se* increased Bcl-2 expression and decreased Bax and Bax/Bcl-2 in SAL groups (SAL+TM and SAL+FW *vs.* SAL+SED). Both exercise models decreased cofilin expression in DOX treated groups (DOX+TM and DOX+FW *vs.* DOX+SED). Moreover, TM and FW prevented DOX-induced increased Bax and Bax/Bcl-2. No significant alterations were found between groups in CypD and ANT expression.




**Chapter 3.3 - Figure 4.** Effect of exercise and sub-chronic DOX treatment on heart mitochondrial (A) Bax, (B) Bcl2, (C) Bax/Bcl2, (D) Cofilin (E) CypD and (F) ANT. Data are means±SEM obtained from different mitochondrial preparations for each group. The results were corrected for TOM20 content and expressed as percentage of SAL+SED group. Typical immunoblots are presented below the histograms. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=4 *per* group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED; #SAL+SED vs. DOX+SED (p≤0.05). Significant (p≤0.05) effects of Exercise and Treatment (E, T) and Non Significant effects (NS, p>0.05) are indicated.

*Mitochondrial dynamics*

Proteins associated with mitochondrial fusion (Mfn1, Mfn2 and OPA1) and fission (DRP1) were semi-quantified by western blotting (Figure 5). As shown, DOX treatment increased DRP1 and decreased all the three fusion-related proteins (DOX+SED *vs.* SAL+SED). TM *per se* increased Mfn2 and both exercise models increased OPA1 expression (SAL+TM and SAL+FW *vs.* SAL+SED). Both models of exercise decreased DRP1 and increased Mfn1, Mfn2 and OPA1 content in DOX treated groups (DOX+TM and DOX+FW *vs.* DOX+SED).

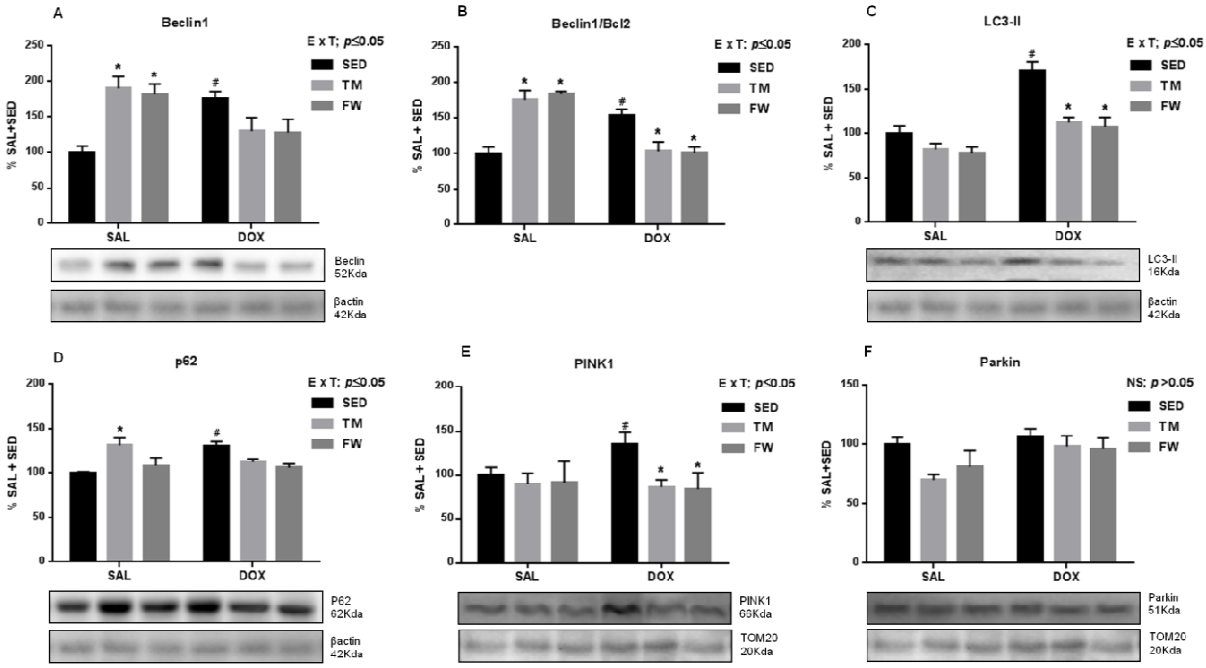



**Chapter 3.3 - Figure 5.** Effect of exercise and sub-chronic DOX treatment on heart mitochondria (A) Mfn1, (B) Mfn2, (C) OPA1 (upper band) and (D) cardiac DRP1. Data are means±SEM obtained from different preparations. The results were expressed as percentage of SED group. DRP1 content was normalized against TOM20 content. TOM20 and β-actin were used for mitochondrial and tissue protein loading controls, respectively. Typical immunoblots are presented below the histograms. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=4 *per* group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED; $^{\#}$SAL+SED vs. DOX+SED (p≤0.05). Significant (p≤0.05) effects of Exercise and Treatment (E, T) and interaction effect of Exercise and Treatment (E x T) are indicated.

*Cellular and mitochondrial quality control*

Intracellular quality control (autophagy and mitophagy) was assessed by measuring the content of Beclin1, a marker of initiation of autophagosome formation, Beclin1-to-Bcl-2 ratio, an end-point for the inhibition of beclin-dependent autophagy, LC3, a marker of the

elongation and formation of the autophagosome and Pink, Parkin and p62, proteins associated with mitophagy regulation (Figure. 6). DOX treatment *per se* increased Beclin1, Beclin1-to-Bcl-2 ratio, LC3-II, p62 and PINK-1 content (DOX+SED *vs.* SAL+SED). Both chronic exercise models increased Beclin1 expression and Beclin1-to-Bcl-2 ratio in saline groups (SAL+TM and SAL+FW *vs.* SAL+ SED) and only TM increased p62 content (SAL+TM *vs.* SAL+SED). TM and FW decreased Beclin1-to-Bcl-2 ratio and LC3-II as well as PINK1 content in DOX-treated groups (DOX+TM and DOX+FW *vs.* DOX+SED).




**Chapter 3.3 - Figure 6.** Effect of exercise and sub-chronic DOX treatment on heart tissue (A) Beclin1, (B) Beclin1/Bcl-2 (C) LC3-II (D) p62 and mitochondrial (E) PINK1 and (F) Parkin. Data are means±SEM for heart obtained from different preparations. The results were corrected for TOM20 or β-actin and expressed as percentage of SAL+SED group. Typical immunoblots are presented below the histograms. SAL+SED – saline sedentary, SAL+TM – saline treadmill, SAL+FW – saline free wheel, DOX+SED – doxorubicin sedentary, DOX+TM – doxorubicin treadmill, DOX+FW – doxorubicin free wheel, n=4 *per* group, *SAL+TM or SAL+FW vs. SAL+SED and DOX+TM or DOX+FW vs. DOX+SED; $^{\#}$SAL+SED vs. DOX+SED (p≤0.05). Significant (p≤0.05) interaction effect of Exercise and Treatment (E x T) and Non Significant effects (NS, p>0.05) are indicated

## Discussion

The present study demonstrated for the first time that physical exercise performed before and during the course of a sub-chronic clinical relevant DOX treatment (Carvalho et al., 2014; Pereira et al., 2012) decreases the threshold for mPTP induction and apoptotic signaling and modulated mitochondrial dynamics and quality control protein signaling markers in cardiac tissue. This effect is likely to contribute to a protective phenotype against the cardiac side-effects characterizing DOX treatment.

Although mitochondrial dysfunction has been implicated in DOX-induced cardiotoxicity, the exact molecular mechanisms are still unclear. However, the impairment of mitochondrial calcium homeostasis, increased apoptosis, alterations on mitochondrial dynamics and on markers of autophagy and mitophagy induced by DOX treatment were previously described (Pereira et al., 2011). This agrees with our present data, in which a 7-wk sub-chronic DOX administration protocol resulted in: (i) increased mPTP susceptibility, (ii) augmented apoptotic signaling (iii) increased fission and decreased fusion thus impairing mitochondrial dynamics and (iv) increased markers of autophagy and mitophagy.

We previously tested TM and FW, two different types of chronic exercise, performed before and during the course of sub-chronic DOX treatment, as strategies to counteract mitochondrial bioenergetic alterations and oxidative stress (Marques-Aleixo et al., a); however, how physical exercise interfere with the important sensors of DOX-induced cardiac toxicity, the increased mPTP susceptibility and consequent activation of apoptotic signaling, is still undetermined. In addition, given the importance of mitochondrial plasticity as well as the interplay between cellular and mitochondrial renewal/clearance in the context of tissue adaptation and survival, mitochondrial dynamics and quality control signaling markers were for the first time analyzed.

Mitochondria are determinant players in the establishment of cytosolic calcium homeostasis by accumulating calcium in a process that is favored by the electrochemical gradient formed

across the inner mitochondrial membrane (Gustafsson and Gottlieb, 2008). Cardiac mitochondria from sub-chronic DOX-treated animals have a lower capacity to accumulate and retain calcium, due to increased mPTP formation, leading to the release of pro-apoptotic proteins and resulting in apoptotic-driven cardiomyocyte death (Oliveira et al., 2004; Oliveira and Wallace, 2006; Santos et al., 2002; Wallace, 2007; Zhou et al., 2001b; Zhou et al., 2001a). As endurance training decreases cardiac mPTP susceptibility (Ascensao et al., 2011c), we hypothesize that chronic exercise performed before and during sub-chronic DOX-treatment counteracts increased mPTP occurrence. The results from the present study showed that both exercise models were able to mitigate DOX-induced increased susceptibility to mPTP induction (Figure 2).

Calcium-induced mPTP opening is modulated by a variety of physiological effectors. For example, increased Bax expression and mitochondrial translocation sensitizes mitochondria PTP opening, being antagonized by anti-apoptotic Bcl-2 family proteins (Szigeti et al., 2010). Our results confirm previous findings reporting increased Bax-to-Bcl-2 ratio after acute DOX treatment (Ascensao et al., 2005b; Childs et al., 2002; Wang et al., 2013). Of relevance, both TM and FW decreased Bax-to-Bcl-2 ratio in sub-chronically DOX-treated animals (Figure 4).

Cofilin has been emerging as an important protein in the regulation of cellular homeostasis (Bernstein and Bamburg, 2010). Mitochondrial cofilin translocation appears to be necessary for the apoptotic signaling interacting with mPTP opening susceptibility and subsequent release of apoptotic proteins (Klamt et al., 2009). Li and co-workers (2013) observed an increase in cofilin oxidation in a rat cardiomyocyte cell line following DOX incubation. However, we did not find alterations in cardiac mitochondrial cofilin content in sub-chronic DOX-treated animals. Of note are also the lower levels of this protein in exercised animals sub-chronically treated with DOX, suggesting that possible lower cofilin and consequent diminished translocation into mitochondria might contribute for the observed protection against DOX-induced apoptotic signaling (Figure 4).

As mentioned, DOX-induces increased mPTP susceptibility and the release of mitochondrial enclosed pro-apoptotic proteins to the cytoplasm, thus contributing to trigger cell death through activation of caspase signaling (Childs et al., 2002; Green and Leeuwenburgh, 2002; Jang et al., 2004; Kumar et al., 2001; Wang et al., 2001). In the present study, the observed increased caspase 3, 8 and 9 activity-like in hearts of sedentary animals sub-chronically treated with DOX was prevented by exercise (Figure 3), which agrees with the decreased susceptibility to mPTP and Bax-to-Bcl-2 ratio as well as with previous data using other exercise models and DOX-treatment schedules (Ascensao et al., 2005b; Ascensao et al., 2011b; Ascensao et al., 2012).

Mitochondria are dynamic organelles that continually modify their morphology, from elongated interconnected networks to fragmented disconnected units, in a continuous balance between local fission and fusion events (Chan, 2006a; Lee et al., 2004). In cardiomyocytes, mitochondrial dynamics is emerging as a fundamental cell biological process, not only for mitochondrial quality control and shape, but also for functioning, regulation of mPTP susceptibility, apoptotic signaling and cell survival (Campello and Scorrano, 2010; Nogueira et al., 2005; Wasilewski and Scorrano, 2009). DOX-induced alterations in mitochondrial dynamics are not well documented; however, it has been suggested that both *in vivo* (Marechal et al., 2011) and in cultured cardiomyocytes (Parra et al., 2008), DOX induces mitochondrial network fragmentation and dysfunction. The same was observed in the present study, suggesting that sub-chronic DOX treatment induced alterations in mitochondrial dynamics through the stimulation of mitochondrial fission and the inhibition of mitochondrial fusion signaling (Figure 5). Excessive fragmentation can cause deleterious alterations in mitochondrial metabolism, mitochondrial membrane potential generation, in the content of complex I, IV, and V subunits (Chan, 2006b; Liesa et al., 2009; Pich et al., 2005), and excessive DRP1-mediated mitochondrial fission contributing to apoptotic cell death (Frank et al., 2001; Parone et al., 2006). Moreover, cardiomyocytes partially deficient in Opa1 (Piquereau et al., 2012) or totally deficient in Mfn1 or Mfn2

(Papanicolaou et al., 2011; Papanicolaou et al., 2012a; Papanicolaou et al., 2012b) exhibit delayed calcium-induced mPTP opening. However, others argue that mitochondrial fusion, are a pre-requisite for increased resistance to mPTP and apoptosis (Ong et al., 2010; Whelan et al., 2012). Accordingly, the results presented here demonstrated that DOX-induced mitochondrial fragmentation was accompanied by increased susceptibility to calcium-induced mPTP opening.

Our results suggest that exercise *per se*, particularly TM, increased the expression of fusion-related proteins in cardiac mitochondria (Figure 6), suggesting an adaptive mechanism to increase mitochondrial metabolic efficiency, which agrees with previous observations (SUN). Moreover, both TM and FW reestablished sub-chronic DOX-induced decreased mitochondrial fusion- and increased the expression of fission-related proteins (Figure 5), which agree with recent data showing that exercise during sub-chronic DOX treatment increased Mfn1/2 content in mice (Dolinsky et al., 2013).

Another key physiological process closely associated with mitochondrial dynamics and apoptotic signaling and essential for tissue adaptation and renewal is auto(mito)phagy. Autophagy is a cytoplasmic quality control process by which the cell recycles damaged macromolecules or non-functional organelles, promoting cell survival. However, under pathological conditions, autophagy can be greatly increased in the myocardium to promote a deleterious rate of protein removal, which can result in the elimination of vital cellular organelles and proteins and can also result in crosstalk with other proteolytic systems to increase cell death (Sciarretta et al., 2011; Zhang et al., 2009; Zheng and Wang, 2010). Autophagy might be triggered by energy depletion, oxidative stress, accumulation of misfolded/oxidized proteins, mitochondrial depolarization and mPTP induction (Carreira et al., 2010; Gottlieb and Carreira, 2010; Scherz-Shouval and Elazar, 2011), common events that are activated by DOX treatment. Although DOX treatment is associated with elevated autophagic activity, suggesting that increased autophagy mediates DOX-induced cardiotoxicity (Dimitrakis et al., 2012; Kobayashi et al., 2010; Lu et al., 2009; Xu et al., 2012;

Zhang et al., 2009), other reports also suggest that autophagy can be an adjuvant therapy to mitigate DOX-induced myocardial damage (Gharanei et al., 2013; Sishi et al., 2013). Our data show that sub-chronic DOX administration increased the Beclin1 content and Beclin1/Bcl-2 ratio (Figure 7). As Bcl-2 can form a complex with Beclin-1 to inhibit autophagy (Kubli and Gustafsson, 2012b; Marquez and Xu, 2012), the observed DOX-induced increased in Beclin1/Bcl-2 ratio could facilitate the activation of autophagy. In addition, the LC3-II, a marker of LC3 cleavage and activation, was increased in DOX-treated animals (Figure 6), collectively suggesting that sub-chronic DOX administration increased cardiac autophagic signaling.

In the present study, cardiac autophagy markers in both exercised groups sub-chronically treated with DOX were not so high as in DOX+SED, as confirmed by decreased Beclin1 expression, Beclin1/Bcl-2 ratio and LC3-II (Figure 6). This may suggest that upstream adaptations occurring in cardiac myocytes, namely the up-regulation of cell defense systems, might blunt autophagy activation induced by DOX in exercised animals. Previous data indicated that short-term preconditioning exercise before DOX administration inhibited increased autophagic signaling in skeletal (Smuder et al., 2011) and in cardiac muscle (Smuder et al., 2013).

A specific form of autophagy can target mitochondria in a process known as mitochondrial autophagy or mitophagy. Dysfunctional mitochondria are segregated from the network and consequently eliminated by mitophagy in an attempt to maintain overall mitochondrial integrity and function (Kubli and Gustafsson, 2012b; Piquereau et al., 2013). In the present study, an increased expression of mitophagy-related proteins PINK1 and p62 in sub-chronic DOX treated animals was observed (Figure 6). These results contradict the findings of Hoshino et al. (2013), who demonstrated that inhibition of Parkin translocation to mitochondria in cardiomyocytes and mouse hearts submitted to DOX effect worsened cardiac and mitochondrial phenotype.

Our data showed that the measured markers of cardiac mitophagy signaling were not altered with chronic exercise (Figure 6). Despite TM *per se* increased p62 expression, it has been suggested that p62 recruitment to mitochondria alone is not sufficient for mitophagy to ensue (Narendra et al., 2010). However, we report here for the first time that the mitophagy-related protein PINK1 in exercised groups treated with DOX was lower than in the DOX+SED group (Figure 6). Furthermore, mitochondrial dynamics signaling-related proteins can coordinately be important players in mitophagy, since the selective clearance of mitochondria seems to be preceded by fission (Gomes and Scorrano, 2008; Lee et al., 2011; Twig et al., 2008). The degradation of Mfn1/2 might switch the balance of mitochondrial dynamics toward fission to facilitate mitophagy, preventing damaged mitochondria from fusing with healthy mitochondria (Clark et al., 2006; Tanaka et al., 2010). Fission produces smaller mitochondrial fragments that can more easily be engulfed by autophagosomes (Kubli and Gustafsson, 2012b), being DRP1-mediated fission a pre-requisite for mitophagy. Mitochondrial fission originates two metabolically different structures, characterized by biochemical, morphological and functional features. In this regard, the mitochondrial fragments with decreased Opa1 content, reduced size and reduced membrane potential are more likely to be targeted by mitophagy-related mechanisms (Twig et al., 2008). DOX-induced autophagy and mitophagy observed in the present study was accompanied by an increase in DRP1 and a decrease in OPA1 levels, alterations that were not observed in exercised groups treated with DOX (Figure 5 and 6).

In summary, our results confirm that the increased susceptibility to mPTP and apoptotic signaling may be a critical step of DOX-induced deterioration of cardiac function and onset of chronic clinical cardiotoxicity. Moreover, autophagy and apoptosis can act synergistically in the regulation of cardiomyocyte death in sub-chronic DOX-treated rats. The observed preventive role of chronic exercise can be associated with an increased resistance of mitochondria to undergo the permeability transition and to apoptotic signaling activation. From our data, it is possible that the regulation of mitochondrial dynamics and quality control signaling may also contribute to the protective mitochondrial phenotype observed against the

deleterious DOX effects. Considering that DOX can result in cardiac mitochondrial impairment with consequent loss of organelle and tissue functions, physical exercise performed during the course of sub-chronic DOX treatment, independently of the intensity, seems to afford protection against this mitochondrionopathy.

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# 3.4

[Study 3]

## Physical exercise improves brain cortex and cerebellum mitochondrial bioenergetics and alters apoptotic, dynamic and auto(mito)phagy markers

Marques-Aleixo I[1], Santos-Alves E[1,2], Balça MM[1], Moreira PI[23], Oliveira PJ[2], Magalhães J[1], Ascensão A[1]

[1]CIAFEL - Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Portugal

[2]CNC - Centre for Neuroscience and Cell Biology, Department of Life Sciences, University of Coimbra, Portugal

[3] Institute of Physiology, Faculty of Medicine, University of Coimbra, Portugal

**ABSTRACT**

We aimed to investigate the effect of two exercise modalities (endurance treadmill training-TM and voluntary free-wheel activity-FW) on brain cortex and cerebellum mitochondrial bioenergetics, permeability transition pore (mPTP), oxidative stress, and proteins involved in mitochondrial biogenesis, apoptosis and quality control.

Eighteen male rats were divided into sedentary-SED, TM and FW. Behavioural alterations and post-sacrifice brain mitochondrial function endpoints were assessed. Oxidative phosphorylation (OXPHOS) proteins, ANT, oxidative stress markers or related proteins (SIRT3, p66shc, UCP2, carbonyls, MDA, -SH, aconitase, Mn-SOD), as well as proteins involved in mitochondrial biogenesis (PGC1α, TFAM) were evaluated. Apoptotic signaling was followed through caspase 3, 8 and 9-like activities, Bax, Bcl2, CypD, and cofilin expression. Mitochondrial dynamics (Mfn1/2, OPA1 and DRP1) and auto(mito)phagy (LC3II, Beclin1, Pink1, Parkin, p62)-related proteins were also measured by western blotting.

Only the TM exercise group showed increased spontaneous alternation and exploratory activity. Both exercise regimens improved mitochondrial respiratory activity, increased OXPHOS complex I, III and V subunits in both brain subareas and decreased oxidative stress markers. Generally, increased resistance to mPTP and decreased apoptotic signalling were observed in brain cortex from TM and in cerebellum from TM and FW. Increased mitochondrial biogenesis, autophagy and fusion signalling markers and decreased fission (DRP1) were observed after exercise.

In conclusion, physical exercise improves brain cortex and cerebellum mitochondrial function, increasing the resistance to oxidative stress and apoptosis. It is also possible that favourable alterations in mitochondrial biogenesis, dynamics and autophagy signalling induced by exercise contributed to increased mitochondrial plasticity leading to a more robust phenotype.

**Keywords:** Exercise, brain, mitochondrial metabolism, mitochondrial quality control

## Introduction

Regular physical exercise accomplishes significant outcomes regarding cognitive function in both epidemiological and animal studies (Cotman et al., 2007; Gomes Da Silva et al., 2012; Hillman et al., 2008; Hopkins et al., 2011; Radak et al., 2001a). Moreover, physical exercise protects neurons against deleterious alterations associated with the aging process (Boveris and Navarro, 2008a; Cotman et al., 2007; Dishman et al., 2006; Radak et al., 2008) and the onset of neurodegenerative disorders (Adlard et al., 2005; Lau et al., 2011; Um et al., 2008; Um et al., 2011; Van Praag, 2008). However, the cellular and molecular mechanisms underlying this protective phenotype induced by exercise in brain physiology is still elusive.

In opposition to contractile tissues such as skeletal and cardiac muscles, in which physical exercise exerts multiple contractile-dependent effects, adaptations in brain tissue are related to other systemic alterations that occur during and after exercise. Mitochondrial activity, including ATP synthesis, regulation of redox balance, calcium homeostasis, cell signaling and fate may explain the observed protective phenotype (Marques-Aleixo et al., 2012). Generally, studies report that exercise-induced brain mitochondrial adaptations include increased content and/or activity of several enzymes involved in aerobic energy production (Dietrich et al., 2008; Ding et al., 2006; Kirchner et al., 2008), increased activity of mitochondrial complexes I, III and IV (Navarro and Boveris, 2004), decreased expression/activation of several proapoptotic proteins (Cho et al., 2010; Um et al., 2011) and increased mitochondrial biogenesis (Steiner et al., 2011) and antioxidant capacity (Camiletti-Moiron et al., 2013). However, other still unknown mitochondrial-mediated mechanisms may also be involved, contributing to the improved functional phenotype associated to exercise. These mechanisms may involve altered susceptibility to the mitochondrial permeability transition (mPT), mitochondrial dynamic plasticity and quality control. In fact, as a result of calcium accumulation and oxidative stress, mitochondria may undergo mPT pore (mPTP) opening, leading to bioenergetic failure, release of proapoptotic proteins into the cytoplasm,

and ultimately to mitocondrial-mediated apoptotic cell death (Toman and Fiskum, 2011). In addition, neurons are particularly sensitive to changes in mitochondrial dynamics. Also, impairments of mitochondrial physiology and quality control have been reported in several neuropathological conditions (Knott and Bossy-Wetzel, 2008; Twig and Shirihai, 2011). Regulation of mitochondrial network structure, resulting from a dynamic mitochondrial fusion, fission, auto(mito)phagy and mitochondrial biogenesis interaction, is necessary for a systematic response to the high-energy requirements of nervous tissue (Chen and Chan, 2006; Gottlieb and Carreira, 2010). Therefore, the present study is a further step into the understanding of the molecular mechanisms related to exercise-induced mitochondrial-mediated neuroprotection.

We thus aimed to analyze the effects of two long-term exercise modalities on brain cortex and cerebellum mitochondrial activity, susceptibility to mPTP opening and apoptotic signaling. Oxidative stress, OXPHOS subunits, molecular markers of mitochondrial biogenesis, mitochondrial fission and fusion as well as auto(mito)phagy signaling were also investigated. The further understanding of the mechanisms by which exercise exerts beneficial effects on brain function may improve the potential of this preventive and therapeutic strategy against neurodegenerative diseases.

## Results

*Characterization of animals*

As shown in Table I, no significant differences between groups were observed regarding the initial body weight, brain cortex and cerebellum weight, and the yield of brain cortex and cerebellum mitochondrial isolation. However, both chronic exercise modalities decreased final body weight and increased brain cortex and cerebellum weight to body weight ratios. Animals from FW ran significantly more than TM group, whereas TM, but not FW, increased the activity of *soleus* CS. The lack of increased *soleus* CS activity in FW group might

possible result from distinct responses of skeletal muscle to chronic exercise modalities (Liu et al., 2009; Noble et al., 1999).

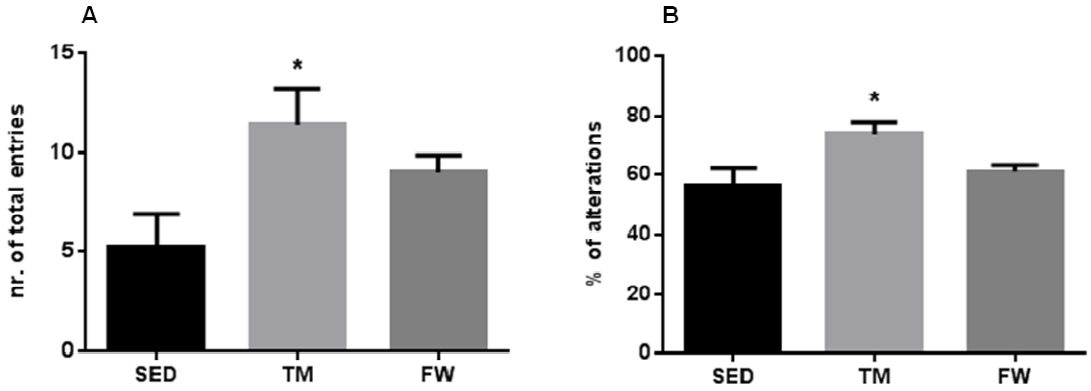
**Chapter 3.4 - Table 1.** Animal data and yield of mitochondrial isolation

| | SED | TM | FW |
|---|---|---|---|
| Initial body weight (g) | 207±3.91 | 214±3.76 | 211±1.55 |
| Final body weight (g) | 598±10.58 | 522±9.87* | 498±5.98* |
| Distance (m/day) | - | 1437±65 | 3920±163$^{\#}$ |
| Brain cortex weight (g) | 1.28±0.04 | 1.30±0.04 | 1.31±0.02 |
| Brain cortex weight/body weight (mg.g$^{-1}$) | 2.13±0.05 | 2.48±0.06* | 2.49±0.09* |
| Brain cortex mitochondrial isolation yield (mg protein.g tissue$^{-1}$) | 22.74±3.82 | 24.76±2.86 | 23.57±4.96 |
| Cerebellum weight (g) | 0.60±0.01 | 0.64±0.01 | 0.63±0.01 |
| Cerebellum weight/body weight (mg.g$^{-1}$) | 0.99±0.03 | 1.24±0.01* | 1.27±0.03* |
| Cerebellum mitochondrial isolation yield (mg protein.g tissue$^{-1}$) | 27.91±2.18 | 28.92±4.81 | 28.20±5.64 |
| *Soleus* citrate synthase activity (mM. min$^{-1}$.mg$^{-1}$) | 10.94±2.07 | 23.34±1.79* | 11.22±2.65$^{\#}$ |

Data are means±SEM (n=6 per group). SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. **vs*. SED; $^{\#}$*vs*. TM ($p \leq 0.05$).

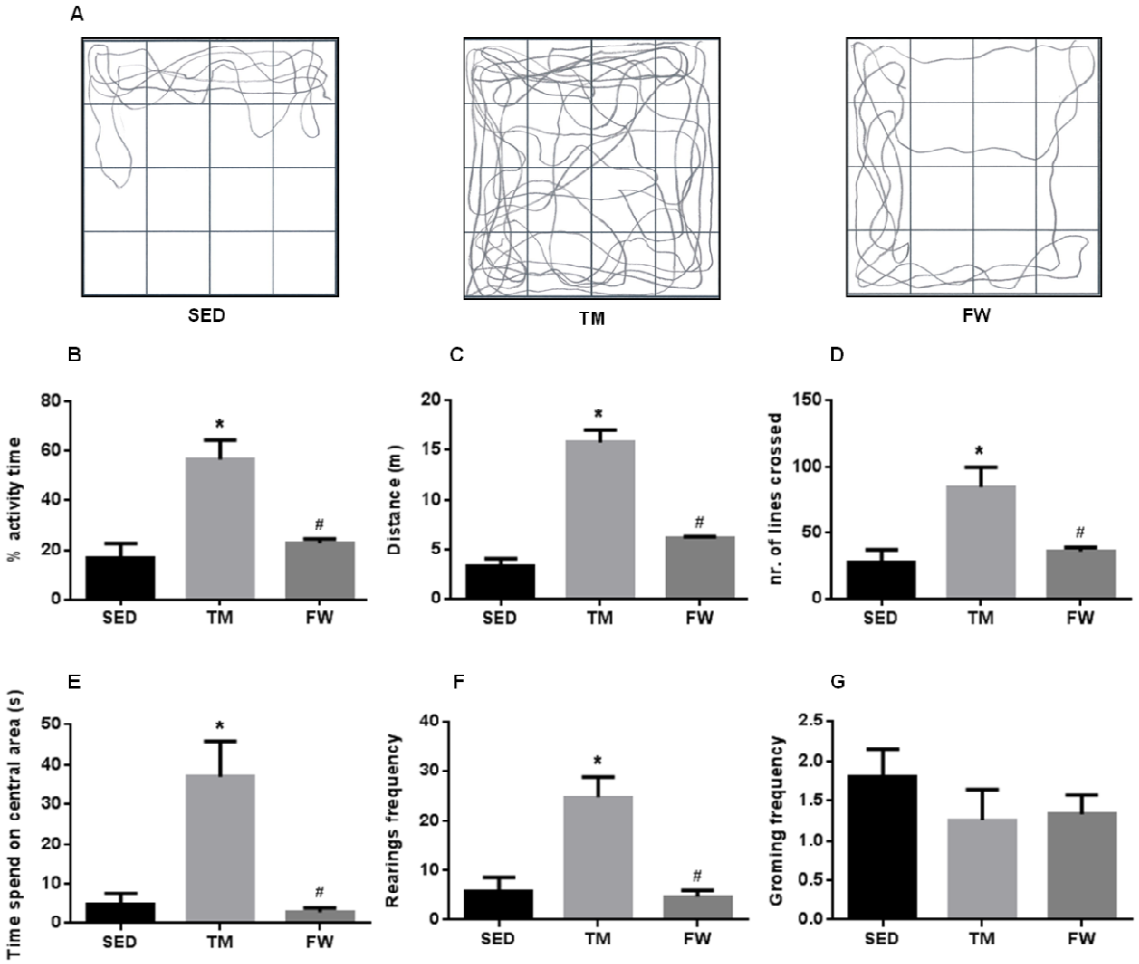
*Behavioral tests*

Animals from SED, TM and FW groups were tested for spontaneous alternation in the Y maze (Figure 1). TM significantly increased the number of total entries and the percentage of alteration suggesting a higher willingness to explore new environments.




**Chapter 3.4 - Figure 1.** Effect of exercise on Y-maze behavior (A) number of total entries and (B) % of alterations. Data are means±SEM (n=6 per group). Experimental details are provided in methods. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity.*vs. SED ($p \leq 0.05$).

Alterations on general activity and exploratory behavior induced by both models of chronic physical activity were analyzed using an open field box (Figure 2). As shown, TM increased % activity time, the locomotive distance traveled, the number of lines crossed, the time spend on central area and rearings. No significant alterations between groups were observed regarding grooming frequency.

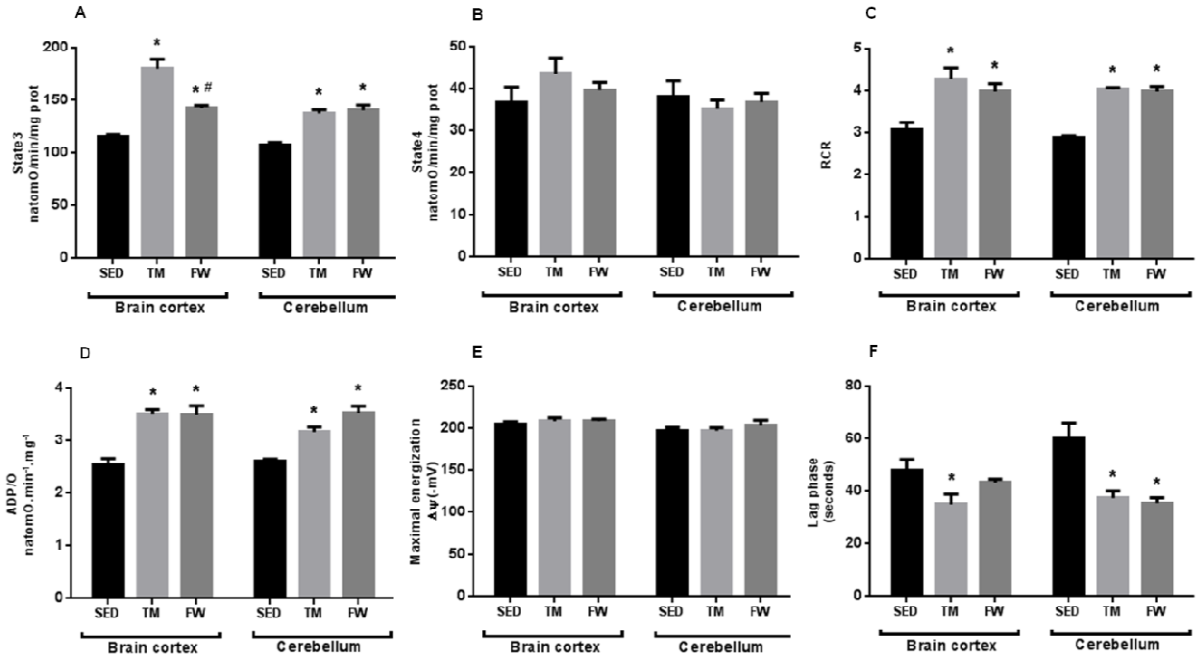



**Chapter 3.4 - Figure 2.** Effect of exercise on Open Field behavior: (A) illustrative example of a SED, TM and FW animal travel path during a 5 min exploration, (B) % activity time, (C) locomotive distance traveled, (D) number of lines crossed, (E) time spend on central area and (F and G) number of rearings and grooming. Data are means±SEM (n=6 per group). Experimental details are provided in methods. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. *$vs.$ SED; $^{\#}vs.$ TM ($p \leq 0.05$).

*Mitochondrial oxygen consumption and transmembrane electric potential*

Mitochondrial respiration and $\Delta\psi$ fluctuations associated with ADP phosphorylation activity were measured to identify exercise-dependent effects on brain cortex and cerebellum mitochondria (Figure 3). Both exercise modalities increased state 3 respiratory rate, the coupling between oxygen consumption and ADP phosphorylation (RCR) and the efficiency of ATP synthesis (ADP/O) in brain cortex and in cerebellum mitochondria. The increase in state 3 induced by TM in brain cortex mitochondria was significantly higher than the increase

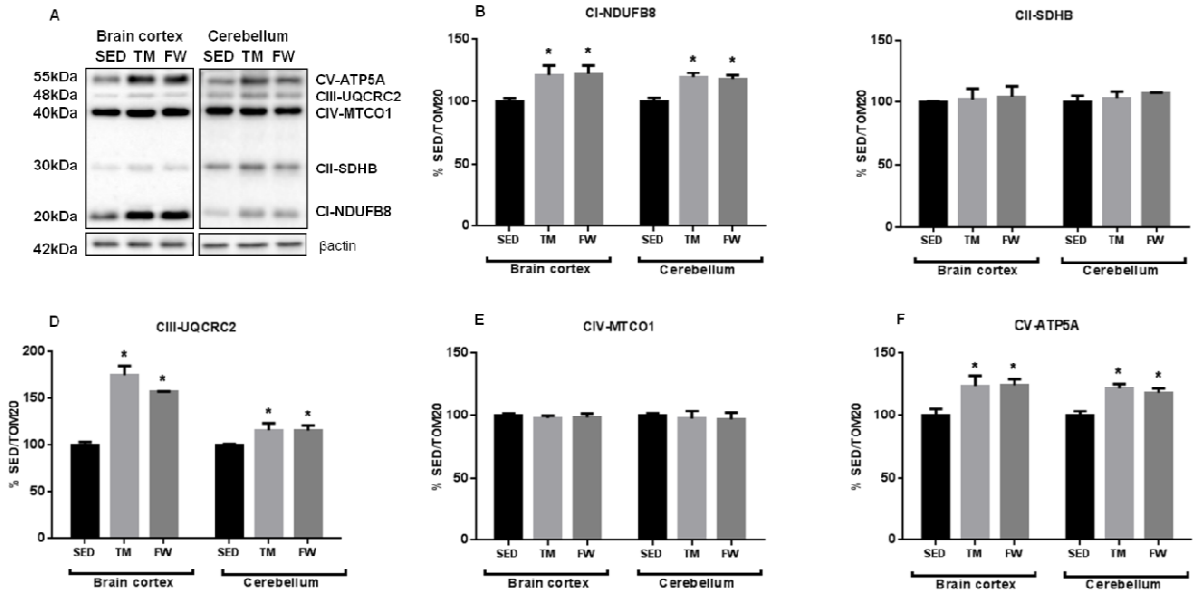
caused by FW. Although no alterations were observed regarding the maximal Δψ in both studied tissue-derived mitochondria, TM decreased the ADP lag phase in brain cortex and both exercise types decreased the ADP lag phase in cerebellum mitochondria.




**Chapter 3.4 - Figure 3.** Effect of exercise on brain cortex and cerebellum (A) state 3 respiration, (B) state 4 respiration, (C) RCR, (D) ADP/O, (E) maximal Δψ and (F) ADP phosphorylation lag phase. Data are means±SEM obtained from six different mitochondrial preparations (0.8 mg/mL protein) for each group. Oxygen consumption and Δψ were measured with G/M as substrates. RCR, respiratory control ratio (state 3/state 4); ADP/O, number of nmol ADP phosphorylated by natom of oxygen consumed; lag phase, the time elapsed in the depolarization/repolarization cycle during ADP phosphorylation. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. **vs.* SED; $^{\#}$*vs.* TM ($p \leq 0.05$).

*OXPHOS complex subunit semi-quantification by Western Blotting*

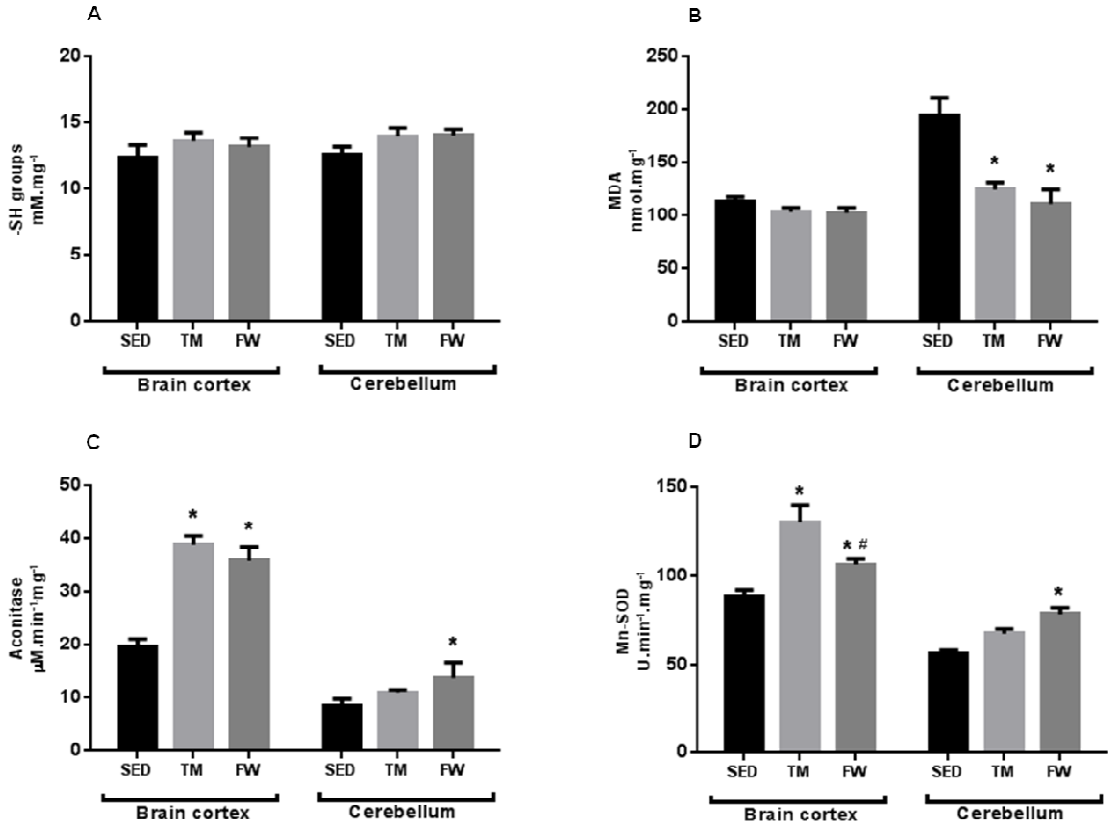
The analysis of brain cortex and cerebellum mitochondria OXPHOS subunits (Figure 4) showed that both exercise models increased the content of complex I, III and V subunits in brain cortex and in cerebellum mitochondria.




**Chapter 3.4 - Figure 4.** Effect of exercise on brain cortex and cerebellum mitochondrial OXPHOS subunits: (A) typical immunoblots; (B) complex I CI-NDUFB8 subunit; (C) complex II CII-SDHB subunit; (D) complex III CIII-UQCRC2 subunit; (E), complex IV CIV-MTCO1 subunit and (F) complex V CV-ATP5A subunit. Data are means±SEM (n=4 per group) for brain cortex and cerebellum. The results were expressed as percentage of SED group. Each OXPHOS subunit was normalized against TOM20 content. β-actin was used for tissue protein loading control. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. **vs.* SED ($p \leq 0.05$).

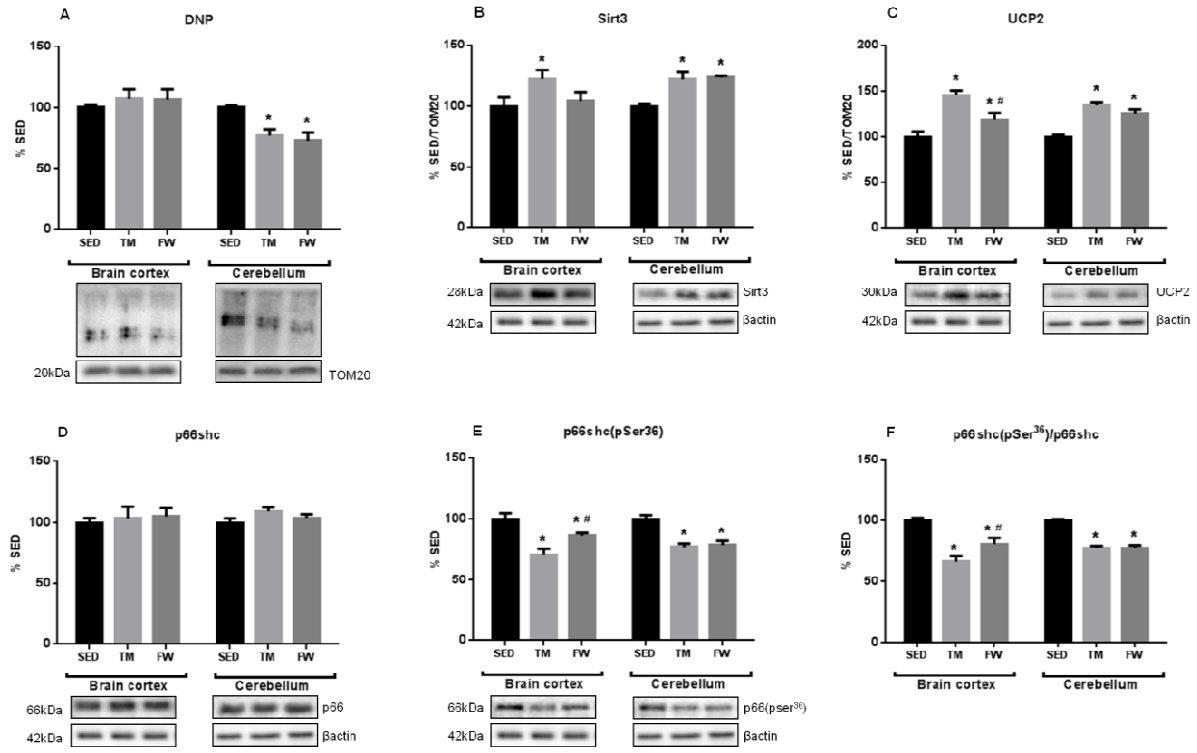
*Mitochondrial oxidative damage and antioxidants*

The present work also analyzed the effect of TM and FW on basal brain cortex and cerebellum mitochondrial oxidative stress makers and intrinsic antioxidant defenses (Figure 5). Mitochondrial MDA levels were not different between groups in brain cortex, whereas both models of physical exercise decreased MDA content in cerebellum mitochondria. An increase in aconitase activity, a Krebs cycle enzyme particularly susceptible to ROS-induced damage and inactivation (Melov et al., 1999), was observed in brain cortex mitochondria from both exercise groups and in cerebellum mitochondria from FW group. Brain cortex Mn-SOD activity was higher in TM group, whereas in cerebellum was higher in FW group. No significant alterations were observed on mitochondrial -SH content in all tested groups.




**Chapter 3.4 - Figure 5.** Effects of exercise on brain cortex and cerebellum mitochondrial (A) -SH content, (B) MDA levels and (C) aconitase and (D) Mn-SOD activities. Data are means±SEM (n=6 per group) for brain cortex and cerebellum mitochondria. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. **vs.* SED ($p \leq 0.05$).

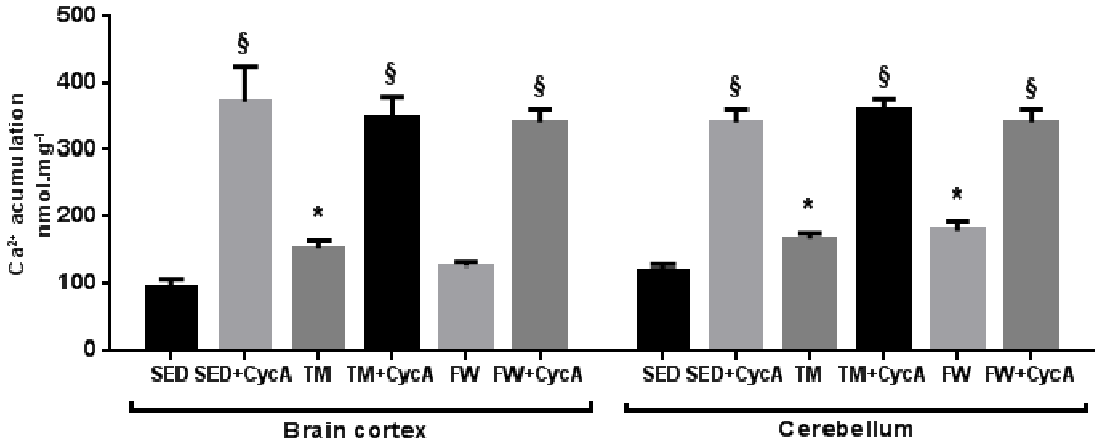
Alterations in brain cortex and cerebellum mitochondrial carbonylated proteins as well as in Sirt3, UCP2 and p66shc induced by exercise are shown in Figure 6. Exercise decreased carbonylated proteins in cerebellum but not in brain cortex. Both exercise modalities increased Sirt3 (except for brain cortex in the FW group) and UCP2 contents and decreased the p66shc($ser^{36}$)/p66shc ratio. This decrease resulted from the observed diminished content of the phosphorylated form p66shc. Of notice is the fact that in brain cortex, the increase in UCP2 and the decrease in p66shc($ser^{36}$)/p66shc were higher in the TM than in the FW group.




**Chapter 3.4 - Figure 6.** Effect of exercise on brain cortex and cerebellum mitochondrial (A) carbonyl groups (B) SIRT3 (C) UCP2 (D) and tissue p66shc(pSer$^{36}$), (E) p66shc and (F) p66shc(pSer$^{36}$)/p66shc ratio. Typical immunoblots are presented below the histograms. Data are means±SEM (n=4 per group). The results were expressed as percentage of SED group. Sirt3 and UCP2 contents were normalized against TOM20 content. TOM20 and β-actin were used for mitochondrial and tissue protein loading controls, respectively. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. *$^{*}$*vs.* SED; $^{\#}$*vs.* TM ($p \leq 0.05$).

*Calcium-induced mitochondrial permeability transition*

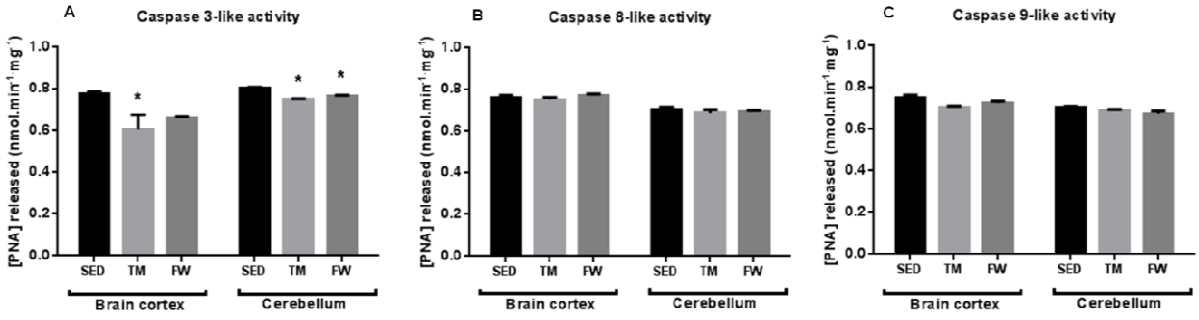
We next questioned whether any of the two exercise regimens altered the amount of calcium needed to induce the mPTP in isolated brain cortex and cerebellum mitochondria. Figure 7 shows the maximum amount of calcium accumulated in brain cortex and cerebellum mitochondria until mPTP opening occurred, seen as an irreversible fall in $\Delta\psi$. As shown, the TM group significantly accumulated more $Ca^{2+}$ before mPTP induction than the SED group in brain cortex mitochondria, whereas in cerebellum both models of exercise increased the tolerance to calcium. Cyclosporin A was able to significantly increase the accumulation of calcium in all groups.




**Chapter 3.4 - Figure 7.** Effects of exercise on brain cortex and cerebellum mitochondrial calcium-induced membrane depolarization. Data are means±SEM for brain cortex and cerebellum mitochondria (0.8 mg protein/mL) obtained from six different mitochondrial preparations in each experimental group. The figure shows the amount of calcium accumulated before the irreversible fall in Δψ and the respective negative control using cyclosporin-A (CycA, 1μM). Succinate-energized mitochondria were used at 30 ºC in a total volume of 1 mL. Mitochondrial Δψ was followed by a $TPP^+$ selective electrode. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. **vs.* SED (p≤0.05); $^{\#}$*vs.* TM (p≤0.05); $^{\S}$*vs.* without CycA (p≤0.05).

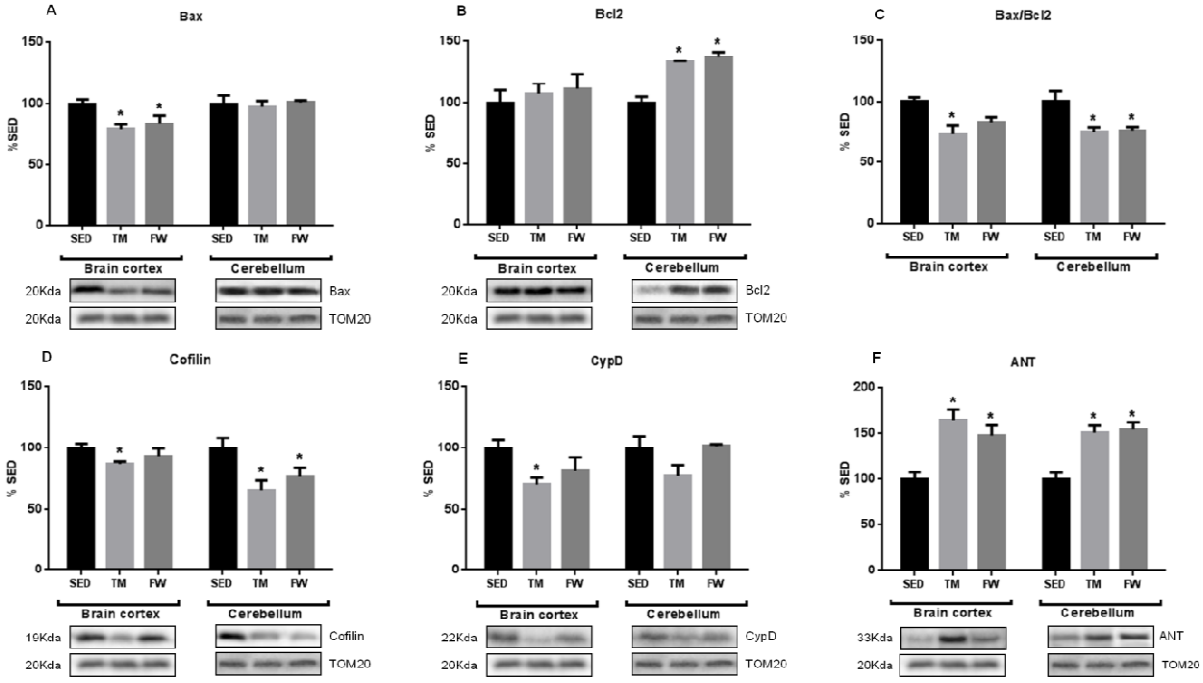
*Apoptotic signaling*

Caspase 3, 8 and 9-like activities were determined as markers for apoptotic signaling activation. As seen in Figure 8, caspase 3-like activity decreased in brain cortex only after TM and in cerebellum after both exercise modalities.




**Chapter 3.4 - Figure 8.** Effects of exercise on brain cortex and cerebellum caspase 3, 8 and 9-like activities. Data are means±SEM (n=6 per group) for brain cortex and cerebellum tissues. Caspase-like activity was measured colorimetrically by pNA release from specific caspase substrates. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. **vs.* SED (p≤0.05).

Following immunoblotting analyses were also performed to semi-quantify mitochondrial proteins involved in apoptotic signaling and/or mPTP regulation. As shown in Figure 9, TM decreased Bax/Bcl2 ratio, cofilin and CypD content in brain cortex. Both exercise models decreased Bax/Bcl2 ratio, cofilin and CypD in cerebellum. While the decrease in Bax/Bcl2 ratio in brain cortex was due to the decreased Bax content, in cerebellum the decrease in the same ratio was a consequence of the increased Bcl2 content. An increase in brain and cerebellum ANT content was also observed in both exercise models.

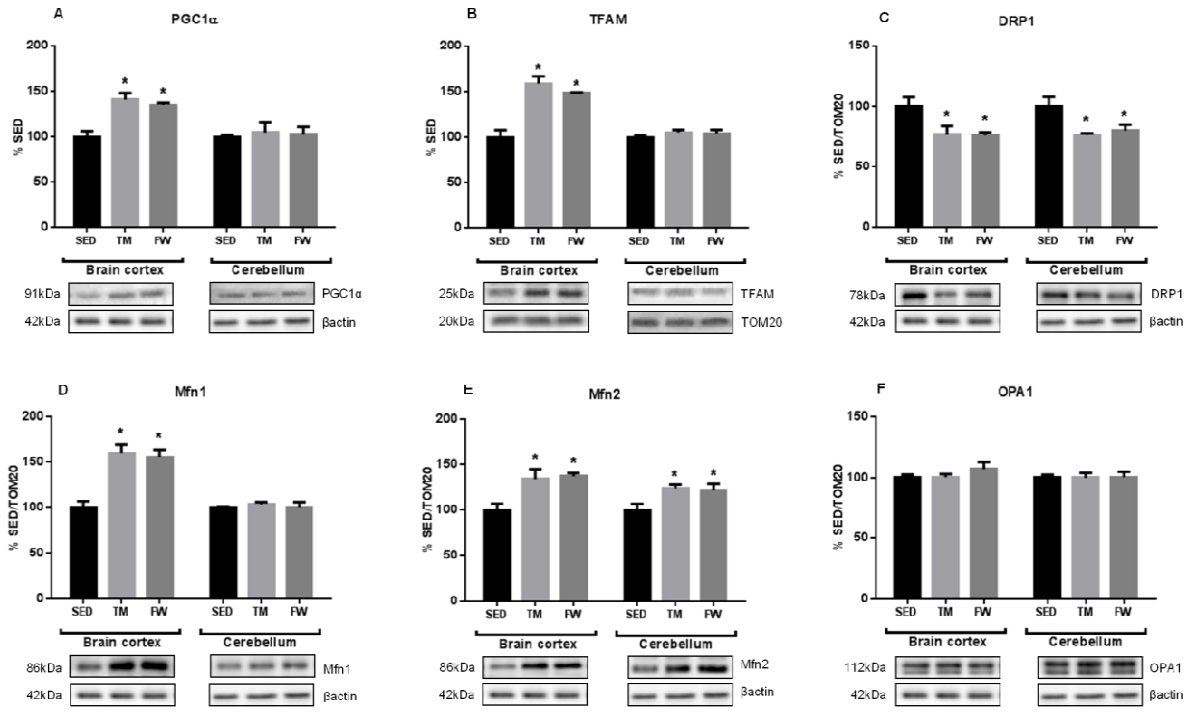



**Chapter 3.4 - Figure 9.** Effect of exercise on brain cortex and cerebellum mitochondrial (A) Bax, (B) Bcl2, (C) Bax/Bcl2, (D) Cofilin (E) CypD and (F) ANT. Data are means±SEM (n=4 per group) obtained from different mitochondrial preparations. The results were expressed as percentage of SED group. TOM20 was used for mitochondrial protein loading control. Typical immunoblots are presented below the histograms. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. **vs.* SED (p≤0.05).

*Mitochondrial biogenesis and dynamics signaling*

The content of proteins associated with mitochondrial biogenesis and dynamics, including fusion and fission were evaluated. PGC1α and TFAM increased in brain cortex in both exercise models, while no alterations were noted regarding the content of these proteins in

cerebellum. Both exercise models increased the content of Mfn2 and decreased DRP1 in both brain areas. In brain cortex an increased Mfn1 was also observed. No differences between groups were observed regarding OPA1.

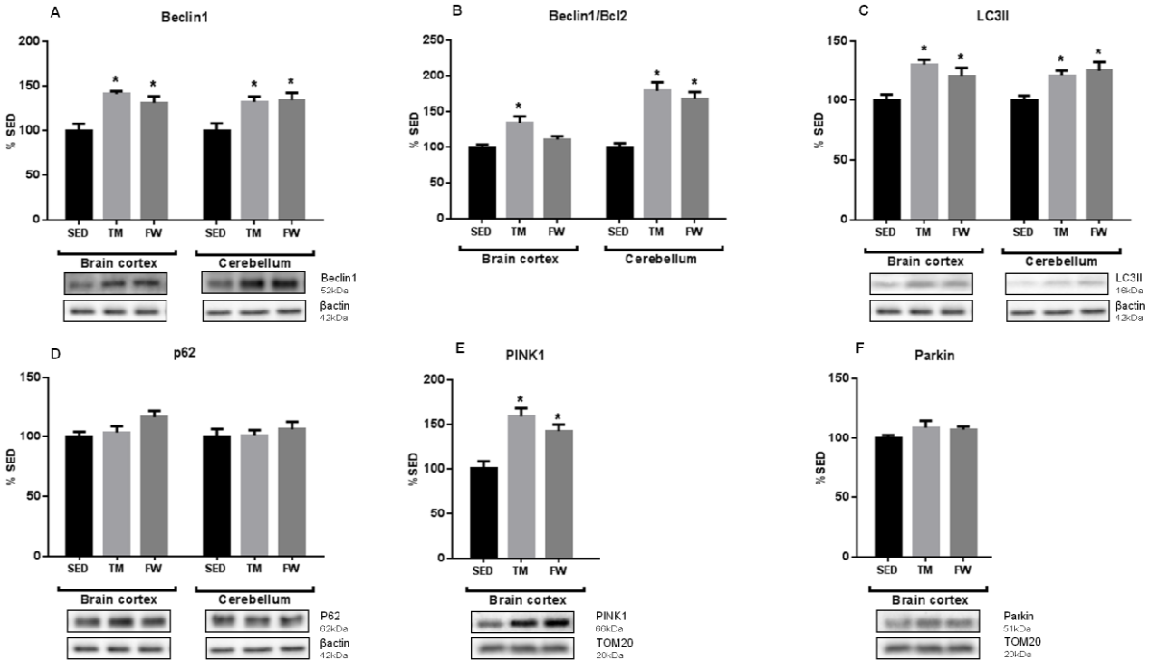



**Chapter 3.4 - Figure 10.** Effect of exercise and sub-chronic DOX treatment on (A) whole tissue PGC1α, (B) mitochondrial TFAM, (C) DRP1 (D) Mfn1, (E) Mfn2 and (F) OPA1. Typical immunoblots are presented below the histograms. Data are means±SEM (n=4 per group) for brain cortex and cerebellum tissue or mitochondria. The results were expressed as percentage of SED group. DRP1, Mfn1, Mfn2 and OPA1 contents were normalized against TOM20. TOM20 and β-actin were used for mitochondrial and tissue protein loading controls, respectively. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. **vs.* SED ($p \leq 0.05$).

*Autophagy and Mitophagy signaling*

Autophagy signaling was assessed through semi-quantification of Beclin1, a marker of the initiation of autophagosome formation; Beclin1-to-Bcl2 ratio, a marker of beclin-dependent autophagy; LC3, a marker for the elongation and formation of the autophagosome and PINK1, Parkin and p62 all associated with mitophagy regulation (Figure. 11). As seen, both exercise modalities resulted in increased content of Beclin1, Beclin/Bcl2 and LC3II in brain

cortex and cerebellum, although Beclin/Bcl2 did not reach statistical significance for brain cortex after FW. TM and FW increased PINK content in brain cortex, while no differences between groups were observed regarding p62 and brain cortex Parkin. Unexpectedly, despite previous reports showing evidence of these proteins in cerebellum (Batlevi and La Spada, 2011; Kageyama et al., 2012; Taymans et al., 2006), we were not able to detect PINK and Parkin staining in membranes loaded with cerebellum mitochondria.




**Chapter 3.4 - Figure 11.** Effect of exercise on brain cortex and cerebellum tissue (A) Beclin1, (B) Beclin1/Bcl2 (C) LC3II, (D) p62 and mitochondrial (E) PINK1 and (F) Parkin. Typical immunoblots are presented below the histograms. Data are means±SEM (n=4 per group) for brain cortex and cerebellum mitochondria and tissue. The results were expressed as percentage of SED group. TOM20 and β-actin were used for mitochondrial and tissue protein loading controls, respectively. SED, sedentary; TM, endurance treadmill training; FW, Free-wheel voluntary physical activity. PINK1 and Parkin were not detected in cerebellum fractions. *_vs._ SED (p≤0.05).

## Discussion

The study of exercise-induced adaptations in the nervous tissue has progressively gained attention due to the effectiveness of physical activity as a preventive and therapeutic tool

against several neurodegenerative diseases. In this regard, mitochondria have assumed particular interest, as they are critical in this high-energy demanding tissue. Moreover, mitochondria are widely recognized important sub-cellular structures in the maintenance of calcium homeostasis and in the control of cellular fate. In order to attain these functions, their plasticity as well as the interplay between dynamics and renewal is essential for the maintenance of cellular quality control.

In the present study, two different exercise strategies, mimicking models of forced endurance training (TM) and voluntary physical activity (FW) programs, were used to determine specific exercise effects on mitochondrial activity and related processes in two brain areas involved in cognition and motor control, the brain cortex and cerebellum. Our data confirmed that physical exercise positively modulated mitochondrial respiratory activity (Dietrich et al., 2008) and showed for the first time that exercise augmented the resistance of brain cortex and cerebellum to mPTP induction. Moreover, the data here presented suggest that alterations in proteins involved in mitochondrial dynamics, apoptosis and autophagic signaling may be associated with the observed functional phenotypes and may impact behavioral performance (Figures 1 and 2), particularly after TM.

Considering the RCR the most important index of mitochondrial functional integrity and fitness (Brand and Nicholls, 2011), our data suggest that exercise improved mitochondrial ability to produce energy (Figure 3). The alterations in respiration resulting from the different exercise regimens may stem from increased expression of complex I, III and V subunits, as well as in ANT (Figure 4). The increased expression of complex I subunit found in the present study is in accordance with the previously reported increased electron transfer activity originating from complex I after long-term endurance training (Navarro et al., 2004). Interestingly, this relationship between OXPHOS complex subunit content and electron transfer activity after chronic exercise was not observed for complex IV (Navarro et al., 2004). Increased OXPHOS subunit III and V after exercise was also previously observed (Bayod et al., 2011). Moreover, the integrity and function of the neural mitochondrial

phosphorylative system is highly dependent on the mitochondrial redox environment, with increased oxidized states being potentially harmful and related to stress-associated neurodegenerative diseases (Mancuso et al., 2006; Migliore and Coppede, 2009; Moreira et al., 2010). Given that physical exercise improves redox balance by decreasing free radical production and/or by up-regulating antioxidant defense systems (Boveris and Navarro, 2008b; Camiletti-Moiron et al., 2013; Marques-Aleixo et al., 2012), here again corroborated by our data in Figure 5, it is likely that these redox-related alterations may had contributed to the observed functional improvements. Accordingly, the mitochondrial metabolic and redox-mediated regulatory proteins p66shc, Sirt3 and UCP2 (Figure 6) were also modulated by both chronic exercise modalities towards a more metabolic and anti-oxidant state possibly favoring a more resistant mitochondrial phenotype.

Another mitochondrial physiological event influenced by increased oxidative stress and calcium overload is the susceptibility to mPTP opening with consequent activation of mitochondrial-driven apoptosis (Norenberg and Rao, 2007; Toman and Fiskum, 2011). A novel finding of the present study was that both TM and FW in cerebellum, and TM only in brain cortex, augmented the resistance to calcium-induced mPTP opening (Figure 7) as well decreased apoptotic markers, as seen by decreased caspase 3-like activity, Bax/Blc2 ratio, and cofilin and CypD expression (Figures 8 and 9), suggesting an important protective effect of chronic exercise on events leading to mitochondrial degeneration and cell death.

The proper balance of mitochondrial biogenesis and clearance/renewal is a key determinant to maintain the overall mitochondrial physiology within the cell (Ghavami et al., 2014). We here hypothesized that the regulation and stimulation of these processes by both studied exercise models may translate into positive outcomes on brain cortex and cerebellum mitochondrial metabolic activity. In fact, in accordance with the previously reported increased PGC1$\alpha$ and SIRT1 (Bayod et al., 2011; Steiner et al., 2011), the improvement in mitochondrial function (Figure 3) was accompanied by increased biogenesis (Figure 9), and by altered mitochondrial fusion and fission markers towards a fused profile (Figure 10),

particularly in brain cortex. As an increase in the selected markers of mitochondrial biogenesis was observed after exercise (particularly in brain), but variations in OXPHOS, Sirt3, UCP2, DRP1, Mfn1/2 and OPA1 content, determined in tissue homogenate and further corrected for TOM20 as a measure of mitochondrial content, still persist, this could reflect an increased mitochondrial quality of the signaling pathways these proteins are involved to, regardless possible increase in mitochondrial mass.

The interplay between mitochondrial dynamics and autophagy is recognized as critical for mitochondrial homeostasis, being fusion, fission and mitochondrial autophagy coordinately linked mechanisms that determine the fate of an individual mitochondrion within the network, and ultimately regulate cellular quality control. In fact, these events determine the architecture of the entire mitochondrial population and are closely related with important features of mitochondrial functions, including respiration, calcium buffering capacity, and apoptosis (Twig and Shirihai, 2011).

Although the classical view suggests that the stimulation of fission facilitates mitochondrial autophagy, i.e., mitophagy and fusion as competing fates of dysfunctional mitochondria, both fusion and mitophagy could also be complementary processes. The selective fusion of healthy mitochondrial population and the segregation of damaged mitochondria from the fusing population not only prevents the migration of damaged components into more fit mitochondria, but also, and equally important, signals dysfunctional components for autophagy (Twig and Shirihai, 2011). Our results suggest that both exercise models promote an increase in brain cortex and cerebellum selective mitochondrial fusion, possibly increasing the healthy network component in the cells. Concomitantly, increased segregation of damaged mitochondria through autophagy in TM and FW animals is also suggested by beclin-1 and LC3II analyses in both tissues and PINK in brain cortex (Figure 11). Therefore, the relevance of exercise as a strategy to prevent and attenuate the deleterious effects characterizing the aged brain and neurodegenerative diseases relies on the ability to improve mitochondrial metabolism through the interaction of cellular physiological processes,

including increased resistance to mPTP and decreased apoptotic signaling, activation of mitochondrial biogenesis, and the modulation of dynamics and autophagy with potentially important implications for tissue performance.

The molecular results of the current study in combination with the observed improved behavioral performance of the animals suggest that one of the neurobiological mechanisms underlying exercise-induced improved cognition may involve improved mitochondrial function. However, depending on exercise intensity and duration, different adaptive responses seem to occur (Camiletti-Moiron et al., 2013; Radak et al., 2001b). In fact, the differential modulator effect of TM exercise when compared with FW on brain cortex MnSOD, SIRT3, UCP2, caspase 3, Bax/Bcl2, cofilin, LC3II and beclin-1/Bcl2 suggests that TM may confer a higher antioxidant and anti-apoptotic potential, and regulates brain cortex autophagy signaling more efficiently than FW. Additionally, several brain area-specific alterations were observed regarding the measured markers. Of these, exercise-induced lower carbonyl groups, MDA and increased Bcl2 levels in cerebellum mitochondria (but not in brain cortex) and the higher TFAM, PGC1$\alpha$, and lower Bax and Mfn1 as a consequence of both exercise models in brain cortex may be highlighted.

In summary, our findings contribute to further understand brain mitochondrial-associated mechanisms targeted by physical exercise. In this context, the expanded use of exercise as a therapeutic strategy to attenuate the negative effects of aging, and the treatment and/or prevention of neurological diseases, particularly in conditions in which mitochondrial dysfunction is closely implicated in the etiology and pathophysiology, is reinforced.

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# 3.5

[Study 4]

## Physical exercise mitigates Doxorubicin-induced brain cortex and cerebellum mitochondrial alterations and cellular quality control signaling

Marques-Aleixo I[1], Santos-Alves E[1,2], Balça MM[1], Moreira PI[23], Oliveira PJ[2], Magalhães J[1], Ascensão A[1]

[1]CIAFEL - Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto, Portugal

[2]CNC - Centre for Neuroscience and Cell Biology, Department of Life Sciences, University of Coimbra, Portugal

[3]Institute of Physiology, Faculty of Medicine, University of Coimbra, Portugal

**ABSTRACT**

Doxorubicin (DOX) is a highly effective anti-neoplastic agent, whose clinical use is limited by a dose-dependent mitochondrial toxicity in non-target tissues, including the brain. We here analyzed the effects of distinct exercise modalities (12-wks endurance treadmill-TM or voluntary free-wheel activity-FW) performed before and during sub-chronic DOX treatment on brain cortex and cerebellum mitochondrial bioenergetics, oxidative stress, permeability transition pore (mPTP), and proteins involved in mitochondrial biogenesis, apoptosis and auto(mito)phagy.

Male Sprague-Dawley rats were divided into saline-sedentary (SAL+SED), DOX-sedentary (DOX+SED; 7-wks DOX ($2mg.kg^{-1}$ *per* wk)), DOX+TM and DOX+FW. Animal behavior and post-sacrifice mitochondrial function were assessed. Oxidative phosphorylation (OXPHOS) subunits, oxidative stress markers or related proteins (SIRT3, p66shc, UCP2, carbonyls, MDA, -SH, aconitase, Mn-SOD), as well as proteins involved in mitochondrial biogenesis (PGC1α and TFAM) were evaluated. Apoptotic signaling was followed through caspases 3, 8 and 9-like activities, Bax, Bcl2, CypD, ANT and cofilin expression. Mitochondrial dynamics (Mfn1, Mfn2, OPA1 and DRP1) and auto(mito)phagy (LC3II, Beclin1, Pink1, Parkin and p62)-related proteins were measured by semi-quantitative Western blotting.

DOX impaired behavioral performance, mitochondrial function, including lower resistance to mPTP and increased apoptotic signaling, decreased the content in OXPHOS complex subunits and increased oxidative stress in brain cortex and cerebellum. Molecular markers of mitochondrial biogenesis, dynamics and autophagy were also altered by DOX treatment in both brain subareas. Generally, TM and FW were able to mitigate DOX-related impairments in brain cortex and cerebellum mitochondrial activity, mPTP and apoptotic signaling.

We conclude that the alterations in mitochondrial biogenesis, dynamics and autophagy markers induced by exercise performed before and during treatment may contribute to the observed protective brain cortex and cerebellum mitochondrial phenotype, which is more resistant to oxidative damage and apoptotic signaling in sub-chronically DOX treated animals.

**Keywords**: Adriamycin, exercise, brain bioenergetics, apoptosis, mitochondrial dynamics, autophagy signalling

## Introduction

Doxorubicin (DOX) is a highly effective antibiotic used to treat several types of malignancies. Despite its high efficacy, DOX clinical use is limited by dose-related severe adverse effects on non-target tissues, including the heart and the brain (Cardoso et al., 2008; Carvalho et al., 2009; Wallace, 2007). Although it is considered that DOX does not cross the blood-brain barrier (Bigotte and Olsson, 1982; Ohnishi et al., 1995), several studies in patients undergoing DOX-based chemotherapy reported persistent changes in cognitive functions, commonly referred to as "chemo brain", sometimes lasting years after cessation of chemotherapy (Aluise et al., 2010).

Although the biochemical basis for DOX toxicity resulting in cognitive impairments requires further studies, the production of pro-inflammatory mediators with consequent increased oxidative damage and mitochondrial dysfunction is implicated (Seruga et al., 2008; Tangpong et al., 2006). In addition, these events may lead to the increased susceptibility to neuronal mPTP opening (Cardoso et al., 2008) and possibly activation of apoptotic signaling (Joshi et al., 2010; Pal et al., 2012; Tangpong et al., 2006; Tangpong et al., 2007) with subsequent deterioration of cognitive function.

Physical exercise is a neuroprotective strategy to mitigate impairments on brain function as it inhibits oxidative stress and apoptotic signaling, resulting in beneficial adaptations and in a healthier neuronal phenotype. Together with increased mitochondrial biogenesis and OXPHOS activity, the data emphasize the role of mitochondria as plastic organelles central for the adaptations resulting from physical exercise (Marques-Aleixo et al., 2012). Recently, our group reported that treadmill and voluntary free wheel running improved behavioral performance, brain cortex and cerebellum mitochondrial fitness including OXPHOS capacity, and altered oxidative status, mPTP susceptibility, apoptotic signaling, mitochondrial dynamics and quality control signaling (Marques-Aleixo et al., c).

Although the effects of chronic physical exercise against DOX-induced impairments in the cardiovascular system, including deterioration in cardiac hemodynamics, mitochondrial bioenergetics and associated pathways have been previously investigated (Ascensao et al., 2007;

Ascensao et al., 2011c; Ascensao et al., 2012), whether brain cortex and cerebellum mitochondrial alterations associated with sub-chronic DOX administration are limited by long-term physical exercise has yet to be determined. Our objective was to analyze the effects of two modalities of long-term exercise with distinct characteristics, treadmill and voluntary activity, performed before and during the course of sub-chronic DOX administration, on brain cortex and cerebellum mitochondrial respiratory activity, OXPHOS components and biogenesis, susceptibility to mPTP opening, apoptotic signaling, oxidative stress, as well as mitochondrial dynamics and auto(mito)phagy signaling. The integrated analysis of these mitochondrial-related alterations with behavioral performance may contribute to understand the mechanisms by which physical exercise is useful for improving cognitive function in patients undergoing chemotherapy with DOX.

## Results

*Characterization of animals and exercise protocols*

DOX treatment decreased animal body weight and increased brain cortex and cerebellum to body weight ratios (DOX+SED, DOX+TM and DOX+FW *vs.* SAL+SED). No significant differences were observed between groups regarding the initial body weight, brain cortex and cerebellum weights and mitochondria isolation yield. Animals from DOX+FW ran significantly more than the DOX+TM group. TM increased the activity of *soleus* citrate synthase (DOX+TM vs. SAL+SED, DOX+SED and DOX+FW). The lack of increased *soleus* CS activity in FW group might possible result from distinct responses of skeletal muscle to chronic exercise modalities (Liu et al., 2009; Noble et al., 1999) (Table 1).

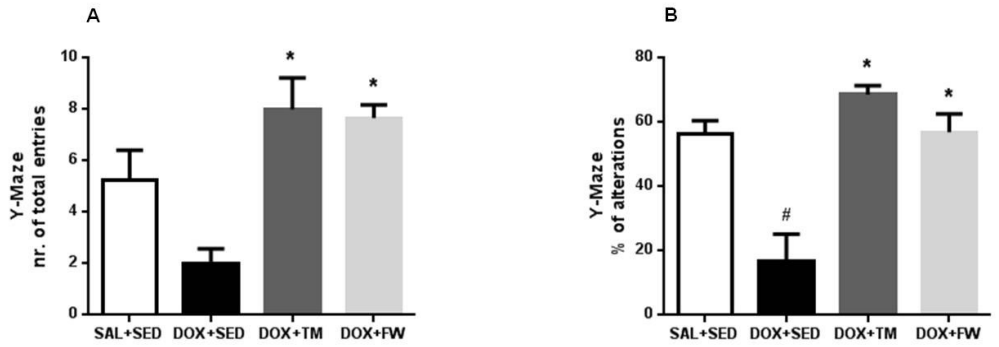
**Chapter 3.5 - Table 1.** Animal data and yield of mitochondrial protein isolation

| | SAL+SED | DOX+SED | DOX+TM | DOX+FW |
|---|---|---|---|---|
| Initial body weight (g) | 207.4±3.9 | 208.9±4.7 | 207.0±4.6 | 208.8±2.6 |
| Final body weight (g) | 598.5±10.6 | 438.00±6.2$^{\#}$ | 426.00±10.8$^{\#}$ | 428.50±16.9$^{\#}$ |
| Distance (m/day) | - | - | 1326±50 | 2122±169$^{\S}$ |
| Brain cortex weight (g) | 1.28±0.04 | 1.23±0.04 | 1.28±0.03 | 1.27±0.05 |
| Brain cortex weight/body weight ($mg.g^{-1}$) | 2.13±0.05 | 2.88±0.07$^{\#}$ | 3.02±0.07$^{\#}$ | 3.01±0.11$^{\#}$ |
| Brain cortex mitochondrial isolation yield (mg protein.g $tissue^{-1}$) | 22.74±3.82 | 25.67±3.20 | 20.68±1.25 | 26.14±3.26 |
| Cerebellum weight (g) | 0.60±0.01 | 0.57±0.04 | 0.60±0.02 | 0.60±0.02 |
| Cerebellum weight/body weight ($mg.g^{-1}$) | 0.99±0.03 | 1.23±0.09$^{\#}$ | 1.36±0.06$^{\#}$ | 1.30±0.05$^{\#}$ |
| Cerebellum mitochondrial isolation yield (mg protein.g $tissue^{-1}$) | 27.91±2.18 | 25.70±2.00 | 23.71±1.44 | 27.17±3.24 |
| *Soleus* citrate synthase activity ($mM.\ min^{-1}.mg^{-1}$) | 10.94±2.07 | 8.69±1.15 | 22.22±1.97$^{\#*}$ | 10.27±1.32$^{\S}$ |

Values are means±SEM (n=6 per group). SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. $^{\#}$*vs*. SAL+SED ($p \leq 0.05$),$^{*}$*vs*. DOX+SED ($p \leq 0.05$), $^{\S}$*vs*. DOX+TM ($p \leq 0.05$).

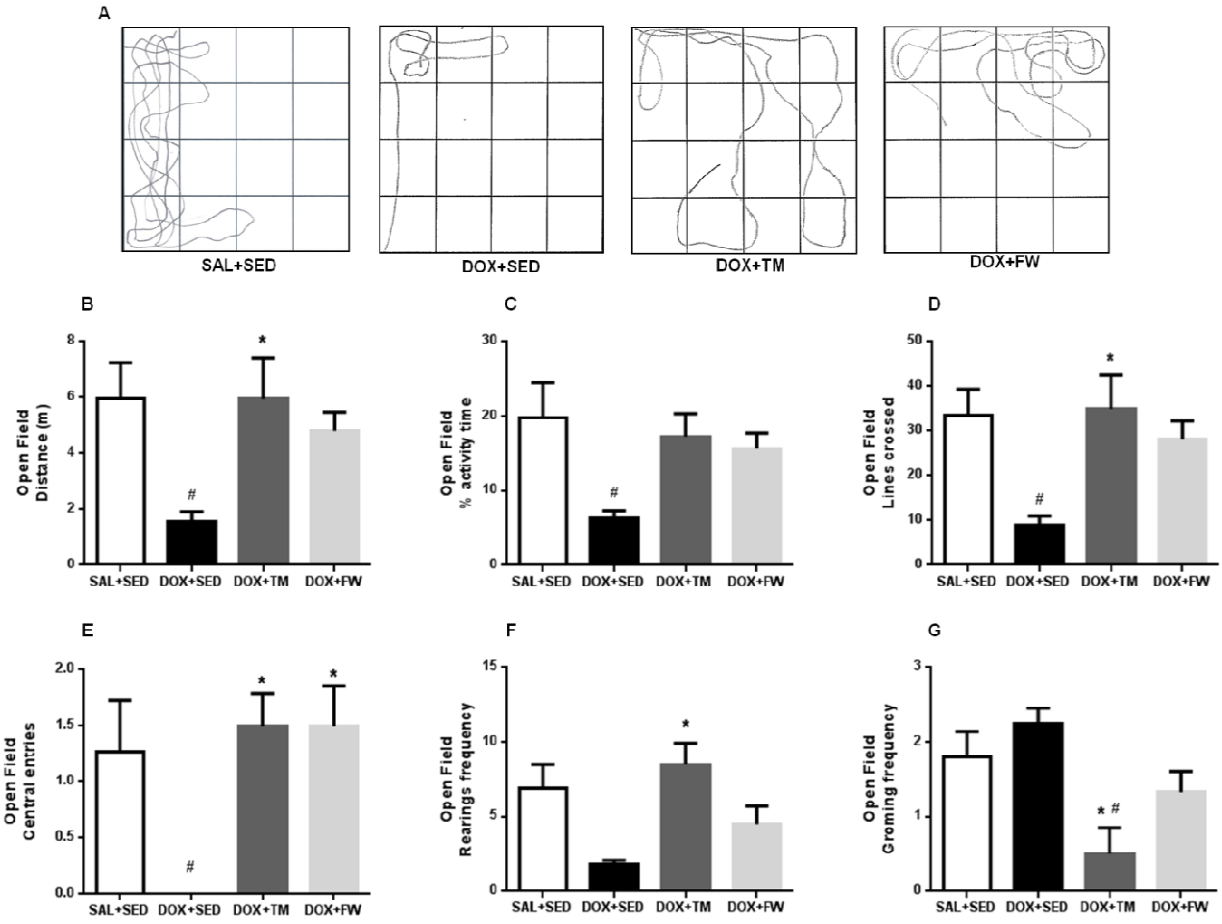
*Behavioral tests*

Figure 1 shows alterations in spontaneous alternation in the Y maze induced by DOX treatment per se and in combination with both modalities of chronic exercise. As it can be observed, DOX treatment decreased the percentage of alternation in arm entrance (DOX+SED *vs*. SAL+SED). Both exercise modalities increased the number of total entries and percentage of alteration (DOX+TM and DOX+FW *vs.* DOX+SED), suggesting a higher willingness to explore new environments, similarly to the SAL group.




**Chapter 3.5 - Figure 1.** Effect of exercise and sub-chronic DOX treatment on Y-maze behavior (A) number of total entries and (B) % of alternation. Data are means ± SEM (n=6 per group). Experimental details are provided in methods. SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. $^{\#}$*vs.* SAL+SED,$^{*}$*vs.* DOX+SED ($p \leq 0.05$).

Regarding motor activity, as shown in Figure 2, DOX treatment decreased the overall locomotor distance traveled, % activity time, the number of lines crossed and the number of central entries on the open field behavioral test (DOX+SED *vs.* SAL+SED). TM exercise increased the distance traveled, the number of lines crossed and rearings when compared with DOX+SED animals (DOX+TM *vs.* DOX+SED) and decreased grooming frequency compared with SAL+SED and DOX+SED groups (DOX+TM *vs.* SAL+SED and DOX+SED), generally suggesting an increase in activity and exploratory behavior.

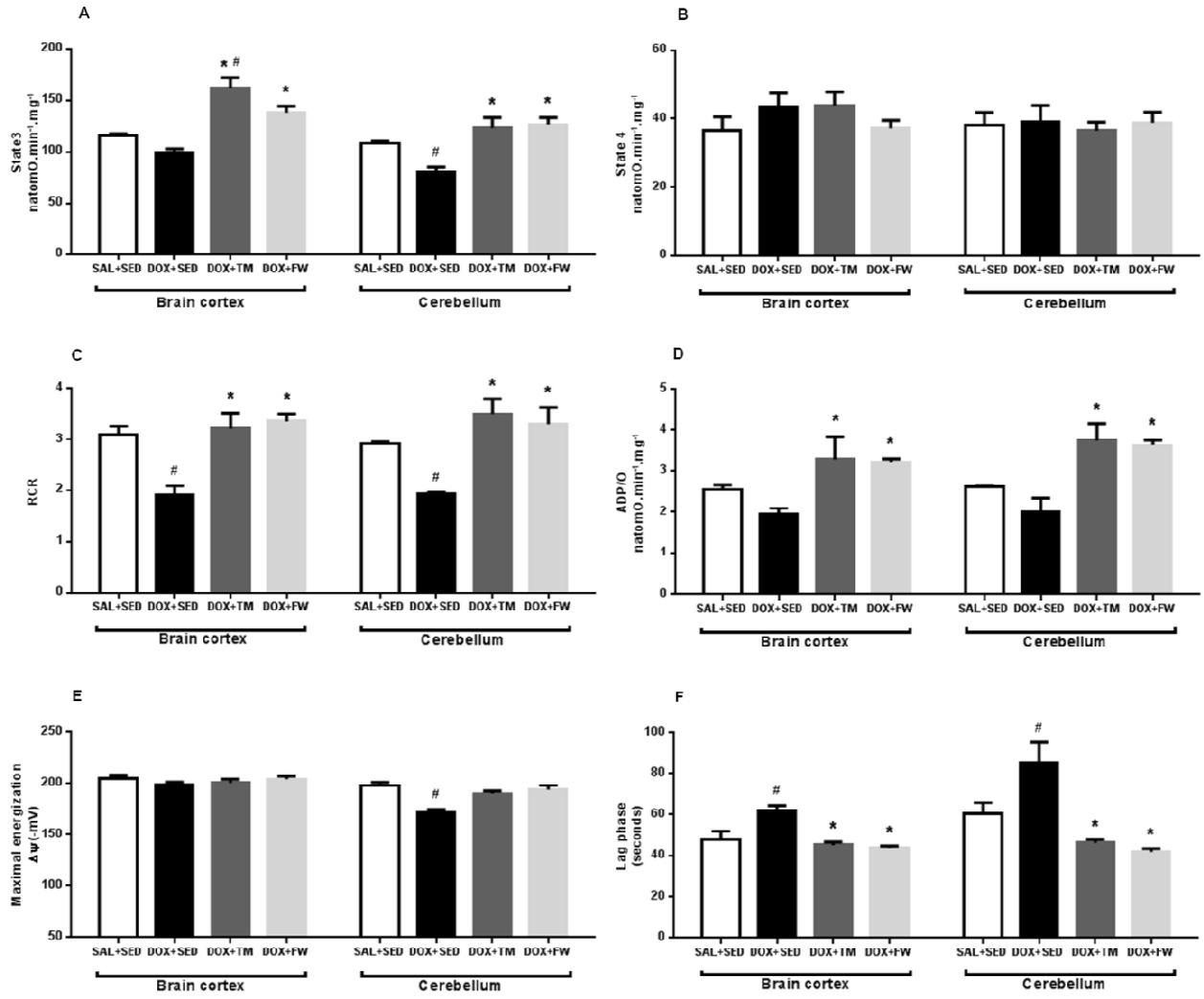



**Chapter 3.5 - Figure 2.** Effect of exercise and sub-chronic DOX treatment on Open Field behavior; (A) illustrative example of animal travel path during a 5 min exploration (B) % activity time, (C) distance traveled, (D) number of lines crossed, (E) number of central entries (F and G) number of rearings and grooming performed. Data are means ± SEM (n=6 per group). Experimental details are provided in methods. SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. $^{\#}$*vs.* SAL+SED, **vs.* DOX+SED ($p \leq 0.05$).

*Brain cortex and cerebellum mitochondrial oxygen consumption and electric transmembrane potential*

Mitochondrial respiration and $\Delta\psi$ fluctuations associated with ADP phosphorylation activity were measured to identify possible exercise-dependent effects on brain cortex and cerebellum in DOX treated animals (Figure 3). Sub-chronic DOX treatment in the sedentary group decreased state 3 respiration and maximal $\Delta\psi$ in cerebellum, decreased the coupling between oxygen consumption and ADP phosphorylation (RCR) and increased the ADP-lag phase in brain cortex and cerebellum mitochondria (DOX+SED *vs.* SAL+SED). When associated with DOX treatment, TM and FW

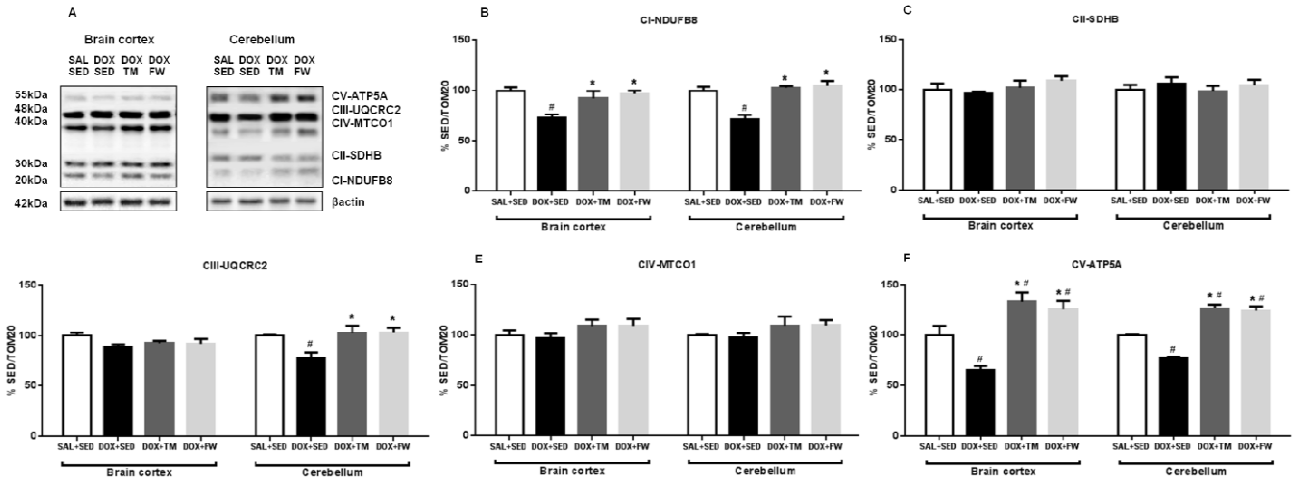
increased state 3 respiration, RCR, the efficiency of ATP synthesis (ADP/O) and decreased the ADP-lag phase in brain cortex and in cerebellum mitochondria (DOX+TM and DOX+FW vs. DOX+SED).




**Chapter 3.5 - Figure 3.** Effect of exercise and sub-chronic DOX treatment on brain cortex and cerebellum (A) state 3 respiration, (B) state 4 respiration, (C) RCR, (D) ADP/O, (E) maximal ∆ψ and (F) ADP phosphorylation lag phase. Data are means ± SEM obtained from different six mitochondrial preparations (0.8 mg/mL protein) for each group. Oxygen consumption and ∆ψ were measured with G/M as substrates. RCR, respiratory control ratio (state 3/state 4); ADP/O, number of nmol ADP phosphorylated by natom of oxygen consumed; lag-phase, the time elapsed in the depolarization/repolarization cycle during ADP phosphorylation SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. #*vs.* SAL+SED, **vs.* DOX+SED ($p \leq 0.05$).

*OXPHOS complex subunits semi-quantification by western blotting*

The analysis of brain cortex and cerebellum mitochondria OXPHOS subunits (Figure 4) showed that DOX decreased complex I and V subunits in brain cortex and cerebellum mitochondria and complex III in cerebellum (DOX+SED *vs.* SAL+SED). Both exercise models increased complex I and V subunits in brain cortex and cerebellum and complex III in cerebellum mitochondria in DOX treated groups (DOX+TM and DOX+FW *vs.* DOX+SED).

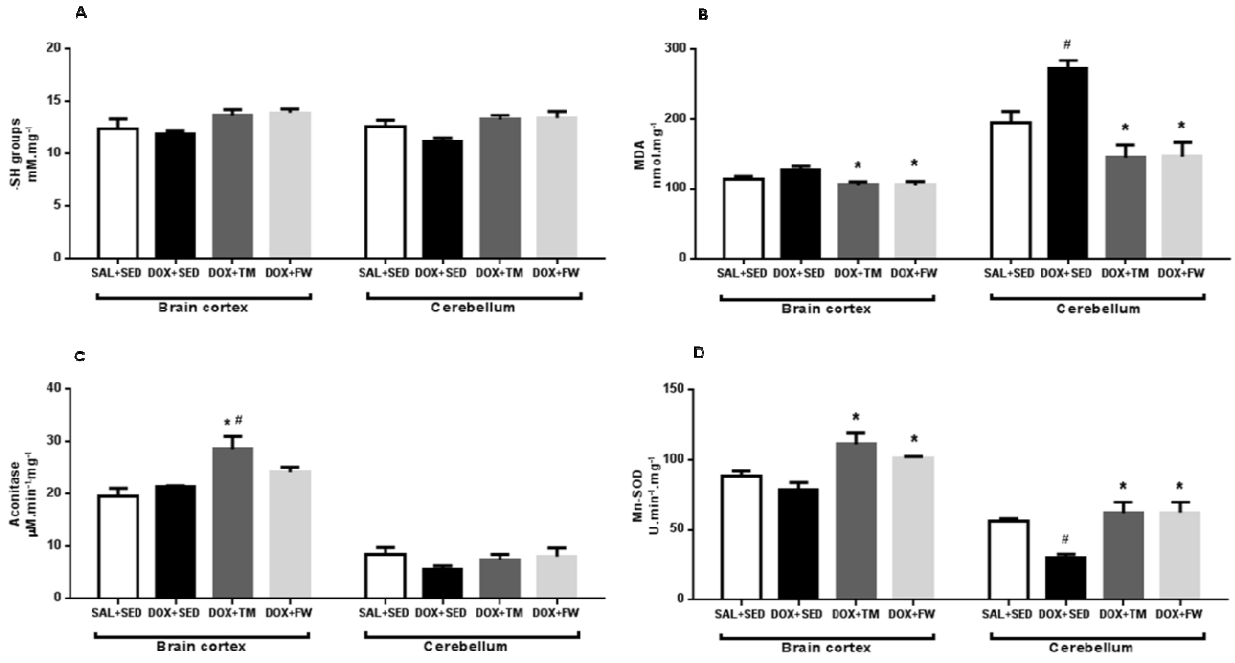



**Chapter 3.5 - Figure 4.** Effect of exercise and sub-chronic DOX treatment on brain cortex and cerebellum mitochondrial OXPHOS subunits: (A) typical immunoblots; (B) complex I CI-NDUFB8 subunit; (C) complex II CII-SDHB subunit; (D) complex III CIII-UQCRC2 subunit; (E), complex IV CIV-MTCO1subunit and (F) complex V CV-ATP5A subunit. Data are means±SEM (n=4 per group). The results were expressed as percentage of SED group. Each OXPHOS subunit was normalized against TOM20 content. β-actin was used for tissue protein loading control. SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. $^{\#}$*vs.* SAL+SED,$^{*}$*vs.* DOX+SED ($p \leq 0.05$).

*Mitochondrial oxidative damage and antioxidants*

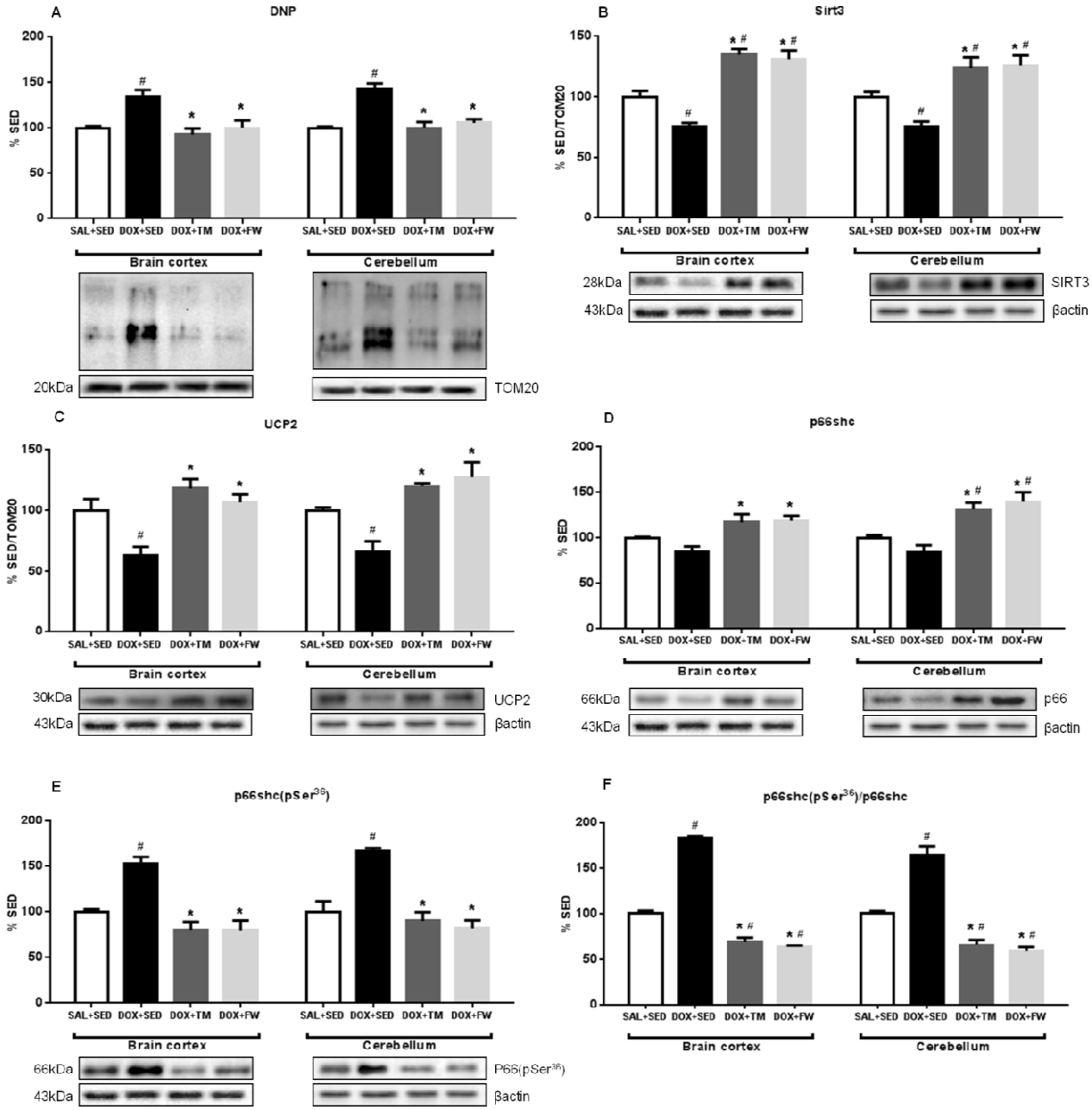
Figure 5 shows the effects of DOX, TM and FW exercise modalities in DOX-treated animals regarding the extent of brain cortex and cerebellum mitochondrial oxidative stress markers. DOX treatment increased MDA levels and decreased Mn-SOD activity in cerebellum mitochondria (DOX+SED *vs*. SAL+SED). Both exercise modalities prevented DOX-induced alterations in MDA levels and Mn-SOD activity in cerebellum, decreased MDA levels and increased Mn-SOD activity

in brain cortex mitochondria. Despite the unexpected lack of alterations in aconitase activity in both brain areas of DOX+SED group, an increase in DOX+TM brain cortex mitochondria was observed (DOX+TM vs. SAL+SED and DOX+SED). No alterations were observed in mitochondrial -SH content in both studied brain areas neither in aconitase activity in cerebellum.




**Chapter 3.5 - Figure 5.** Effects of exercise and sub-chronic DOX treatment on brain cortex and cerebellum mitochondrial (A) -SH content, (B) MDA levels and (C) aconitase and (D) Mn-SOD activities. Data are means ± SEM (n=6 per group). SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity $^{\#}$*vs.* SAL+SED, ***vs.* DOX+SED ($p \leq 0.05$).

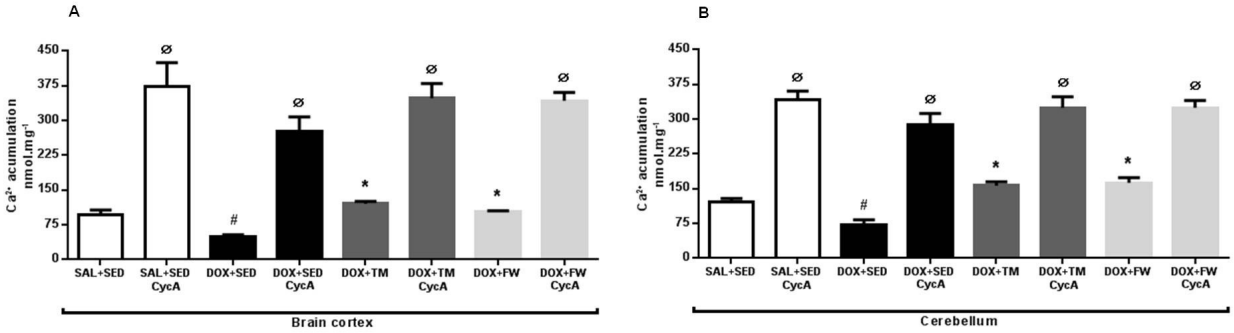
Immunoblotting analyses were performed to semi-quantify mitochondrial protein carbonylation as well as proteins involved in the modulation of ROS production. As shown in Figure 6, DOX increased the amount of carbonylated proteins, the p66shc(ser$^{36}$)/p66shc ratio and decreased SIRT3 and UCP2 protein content in cerebellum and brain cortex mitochondria (DOX+SED *vs.* SAL+SED). Both exercise modalities mitigated DOX-induced alterations in the mentioned markers (DOX+TM and DOX+FW *vs.* DOX+SED).




**Chapter 3.5 - Figure 6.** Effect of exercise and DOX treatment on brain cortex and cerebellum mitochondrial (A) carbonyls (B) SIRT3 (C) UCP2 (D) tissue p66shc (E) p66shc(pSer$^{36}$) and (F) p66shc(pSer$^{36}$)/p66shc ratio. Typical immunoblots are presented below the histograms. Data are means ± SEM (n=4 per group). The results were expressed as percentage of SED group. SIRT3 and UCP2 contents were normalized against TOM20 content. TOM20 and β-actin were used for mitochondrial and tissue protein loading controls, respectively. SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. $^{\#}$*vs.* SAL+SED, ***vs.* DOX+SED (p≤0.05).

*Calcium-induced mitochondrial permeability transition*

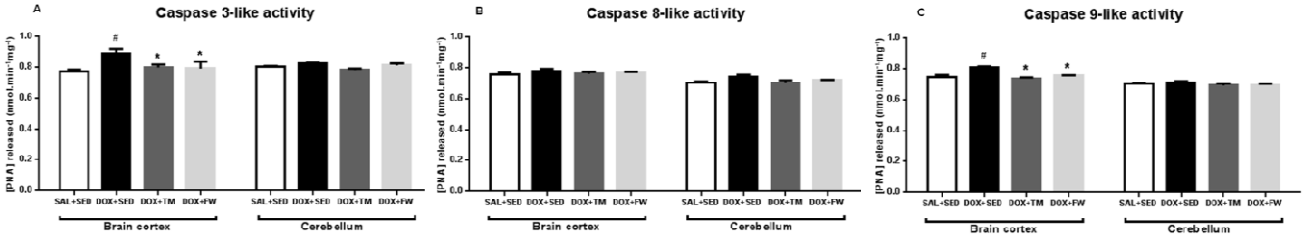
We next questioned whether exercise altered the amount of calcium needed to induce the mPTP, a condition associated with cell dysfunction and loss of viability (Bernardi, 2013). Figure 7 shows the maximum amount of brain cortex and cerebellum mitochondrial $Ca^{2+}$ accumulated until mPTP opening was observed as an irreversible drop in $\Delta\psi$. DOX+SED group accumulated significantly less $Ca^{2+}$ before mPTP induction in both studied brain areas (DOX+SED vs. SAL+SED). Both chronic exercise modalities reverted DOX-induced decreased capacity to accumulate $Ca^{2+}$ (DOX+TM and DOX+FW vs. DOX+SED).




**Chapter 3.5 - Figure 7.** Effects of exercise and sub-chronic DOX treatment on brain cortex and cerebellum mitochondria calcium-induced membrane depolarization. Data are means ± SEM for brain cortex and cerebellum mitochondria (0.8 mg protein/mL) obtained from different six mitochondrial preparations in each group. The figure shows the amount of calcium accumulated before the irreversible fall in $\Delta\psi$ and the respective negative control using cyclosporine-A (CycA, 1µM) to inhibit mPTP induction by $Ca^{2+}$. Succinate-energized mitochondria and a $TPP^+$ selective electrode at 30 ºC in a total volume of 1 mL were used. SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. $^{\#}$*vs.* SAL+SED, **vs.* DOX+SED, $^{\varnothing}$*vs.* without CycA ($p \leq 0.05$).

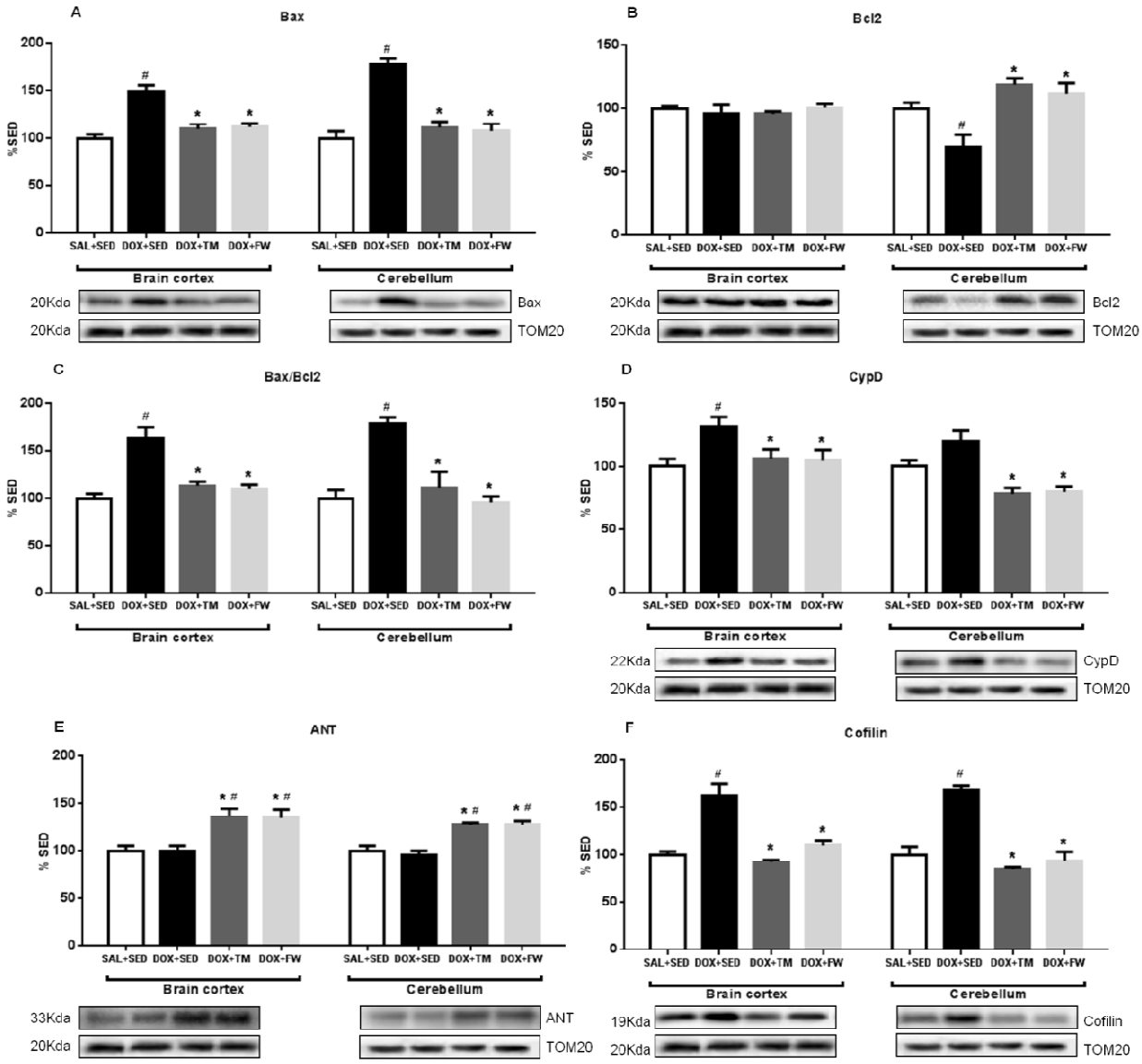
*Apoptotic signaling*

Caspases 3, 8 and 9-like activities were determined as markers of apoptotic signaling activation. As seen in Figure 8, increased caspases 3 and 9-like activities were observed in brain cortex from DOX+SED group (DOX+SED *vs.* SAL+SED) and both exercise modalities decreased these increments (DOX+TM and DOX+FW *vs.* DOX+SED). No treatment-related alterations were observed in cerebellum samples.




**Chapter 3.5 - Figure 8.** Effects of exercise and sub-chronic DOX treatment on brain cortex and cerebellum caspases 3, 8 and 9-like activities. Data are means ± SEM (n=6 per group) for brain cortex and cerebellum tissues. Caspase-like activity was measured colorimetrically by pNA release from specific caspase substrates. SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. $^{\#}$*vs.* SAL+SED, $^{*}$*vs.* DOX+SED ($p \leq 0.05$).

Immunoblotting analyses were performed to semi-quantify mitochondrial proteins involved in apoptotic signaling and/or mPTP regulation. ANT and cofilin content were also semi-quantified. As shown in Figure 9, DOX increased Bax/Bcl2 and cofilin in brain cortex and cerebellum and CypD in brain cortex mitochondria (DOX+SED *vs.* SAL+SED). Both exercise modalities restored Bax/Bcl2, CypD, cofilin and increased ANT content (DOX+TM and DOX+FW *vs.* DOX+SED).

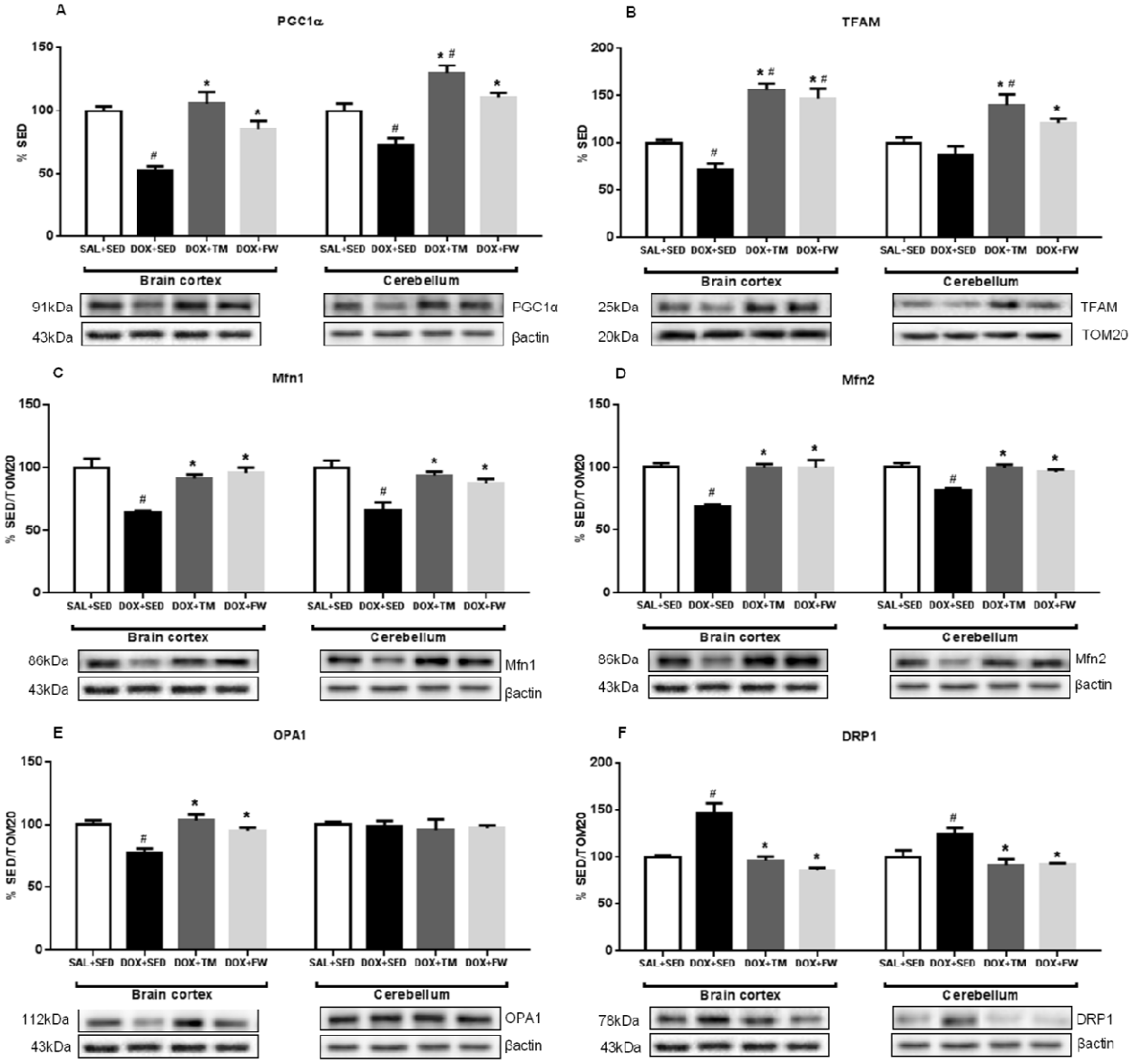



**Chapter 3.5 - Figure 9.** Effect of exercise and sub-chronic DOX treatment on brain cortex and cerebellum mitochondrial (A) Bax, (B) Bcl2, (C) Bax/Bcl2, (D) CypD (E) ANT and (F) cofilin. Typical immunoblots are presented below the histograms. Data are means ± SEM (n=4 per group). The results were expressed as percentage of SED group. TOM20 was used for mitochondrial protein loading control. SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. $^{\#}$*vs.* SAL+SED,$^{*}$*vs.* DOX+SED (p≤0.05).

*Mitochondrial biogenesis and dynamics signaling*

The content of proteins associated with mitochondrial dynamics including fusion, fission and biogenesis was evaluated by western blotting (Figure 10). DOX treatment decreased PGC1α, Mfn1 and 2 in brain cortex and cerebellum, decreased TFAM and OPA1 only in brain cortex and

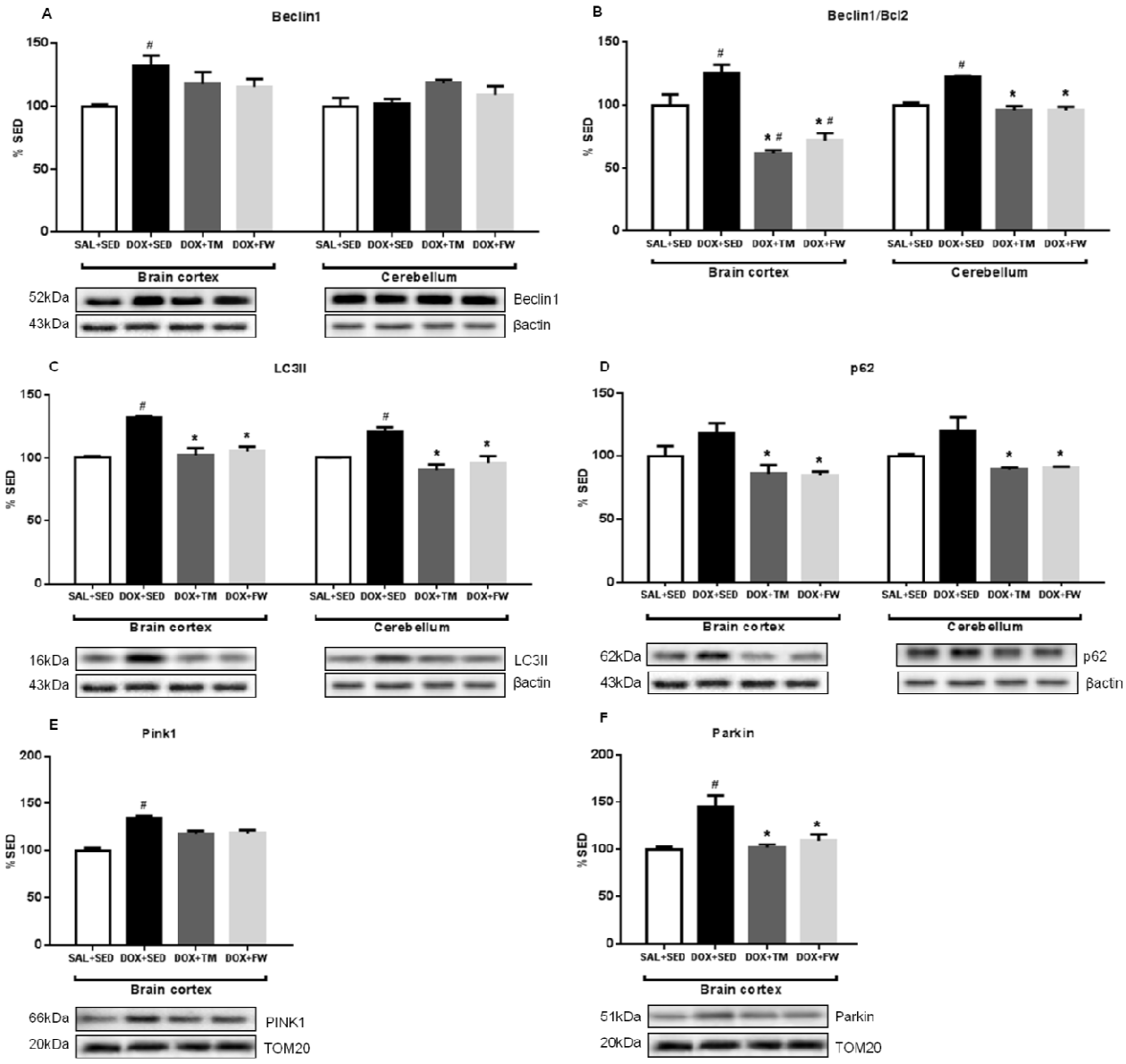
increased DRP1 in both brain areas (DOX+SED *vs.* SAL+SED). Both exercise models mitigated DOX-induced alterations in PGC1α and TFAM, Mfn1 and 2 and DRP1 levels in both brain areas. DOX-increased OPA1 levels in the brain cortex were mitigated by exercise (DOX+TM and DOX+FW *vs.* DOX+SED).




**Chapter 3.5 - Figure 10.** Effect of exercise and sub-chronic DOX treatment on (A) tissue PGC1α, (B) mitochondrial TFAM, (C) DRP1 (D) Mfn1, (E) Mfn2 and (F) OPA1. Typical immunoblots are presented below the histograms. Data are means ± SEM (n=4 per group). The results were expressed as percentage of SED group. DRP1, Mfn1, Mfn2 and OPA1 contents were normalized against TOM20. TOM20 and β-actin were used for mitochondrial and tissue protein loading controls, respectively. SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. $^{\#}$*vs.* SAL+SED,$^{*}$*vs.* DOX+SED ($p \leq 0.05$).

*Markers of autophagy and mitophagy signaling*

Markers of auto(mito)phagy signaling were assessed through the expression of Beclin1, a marker of the initiation of autophagosome formation; Beclin1-to-Bcl2 ratio, a marker of inhibition of Beclin-dependent autophagy; LC3, a marker of the elongation and formation of the autophagosome and PINK1, Parkin and p62, known proteins associated with mitophagy regulation (Ashrafi and Schwarz, 2013) (Figure. 11). As seen, sub-chronic DOX treatment increased the expression of Beclin/Bcl2 and LC3II in brain cortex and cerebellum; Parkin and Pink1 increases were only observed in brain cortex (DOX+SED *vs.* SAL+SED). Both exercise modalities mitigated DOX-induced alterations in autophagy related proteins in both studied brain areas as well as Parkin expression in brain cortex (DOX+TM and DOX+FW *vs.* DOX+SED). As can be inferred from the figure, we were not able to detect PINK and Parkin staining in membranes loaded with cerebellum mitochondria, despite previous reports showing evidence of these proteins in cerebellum (Batlevi and La Spada, 2011; Kageyama et al., 2012; Taymans et al., 2006).




**Chapter 3.5 - Figure 11.** Effect of exercise on brain cortex and cerebellum tissue (A) Beclin1, (B) Beclin1/Bcl2 (C) LC3II (D) p62 and mitochondrial (E) PINK1 and (F) Parkin. Typical immunoblots are presented below the histograms. Data are means ± SE (n=4 per group). The results were expressed as percentage of SED group. TOM20 and β-actin were used for mitochondrial and tissue protein loading controls, respectively. SAL+SED, saline sedentary; DOX+SED, DOX sedentary; DOX+TM, DOX endurance treadmill training; DOX+FW, DOX Free-wheel voluntary physical activity. # *vs.* SAL+SED, **vs.* DOX+SED ($p \leq 0.05$).

## Discussion

*Overview of findings*

Treatments with DOX often resulted in several central nervous system perturbations including brain toxicity with consequent cognitive and behavioral impairments (Christie et al., 2012; Merzoug et al., 2011). Therefore, strategies aiming at limiting these declines, including physical exercise are warranted. The experiments of the present study provide new and important information in what concerns the effects of sub-chronic DOX administration on markers of mitochondrial biogenesis, dynamics and autophagic quality control signaling in brain cortex and cerebellum and also show for the first time an inhibitory effect of the two studied chronic exercise modalities on the referred alterations caused by DOX.

We hypothesized that DOX administration would result in mitochondrial dysfunction, activation of apoptotic signaling, as well as in an increase in autophagy markers, and that chronic exercise would mitigate these effects. Specifically, our results support our hypothesis and show that, when performed before and during the course of sub-chronic DOX administration, physical exercise mitigated the behavioral alterations and the compromised mitochondrial bioenergetic, biogenesis, the enhanced oxidative damage and mPTP susceptibility with the consequent increased apoptotic signaling caused by DOX in brain cortex and cerebellum. Moreover, both exercise modalities modulated markers of mitochondrial dynamics and auto(mito)phagy, possibly contributing to the observed protective behavioral and mitochondrial phenotypes that were significantly affected by DOX treatment. Collectively, data reinforce the remodeling effects observed in mitochondria and whole brain cortex and cerebellum in an attempt to counteract the harmful consequences of DOX treatment, particularly related to alterations in markers of mitochondrial dynamics and quality control and that both modalities of chronic exercise positively altered the referred signaling processes.

*DOX impairs brain and cerebellum mitochondrial fitness*

DOX-based chemotherapy side effects have been associated with impairments of cognitive function (Jansen et al., 2008; Jansen et al., 2011) and motor disturbances (Tager et al., 2010), which may be, at least in part, associated with a pro-oxidant status and brain mitochondrial dysfunction. Indeed, the observed impaired behavioral performance of animals treated with DOX (Figure 1 and 2) was accompanied by alterations in brain cortex and cerebellum mitochondrial respiratory chain function (Figure 3), suggesting that one of the neurobiological mechanisms underlying DOX-induced cognitive alterations is mitochondrial dysfunction. In line with the present study, previous reports showed decreased brain mitochondrial RCR (when using complex I- but not complex II-linked substrates) (Tangpong et al., 2006; Tangpong et al., 2007) and a reduction of the mitochondrial membrane potential (Pal et al., 2012) after an acute DOX injection. An inhibitory effect on ADP-stimulated respiration was also observed after pre-incubation of brain mitochondria with DOX (Tokarska-Schlattner et al., 2007). However, a study using brain crude mitochondria did not found differences in respiratory states after sub-chronic DOX administration, which can be explained by differences in experimental design or protocol and/or be tissue specific (Cardoso et al., 2008).

The impairment in the expression of electron transport chain complexes I and V (and III only in cerebellum) subunits could have contributed, at least partially, to the observed decline in brain and cerebellum mitochondrial activity in DOX treated animals (Figure 4). Additionally, as increased oxidative damage is known to be implicated in OXPHOS functionality, the observed pro-oxidant status induced by sub-chronic DOX treatment (Figure 5 and 6) could also be associated with decreased activity, content and/or organization of OXPHOS complexes or other proteins of the phosphorylation system (Pereira et al., 2011). Although enhanced lipid peroxidation and decreased Mn-SOD activity were only seen in cerebellum mitochondria from DOX-treated animals (Figure 5), increased protein carbonylation and p66shc(ser$^{36}$)/p66shc and decreased SIRT3 and UCP2 expression observed in cerebellum and brain cortex mitochondria (Figure 6) suggest a role of oxidative stress in DOX-induced brain and cerebellum mitochondrial damage. Previous studies also reinforced the idea that DOX alters brain mitochondrial redox homeostasis as seen by Mn-

SOD inactivation and reduced expression (Tangpong et al., 2007), enhanced ROS production, increased MDA levels and protein carbonylation and down-regulating other antioxidant enzymes (Pal et al., 2012) after acute DOX treatment. Decreased GSH content and aconitase activity and increased TBARS were also reported in brain mitochondria after sub-chronic DOX treatment (Cardoso et al., 2008). Hippocampal cell cultures incubated with DOX showed increased superoxide and hydrogen peroxide production resulting in increased lipid peroxidation (Howard et al., 2002), suggesting a clear role of oxidative stress on DOX-induced neurotoxic effects.

Enhanced oxidative stress and calcium overload are also known to influence mPTP susceptibility with consequent activation of mitochondrial-driven apoptosis (Norenberg and Rao, 2007; Toman and Fiskum, 2011). Indeed, DOX treatment increases the susceptibility of brain mitochondria to undergo mPTP opening (Cardoso et al., 2008) with consequent activation of apoptotic signaling cascades, including the release of enclosed pro-apoptotic proteins to the cytoplasm, thus contributing to trigger cell death through caspase signaling (Pal et al., 2012; Tangpong et al., 2006). Accordingly, brain and cerebellum mitochondria from DOX-treated animals showed an increased mPTP opening susceptibility and apoptotic signaling seen by Bax/Blc2 and cofilin and by CypD, caspase 3 and 9-like activity only in brain mitochondria (Figure 7, 8 and 9).

Mitochondrial quality control is maintained by a highly dynamic equilibrium between fusion and fission, segregating dysfunctional from healthy mitochondria, which are then targeted for disposal and recycling by mitochondrial autophagy (Gottlieb and Carreira, 2010; Twig and Shirihai, 2011). These processes, together with mitochondrial biogenesis, provide a highly efficient energy transducing machinery necessary for cell adaptation and survival. Indeed, the decreased cortex and cerebellum mitochondrial function in DOX-treated animals was accompanied by decreased biogenesis-related proteins such as PGC1α and TFAM, as well as by alterations in mitochondrial dynamics signaling towards a more fragmented profile e.g., through the stimulation of mitochondrial fission and the inhibition of fusion signaling (Figure 11), although we cannot confirm whether morphologically this effect was indeed observed. These alterations in the markers of dynamics signaling following DOX-treatment, possibly translating into changes in the architecture of mitochondrial population in both studied brain areas, were accompanied by an increased

auto(mito)phagy signaling, as seen by Beclin/Bcl2 and LC3II. Increased Parkin and Pink1 was only observed in brain cortex (Figure 12). For the first time, the alterations reported here in these master molecules of intercellular signaling pathways caused by DOX administration are consistent with other pathological stimuli such as Aβ peptide-induced Alzheimer disease, in which neuronal cells are exposed to toxic stimuli (Shearzadeh et al 2014). Under these harmful circumstances, autophagic-lysossomal pathways target misfolded and aggregated proteins as well as dysfunctional mitochondria acting as micro and macro quality control mechanisms of organelles and proteins with aberrant structure and function in a high-energy dependent tissue such as brain. Moreover, under toxic stressful conditions, the loss of mitochondrial connectivity augment the sensitivity to apoptotic stimuli and/or can facilitate mitochondrial autophagy (Kubli and Gustafsson, 2012b). The present data suggest that apoptosis and autophagy may act synergistically in the regulation of brain and cerebellum mitochondrial and cellular death pathways in an attempt to segregate and remove damaged mitochondria.

*Cross tolerance effect of exercise against DOX-induced brain cortex and cerebellum mitochondrial alterations*

Patients undergoing chemotherapy treatments experience severe fatigue or exercise intolerance that may severely limit their ability to follow exercise regimens (Lucia et al., 2003). Considering this limitation and that exercise regimens with distinct intensity, duration and load lead to different physiological, biochemical and functional adaptations (Camiletti-Moiron et al., 2013; Klaus and Amrein, 2012; Ogonovszky et al., 2005), the present experimental setup included two models of chronic exercise, TM and FW.

We previously reported that physical exercise improves brain cortex and cerebellum mitochondrial function, increasing the resistance to oxidative stress and apoptosis, with favourable alterations in mitochondrial biogenesis, dynamics and autophagy signaling, contributing to increased mitochondrial plasticity and leading to a more resistant phenotype (Marques-Aleixo et al., c). Another novel finding of the present study was that both modalities of physical exercise markedly

mitigated the previous mentioned alterations promoted by DOX treatment in brain cortex and cerebellum reported in sedentary group.

Besides mitochondrial function, including RCR and the lag phase (Figure 3), both exercise modalities were able to restore the expression of OXPHOS complexes I, III and V subunits (Figure 4) and mitochondrial redox homeostasis (Figures 5 and 6) that were decreased by DOX treatment in sedentary animals. As previous suggested, physical exercise improves brain redox balance by decreasing free radical production and/or by up-regulating antioxidant defense systems (Boveris and Navarro, 2008b; Camiletti-Moiron et al., 2013; Marques-Aleixo et al., 2012), which may contribute to counteract the observed mitochondrial dysfunction imposed by DOX. Moreover, increased SIRT3 and p66shc(ser$^{36}$)/p66shc expression as well as decreased lipid peroxidation, enhanced Mn-SOD activity and ANT expression were observed in exercised groups, exceeding the sedentary animals without treatment. These results suggest that brain oxidative status is highly modulated by exercise and the extent of redox-related benefits of both exercise modalities are markedly significant even in DOX-treated animals, possibly favoring a more resistant mitochondrial phenotype.

As mentioned, DOX-induces increased mPTP susceptibility and apoptotic signaling. The present study showed for the first time that physical exercise performed during sub-chronic DOX treatment mitigates the observed increased brain cortex and cerebellum susceptibility to mPTP and apoptotic signaling (Figure 7) as seen by decreased Bax-to-Bcl-2 ratio, CypD and cofilin as well as caspases 3 and 9 like-activities in brain cortex (Figure 8 and 9). Previous data suggested that the decrease of mitochondrial-driven apoptosis in brain cortex is more effective with endurance training than with voluntary exercise (Marques-Aleixo et al., c). However, under the cell stressful conditions caused by DOX treatment, both exercise modalities seem to exert an anti-apoptotic effect sufficient to counteract DOX pro-apoptotic signaling in brain cortex and cerebellum.

Brain mitochondrial biogenesis signaling is modulated by exercise (Bayod et al., 2011; Marques-Aleixo et al., c; Steiner et al., 2011), thus representing a target for favorable adaptations against severe stress. Accordingly, our results showed that increased PCG1α and TFAM resulting from

both exercise modalities counteracted DOX suppression of mitochondrial biogenesis signaling seen in the sedentary group (Figure 10). Moreover, an increased content in dysfunctional mitochondria shift their dynamics toward fission (Santos et al., 2010), which agrees with our data as mitochondrial function and dynamic was severely affected by DOX treatment. Both exercise modalities performed before and during the course of DOX treatment decreased DRP1 levels and increased OPA1 in cerebellum and increased Mfn1/2 in both studied brain areas (Figure 10), which suggest that exercise favorably modulated mitochondrial dynamic balance and mitigates the shift toward fission pattern imposed by sub-chronic DOX administration.

Quality control and mitochondrial dynamics systems are tightly coupled and their proper balance is crucial for cellular adaptation and stress resistance. Therefore, besides biogenesis and dynamics, both exercise modalities also return autophagy signaling back to the levels of sedentary non-treated group, as seen by Beclin and LC3II expression; and Parkin (Figure 11). Moreover, exercise decreased p62 content in both studied brain areas even in the absence of significant alterations caused by DOX (in sedentary group).

As alterations in the selected markers of mitochondrial biogenesis were observed after DOX treatment and exercise, but variations in OXPHOS, SIRT3, UCP2, DRP1, Mfn1/2 and OPA1 content, determined in tissue homogenate and further corrected for TOM20 as a measure of mitochondrial content, still persist, this could reflect an impairment caused by DOX treatment, which was mitigated following exercise, of mitochondrial quality signaling pathways where these proteins are involved, regardless possible increase in mitochondrial mass. Together, these findings suggest that both exercise models performed during treatment reverted behavioral impairments caused by DOX; also, exercise promotes an increase in brain cortex and cerebellum mitochondrial function, mitigating increased apoptotic and/or autophagy signaling activation and possibly contributing to bring back mitochondrial network to a healthier phenotype.

*Study relevance and concluding remarks*

The present study evidenced that brain and cerebellum mitocondrial dysfunction induced by sub-chronic DOX treatment is similar to the described pathways known to contribute to the onset and/or the progression of the most known types of neurodegenerative diseases, including the involvement of mPTP and increased oxidative stress, inappropriate regulation of apoptosis and autophagy systems and excessive mitochondrial fragmentation (Ghavami et al., 2014; Reddy et al., 2011; Toman and Fiskum, 2011). The study provides for the first time evidence that sub-chronic DOX treatment alters biomolecular markers of mitochondrial biogenesis, fusion and fission and autophagy signaling in brain cortex and cerebellum. Moreover, we also demonstrated that chronic physical exercise performed before and during the course of DOX administration mitigated many of the DOX-induced behavioral changes, mitochondrial functional impairments, including mPTP susceptibility and apoptotic signaling. We further provide evidence that the referred improved phenotype was accompanied by alterations in mitochondrial biogenesis, dynamics and autophagic signaling markers. Finally, the results obtained evidenced that exercise performed during the course of sub-chronic DOX treatment is a reliable strategy to attenuate DOX negative effects in central nervous system, in a similar manner to what has been described for cardiac tissue (Ascensao et al., 2005b; Ascensao et al., 2005a; Ascensao et al., 2012).

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# 4.

# GENERAL DISCUSSION

**Setup justification and overview of findings**

Although the mechanisms underlying DOX-toxicity are not still completely determined, it is accepted that the antitumor activity is independent from the toxicity inflicted to non-target tissues, including the heart and the brain. For instance, several studies in patients undergoing DOX-based chemotherapy have reported persistent changes in cognitive functions, commonly referred to as "chemo brain", sometimes lasting years after cessation of chemotherapy (Aluise et al., 2010). Indeed, DOX treatment is followed by a dose-dependent toxicity that is also dependent on the used schedule. One of the most common approaches to monitor cardiac DOX toxicity is to continuously assess cardiac function, since the endomyocardiac biopsy is invasive and obviously impractical (Singal and Iliskovic, 1998). Therefore, animal models are frequently used, not only to assess cardiac (dys)function, but also to analyze the underlines cellular and molecular mechanisms associated with DOX cardiac side effects, thus allowing a more effective development of strategies able to counteract DOX toxicity.

Although several drug schedules have been used to investigate both sub-chronic and acute DOX toxicity, our objective was not to induce a substantial damage to the studied organs, but to mimic biochemical and functional alterations that usually resemble those seen in DOX-treated patients rather than to induce substantial damage to the studied organs. Therefore, animals received low sub-clinical dosages of DOX as the duration of the treatment, the time schedule and the method of administration were as closer as possible compared to a clinical situation and in agreement with the maximal dosage allowed in human chemotherapy (Carvalho et al., 2014; Pereira et al., 2012).

As previous mentioned, although the mechanisms behind heart and brain mitochondrial DOX-toxicity seem to be different, DOX-induced toxicity leads to declined cardiac and brain function, which may be caused, at least in part, by progressive bioenergetic collapse involving mitochondria in the process. Importantly, the sub-chronic DOX treatment

administrated in the present study was previously used to investigate DOX-related mitochondrinopathy (Oliveira and Wallace, 2006; Oliveira et al., 2006; Serrano et al., 1999). The involvement of DOX treatment in alterations of the mitochondrial function as been discussed along the general introduction, theoretical and experimental sections of the present dissertation. Generally, cardiac and brain defective mitochondrial function in DOX-treated animals were previously reported, including inhibition of oxidative phosphorylation, decreased calcium-loading capacity, stimulation of ROS production as well as increased apoptotic signaling (Cardoso et al., 2008; Carvalho et al., 2009; Pal et al., 2012; Sardao et al., 2008; Tangpong et al., 2006; Tangpong et al., 2007; Tokarska-Schlattner et al., 2007; Wallace, 2003). Moreover, despite the previous studied side effects of DOX treatment on alterations of heart mitochondrial dynamics and on markers of autophagy and mitophagy (Pereira et al., 2011), to our knowledge, no previous studies have addressed possible alterations induced by chronic exercise on these mitochondrial quality control markers on heart, brain cortex or cerebellum in the context of DOX toxicity.

Regarding the sedentary groups in the studies comprised in the present dissertation, data agree with previous reports, in which a 7-wk sub-chronic DOX administration protocol resulted in: (i) impaired behavioral performance; (ii) altered heart mitochondrial morphology; (iii) decreased heart and brain cortex mitochondrial respiratory activity; (iv) decreased heart OXPHOS complexes activity and organization, and brain cortex and cerebellum OXPHOS subunits content; (v) decreased mitochondrial biogenesis signaling; (vi) increased oxidative stress; (vii) increased mPTP susceptibility; (viii) augmented apoptotic signaling; (ix) increased fission and decreased fusion signaling markers thus impairing mitochondrial dynamics; and (x) increased markers of autophagy and mitophagy (Chapters 3.2, 3.3 and 3.5).

Importantly, although frequently suggested that DOX-toxicity particularly affects the heart, in the set of studies that comprises this dissertation, no apparent distinction regarding DOX-induced mitochondrial toxicity between heart, brain and cerebellum mitochondria were

observed. Therefore, in the present used model and set-up one cannot suggest that heart mitochondria are more affected by DOX treatment than brain-derived ones.

Therapies aiming at preserving mitochondrial function after and during DOX treatment are imperative. Despite several attempts, it is fairly consensual that most of protective strategies have not achieved full successful outcomes. In this context, research on the role of chronic exercise and physical activity, not only as a useful strategy on cancer prevention, but also on the mitigation of chemotherapeutics side-effects in non-target tissues such as the heart has been a topic of considerable clinical interest over the last years (Ascensao et al., 2011b; Ascensao et al., 2012; Gibson et al., 2013; Hydock et al., 2012; Jensen et al., 2013).

Based on a wealth of evidence suggesting that regular physical activity of moderate intensity is associated with decreased risk for some types of cancer, several studies proposed that physical training can alter biomarkers for cancer risk in susceptible individuals (Cummings et al., 2009; Friedenreich and Orenstein, 2002; Harriss et al., 2009). Moreover, the role of exercise training following the diagnosis of the disease, either during treatment or after the cessation of chemotherapy has also been investigated. It has been suggested that exercise is a feasible and safe supportive intervention, which increases the quality of life and reduces fatigue indexes, increasing also exercise tolerance and muscular strength in cancer populations (Doyle et al., 2006; Jones and Demark-Wahnefried, 2006). An issue that still lacks clarification is if exercise interferes with the completion rates or efficacy of cancer treatment. In one of the few works available, Jones et al (2005) reported that 8 weeks of treadmill running did not interfere with the efficacy of chronic DOX administration in cancer treatment on female mice transplanted with MDA-MB-231 breast xenografts.

Both epidemiological and animal studies reported advantages of exercise against chemotherapy side effects based on hemodynamic and behavioral evidences (Braam et al., 2013; Dolinsky et al., 2013; Jensen et al., 2013; Mishra et al., 2012a, 2012b). However, despite the fully identification of mechanisms in non-target tissues is urgently required,

increasing evidence behind the referred protection induced by exercise has suggested a central role for mitochondria in this cross-tolerance phenomenon (Ascensao et al., 2005a; Ascensao et al., 2005b; Ascensao et al., 2006a; Ascensao et al., 2006b; Ascensao et al., 2006c; Ascensao et al., 2011a; Ascensao et al., 2012). In the particular context of DOX-toxicity, physical exercise of different types and characteristics, including acute bouts of treadmill running, short and long term forced endurance training in the form of treadmill running or swimming as well as voluntary physical activity has been studied (Ascensao et al., 2005a; Ascensao et al., 2011a; Jensen et al., 2013; Kavazis et al., 2010). One of the main justifications for the use of this non-pharmacological strategy to prevent or treat DOX-side effect lies on the fact that both DOX and exercise interfere with mitochondrial function and plasticity. This justifies the reasonable interest in the different mechanisms and targets, in which these organelles could be at least in some extent, central to explain the hopeful beneficial outcome of the combination.

One of the current major concerns regarding the use of physical exercise in patients receiving anti-cancer therapies is related to exercise characteristics (i.e., intensity, duration frequency and the timing between exercise and the treatment) that are most advantageous to elevate important defense systems and thus, to antagonize the toxic effects caused by DOX treatment. Therefore, the experimental studies of the present work aimed to provide additional support on the effects of two different chronic exercise models with distinct duration and intensity characteristics (TM-treadmill endurance training and FW-voluntary physical activity) performed before and during sub-chronic DOX treatment, against the previous mentioned heart and brain mitochondrial dysfunction, seen as sensor of DOX toxicity.

Due to the lack of information on the effects of exercise as a possible modulator of brain mitochondrial function, namely mitochondrial dynamic and auto(mito)phagy signaling, we thought to examine the impact of the two different exercise models *per se* on brain cortex and cerebellum mitochondrial function with particular interest in the referred underexplored

processes. Generally, 12 wk of treadmill exercise training or voluntary physical exercise resulted in: (i) improved behavioral performance; (ii) increased on brain cortex and cerebellum mitochondrial respiration; (iii) increased OXPHOS subunits content; (iv) increased mitochondrial biogenesis signaling; (v) decreased oxidative stress; (vi) decreased mPTP susceptibility and apoptotic signaling; (viii) increased fusion and decreased fission; and (ix) increased markers of autophagy and mitophagy (Chapter 3.4). Importantly, this study suggests that endurance training (TM) may lead to a higher antioxidant and anti-apoptotic potential, and regulates brain cortex autophagy signaling more efficiently than voluntary physical activity (FW).

The heart is one of the most studied tissues as targets of exercise-induced protection, including against DOX-induced toxicity, with several reports showing promising beneficial effects against DOX-induced mitochondrial dysfunction (Ascensao et al., 2011b; Ascensao et al., 2012; Dolinsky et al., 2013; Kavazis et al., 2010). However, the possible cardiac mitochondrial alterations induced by different exercise models performed during sub-chronic DOX treatment are still an underexplored research issue, being one of the aims of the present study. Additionally, the relevance of exercise as a strategy to counteract brain and cerebellum mitochondrial dysfunction induced by sub-chronic DOX treatment was never addressed so far.

The present studies demonstrated that both exercise modalities when performed before and during the course of sub-chronic DOX treatment might have a preventive role, mitigating the associated DOX impairments on heart, brain and cerebellum mitochondria. Indeed, when compared with sedentary, animals engaged in chronic exercise protocols and treated with DOX showed improvements on: (i) behavioral performance; (ii) heart mitochondrial morphology; (iii) heart, brain and cerebellum mitochondrial respiratory activity; (iv) heart OXPHOS complexes activity and organization as well as subunits content in the heart, brain cortex and cerebellum; (v) markers of mitochondrial biogenesis; (vi) threshold for mPTP

induction and decreased apoptotic signaling; and (vii) mitochondrial dynamic and auto(mito)phagy signaling regulation (Chapter 3.2, 3.3 and 3.5).

Collectively, these effects likely contribute to a protective phenotype induced by both modalities of chronic exercise and reinforce the role of exercise on heart, brain cortex and cerebellum mitochondria remodeling in an attempt to counteract the harmful consequences of DOX treatment. This protective effect observed with both exercise models was accompanied by the reestablishment of the alterations on behavioral tests and on heart mitochondrial morphology, anticipating an improved function of the studied organs

**Cross-tolerance effect of exercise against DOX-induced heart, brain cortex and cerebellum mitochondrial alterations**

Exercise, particularly chronic interventions, is known to induce cardioprotection against stressful conditions such as DOX treatment through positive mitochondrial-mediated effects (Ascensao et al., 2011b; Ascensao et al., 2012). Previous work from our lab have suggested that the benefits of exercise on the cardiovascular system of DOX-treated rats include the protection of the heart tissue and mitochondria (Ascensao et al., 2005a; Ascensao et al., 2005b; Ascensao et al., 2006b; Ascensao et al., 2006a; Ascensao et al., 2007; Ascensao et al., 2011b; Ascensao et al., 2011a; Ascensao et al., 2012), which is in agreement with the protective role of exercise on DOX-induced cardiotoxicity and cardiac dysfunction (Chicco et al., 2005; Chicco et al., 2006a; Chicco et al., 2006b; Kanter et al., 1985). These issues are critically reviewed in Chapter 2.1. Furthermore, the results here presented add new findings regarding the possible involvement of metabolic and cellular renewal signaling mechanisms altered by both DOX and exercise and by the combination treatment. These include the modulation of SIRT3 and p66Shc expression (Chapter 3.2), and mitochondrial quality control processes, including mitochondrial dynamic and auto(mito)phagy signaling (Chapter 3.3).

Whereas the knowledge about exercise as a strategy involved in the protection of brain mitochondrial function have been previously studied (detailed on Chapter 2.2), the effects of this non-pharmacological strategy in mitochondrial regulation of mPTP and consequent driven apoptotic signaling, mitochondrial dynamics and quality control further support mitochondria as a key target for exercise-induced neuroprotection (Chapter 3.4). Moreover, the role of exercise against DOX-induced brain and cerebellum mitochondrial dysfunction is explored for the first time in the present work (Chapter 3.5).

**Mitochondrial function, oxidative stress and apoptosis**

Mitochondria are organelles devoted to energy production being OXPHOS system the major source of high-energy compounds in the cell. Generally, the electrons provided by the reducing equivalents such as NADH and succinate are transferred to oxygen through the electron transport chain carriers, leading to the formation of water, being this process coupled with the formation of a proton gradient across the inner membrane. This voltage gradient is used by ATP synthase to generate ATP. However, the extent of mitochondrial mediated mechanisms are far beyond and these highly plastic and dynamic organelles have been implicated in the regulation of several physiological processes involving cell life and death, signaling and renewal.

Reactive oxygen species (ROS) are produced as an inevitable by-product of mitocondrial respiration. These reactive species may play a dual role, deleterious or beneficial, depending on their levels and with important impact on mitochondrial metabolism and function. Low/moderate production of ROS by mitochondria may play a key role as regulator of several enzymes of the tricarboxylic acid (TCA) cycle and OXPHOS reactions as well as on the activation of signaling pathways crucial for the proper functioning of several redox-sensitive cascades, including inflammation and apoptosis. It is known that under abnormal stressful conditions, including cardiovascular and neurodegenerative diseases, the balance

between ROS production and the ability of the antioxidants systems became compromised for neutralizing ROS generated above a healthy threshold (Mailloux et al., 2013). Additionally, it is nowadays accepted that mitochondria are highly sensitive to oxidation and relatively reducing membrane-limited organelles, allowing a low-flux redox signaling and control to occur in association with the plasma membrane, cytoplasm, nucleus and extracellular space. Such compartmentation permit the maintenance of redox states at different steady-state potentials in different subcellular compartments, the disequilibrium of major thiol/disulphide couples in individual subcellular compartments and the evidence for redox signalling via discrete mechanisms in the different compartments. Therefore, the maintenance of redox states at stable, non-equilibrium conditions in different subcellular compartments, including mitochondria, might represent a vital aspect of biologic organization and function (Jones and Go, 2010).

Particularly when one consider the former scenario, in which a proper balance between ROS and antioxidants are considerably compromised, mitochondrial nucleic acids, lipid membranes and proteins may be affected, with consequent loss of mitochondrial integrity and function. Moreover, severe altered redox status and mitochondrial malfunction are believed to interfere with calcium homeostasis leading to an increased mitochondrial susceptibility to calcium induced mPTP and depolarization, liberating signaling proteins from mitochondria to the cytoplasm that in turn activate apoptotic cell death (Vercesi et al., 2006).

As previous mentioned, compromised oxidative phosphorylation and inhibition of ATP synthesis along with increased oxidative stress and apoptotic signaling are known consequences of DOX toxicity. Moderate endurance training mitigated the increased oxidative stress and damage, and apoptotic signaling, as well as the alterations in cardiac mitochondrial morphology caused by the administration of an acute single-dose of DOX (Ascensao et al., 2005b; Ascensao et al., 2006b; Ascensao et al., 2006a; Ascensao et al., 2007). Accordingly, in the present work we showed that exercise afforded protection against

sub-chronic DOX treatment induced heart mitochondrial bioenergetics dysfunction, including decreased functionality of OXPHOS system, RCR and ΔΨ (Chapter 3.2 and 3.5) being this improved mitochondrial ability to produce energy dependent in part of the electron transport chain (ETC) flux. As observed in study 1 (Chapter 3.2), the impairment in heart mitochondrial complex I subunit content and activity (classically associated with DOX-induced cardiotoxicity) and the activation of a serious of cascade events including, stimulation of ROS generation, the interference with mitochondrial calcium regulation, induction of the mPTP and apoptosis (Wallace, 2007) were mitigated by both exercise models (Chapter 3.2 and 3.3). A possible explanation relies on exercise prevention of DOX inactivation of dehydrogenases, providing an increased amounts of reducing equivalents to the mitochondrial ETC and preserving mitochondrial complex activity, namely complex I and V. Moreover, the possible up-regulation of Krebs cycle enzymes induced by exercise (Holloszy et al., 1970) might have a role on the afforded protection against DOX cardiotoxicity. The metabolic regulation, combined with alterations on the pro-oxidant environment induced by DOX, might interfere with cellular calcium homeostasis, contributing to the decreased susceptibility to mPTP induction of exercised animals. Actually, the increased levels of SIRT3 observed in the heart and brain tissues from DOX exercised animals could prevent DOX-induced apoptosis by lowering reactive oxygen species and inhibiting mPTP (Kincaid and Bossy-Wetzel, 2013). SIRT3 is a mitochondrial stress sensor that modulates the activity of several others mitochondrial proteins involved in metabolism through its deacetylase activity and has been suggested to be evolved multiple mechanisms to reduce ROS (Bell and Guarente, 2011). For instances, SIRT3 was shown to deacetylate subunits of complex I (Ahn et al., 2008) and complex II (Cimen et al., 2010), and SIRT3 deficiency may result in reduced efficiency of ECT, thereby increasing the probability of electrons being transferred to molecular oxygen to generate ROS in the process of ATP production. Indeed, $SIRT3^{-/-}$ cells are known to decrease steady-state ATP levels, which may result from a defect in deacetylating electron transport chain components (Ahn et al., 2008). SIRT3 also deacetylates and activates the TCA cycle enzyme isocitrate dehydrogenase 2 (IDH2) (Bell and Guarente, 2011; Sack,

2011), which elevated NADPH necessary for glutathione reductase, thereby converting oxidized glutathione (GSSG) into reduced glutathione (GSH), the electron donor used by mitochondrial glutathione peroxidase (GPX) to detoxify ROS. Additionally, SIRT3 deacetylates and directly activates Mn-SOD in the mitochondrial matrix (Tao et al., 2010), which results in increased specific and enhanced scavenging activity of ROS. According to the results of the present thesis, exercise was able to mitigate DOX-induced decreased the content in mitochondrial SIRT3 and increased acetylation levels (in heart mitochondria), which is in agreement with decreased complex I content, increased oxidative damage and decreased Mn-SOD activity (Chapter 3.2 and 3.5).

In addition, oxidative stress is known to influence mPTP susceptibility with consequent activation of mitochondrial-driven apoptosis (Norenberg and Rao, 2007; Toman and Fiskum, 2011) and it has been reported that SIRT3 activity along with ROS suppression; also inhibits mPTP opening-driven apoptosis. Indeed, Sundaresan et al. (2008) suggested that SIRT3 hinder the translocation of Bax to mitochondria by promoting the sequestration of this pro-apoptotic protein by Ku70 within the cytoplasm. Others showed that SIRT3 stimulates the detachment of hexokinase II from the outer mitochondrial membrane by deacetylating cyclophilin D (CypD - regulatory component of the mPTP), a step necessary for stimulating oxidative phosphorylation (Shulga et al., 2010). Hafner et al. (2010) proposed that SIRT3 deacetylates CypD on lysine 166, adjacent to the binding site of cyclosporine A (a CypD inhibitor) indicating that by regulating the mPTP, SIRT3 ensures metabolic and cell survival signals within the mitochondrial.

Given these perspectives, the decreased of SIRT3 and concomitant increased sensitivity to mPTP formation and increased ROS production in DOX sedentary group possibly resulted in increased levels of mitochondrial acetylated proteins (Chapter 3.2), ultimately leading to mitochondrial dysfunction and apoptosis. In fact, mPTP is a critical event in DOX cardiotoxicity and its inhibition or attenuation induced by exercise possibly translated into amelioration of mitochondrial function and a declined release of mitochondrial enclosed pro-

apoptotic proteins to the cytoplasm, thus contributing to decrease trigger cell death through activation of caspase signaling, as detailed in Chapter 3.3.

Although not evaluated in our studies, it is possible to hypothesize that DOX can modulate endogenous levels of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α) (Seruga et al., 2008; Tangpong et al., 2006). Mitochondria are one of the major target of inflammation-associated injury and therefore TNF-α induces pro-apoptotic effects by the activation of pro-caspase 8 and by the inhibition of the initial segment of the respiratory chain with consequent increases ROS production. It also affects other features of mitochondria functionality, including mitochondrial mPTP opening susceptibility, swelling of mitochondrial matrix, disruption of the outer mitochondrial membrane and cytochrome c release (for refs see Skulachev, 1999). It has been reported that TNF-α directly induces mitochondrial ROS production within cardiac myocytes, causes mitochondrial DNA damage and dysfunction (Suematsu et al., 2003) and potentiates the apoptotic effect of DOX in cardiomyocytes (Chiosi et al., 2007). Moreover, as previous mentioned, it is generally accepted that the blood brain barrier prevent the passage of DOX into the central nervous system, including brain cortex (Ohnishi et al., 1995) and the hippocampus (Bigotte and Olsson, 1982b). Therefore, one of the mechanisms underlying DOX neurotoxic effects has been associated with the ability of TNFα to migrate across the blood brain barrier and stimulate locally its production (Seruga et al., 2008; Tangpong et al., 2006). This brain pro-inflammatory environment caused by DOX inhibit the respiratory chain with consequent increases ROS production by mitochondria (Tangpong et al., 2007) eventually leading to mitochondrial dysfunction and to the activation of mitochondria-driven apoptotic cell death pathways, (Joshi et al., 2010; Pal et al., 2012; Tangpong et al., 2006; Tangpong et al., 2007) at least in part dependent on increased susceptibility to mPTP opening (Cardoso et al., 2008).

An exercise-decreased TNF-α levels has been previously reported in plasma (Moon et al., 2012) and whole brain (E et al., 2013; Patel and White, 2013). This sustain that exercise may

reduce brain inflammation set points, with possible implications on brain mitochondrial responses. Indeed, as detailed in Chapter 2.2, physical exercise is involved with a series of adaptations usually leading to the upregulation of brain protective mechanisms related to improvement in mitochondrial activity, antioxidant networks and decreased apoptotic signaling. Accordingly, in study 3 (Chapter 3.4), important benefits of both exercise models on brain cortex and cerebellum mitochondrial function are reported, possible in an attempt to sustain increased metabolic demands. These favorable adaptations lead to decreased oxidative stress and mPTP susceptibility with consequent decreased apoptotic signaling. This study sustain the previous idea explained in the theoretical background (Chapter 2.2) that the multiple health benefits of exercise might include protection against brain alterations associated with mitochondrial impairments as, for instance, the aging process and the onset of neurodegenerative disorders. Moreover, considering that DOX can result in brain and cerebellum mitochondrial impairment with consequent loss of organelle and tissue functions, which may contribute to the cognitive dysfunction observed in a significant fraction of patients undergoing chemotherapy (Aluise et al., 2010), the relevance of different exercise regimens as tools to prevent and attenuate the deleterious effects characterizing DOX-induced brain toxicity were addressed in the study 4 (Chapter 3.5).

Similar to the protective phenotype provided by both exercise models on heart mitochondria from animals sub-chronically treated with DOX, we here showed that the protection against DOX-induced brain cortex and cerebellum afforded by exercise relies, at least in part, on the ability to improve mitochondrial metabolism. Endurance training and voluntary physical activity counteracted the previous mentioned side effects of DOX treatment including brain mitochondria bioenergetics dysfunction, increased oxidative and mPTP susceptibility, leading to a phenotype more resistance to apoptosis (Chapter 3.5). This suggests that physical exercise might be used as a strategy to restore brain and cerebellum cellular homeostasis reducing the extent of mitochondrial dysfunction and thus enhance neurological outcome following DOX treatment.

**Mitochondrial dynamics and quality control**

As previously mentioned, mitochondria are crucial organelles for energy production and regulation of cell signaling (Ernster and Schatz, 1981); however, mitochondria are also the major source of ROS that may compromise cellular and mitochondrial integrity amplifying apoptotic signaling (Green and Kroemer, 2004). Under adaptive physiological or stressful deleterious conditions, mechanisms of mitochondrial quality control have evolved to avoid cell damage and to maintain the overall fitness of the cell. Indeed, mitochondria are dynamic organelles whose morphology, shape and distribution are in constant adjustment. Biogenesis, dynamic and autophagy are tightly interconnected processes, in order to meet cellular requirements (Gomes and Scorrano, 2013).

Mitochondrial dynamics is regulated by several mitochondrial-shaping proteins and depends on the balance between two processes that are continuously occurring: fission and fusion. The outer mitochondrial membrane protein fission 1 (Fis1) and the GTPase DRP1 are the main elements of the mitochondrial fission machinery (James et al., 2003; Smirnova et al., 2001). Generally, when the fission process occurs, cytosolic DRP1 is recruited into the mitochondrial fission *foci* where it interacts with outer membrane receptors (including Fis1, mitochondrial fission factor (Mff) and mitochondrial dynamics proteins (MiD49/51)). Then, Drp1 oligomerises into spirals around mitochondria at future fission sites, constricting and subsequently dividing the mitochondrion through GTP hydrolysis (Elgass et al., 2013; Serasinghe and Yoon, 2008; Yoon et al., 2003). In contrast, mitochondrial fusion promotes the assembly of individual mitochondria that combine their membranes in a process controlled by Mfn1/2 and OPA1 (Chen et al., 2003). These proteins also have been implicated in diverse cellular functions, including generation of reactive oxygen species (Yu et al., 2006), calcium signaling (Szabadkai et al., 2004), formation of dendritic spines (Li et al., 2004), migration of lymphocytes (Campello et al., 2006), cell cycle (Mitra et al., 2009) and even apoptosis (Frank et al., 2001; Scorrano et al., 2002). For instances, besides its role in mitochondrial fusion, OPA1 is also essential for inner mitochondrial membrane structure

(Frezza et al., 2006; Olichon et al., 2003) and for apoptosis, by altering the size of the cristae junctions (Cipolat et al., 2006; Frezza et al., 2006) that expand to allow cytochrome c release during cell death (Scorrano et al., 2002).

A growing set of evidence is implying mitochondrial morphological changes in the course of another type of cell response, autophagy (Gomes and Scorrano, 2013; Twig and Shirihai, 2011). During autophagy, organelles and parts of cytoplasm are sequestered and subsequently delivered to lysosomes for hydrolysis, in certain conditions, when is important to generate amino acids, and ultimately fuel the tricarboxylic acids cycle to maintain ATP production (Levine and Klionsky, 2004) or to selectively remove damaged or unneeded organelles, including mitochondria (Gomes and Scorrano, 2013; Twig and Shirihai, 2011; Youle and Narendra, 2011). Autophagy has dual functions: under physiological conditions, autophagy is essential for optimal cellular function and survival; however, under pathological conditions, enhanced autophagy has been shown to have a protective role in some cardiac disease states, such as ischemic preconditioning, while it is harmful in others, including cardiac hypertrophy (Sciarretta et al., 2012). Indeed, mitochondrial damage can induce mitophagy and inhibition of autophagy reducing mitochondrial respiration (Twig et al., 2008).

Activation of the autophagic signaling pathway begins with the formation of an isolation membrane, which is the first step of autophagosome formation, in which Beclin1 seems to play an important role by mediating the localization of autophagy proteins to the isolation membrane (Gustafsson and Gottlieb, 2008). The initiation of autophagosome formation is followed by the expansion and maturation of the autophagosome. This process requires the interaction of several autophagy-related proteins, including LC3 (Kubli and Gustafsson, 2012). Endogenous PINK1 is rapidly degraded in healthy mitochondria, whereas it accumulates on the surface of damaged, depolarized organelles and selectively recruits PARKIN from the cytosol specifically to the damaged mitochondria (Narendra et al., 2010). After recruitment, PARKIN ubiquitinates proteins in the outer mitochondrial membrane and mediates selective engulfment of depolarized mitochondria by autophagosomes (Narendra et

al., 2008). Furthermore, despite needed clarification, it has been reported that the ubiquitin-binding adaptor p62 can both aggregate ubiquitylated proteins and recruit ubiquitylated cargo into autophagosomes by binding to LC3 (Pankiv et al., 2007).

Although the growing importance of the autophagy and mitophagy (mitochondrial autophagy) as key mechanisms in mitochondrial quality control, the interconnection between these processes and mitochondrial architecture is still under debate. Mitochondrial fragmentation is a common stress response that is principally required to segregate and eliminate dysfunctional mitochondria. However, excessive and unbalanced fission under a variety of metabolic insults has deleterious effects in several tissues including cardiac and neuronal (Twig and Shirihai, 2011). Evidence suggests that fragmentation is required for mitochondrial autophagy, facilitating engulfment and degradation of the organelle by the autophagosome (Arnoult et al., 2005; Nowikovsky et al., 2007; Youle and Narendra, 2011). However, the relationship between mitochondrial shape and mitophagy seems more complex than a straightforward equation fragmentation-autophagy. Gomes and Scorrano (2008) indicated that fragmentation *per se* is not sufficient to trigger autophagy. Moreover, they suggested that mitochondrial dysfunction can activate autophagy machinery and therefore, mitochondrial dysfunction rather than fragmentation *per se*, determines whether the cell induces a program of autophagy. In agreement, Twig et al. (2008) demonstrated that mitochondrial fusion is selective for polarized active mitochondria and that the selectivity of the fusion machinery makes fusion and mitophagy complementary processes by preventing the migration of damaged and dysfunctional components into more active mitochondria, leading them available for mitophagy (Twig and Shirihai, 2011).

Unbalanced mitochondrial quality control may be associated with aging-related decreases in cardiac contractility and the pathogenesis of specific heart diseases that are specially caused by mitochondrial dysfunction including DOX-induced cardiotoxicity (Zhang et al., 2012). Although increased autophagy has been claimed for assisting tissue survival by removing damaged and dysfunctional molecules, substructures and organelles, most studies showed

that DOX upregulates cardiac autophagy and generally attenuation of autophagy confers a cardioprotective effect (Lu et al., 2009; Xu et al., 2012). DOX may induce autophagy by directly or indirectly affect upstream pathways responsible for the control of essential autophagy regulatory proteins (Atg12, Atg5, Beclin1, Bcl-2, LC3-II and others) (Dirks-Naylor, 2013). Interestingly, the same author suggested that this autophagic response might be species specific, as DOX treatment increased autophagy in primary rat cardiomyocytes and heart, being decreased in mice. Previous research has demonstrated that DOX-induced cardiotoxicity could have a direct effect on mitochondrial fragmentation by up regulating DRP1 levels (Gharanei et al., 2013), which was found to occur simultaneously with the release of cytochrome c and apoptosis (Martinou and Youle, 2006). Moreover, as mentioned, under pathological conditions, this fragmented architectural phenotype is more favorable to mitopahgy. However, confirming the relevance of mitochondrial autophagy for the preservation of cardiac function through stabilization of mitochondrial fitness, others elegantly demonstrated that inhibition of Parkin translocation from cytosol to mitochondria in cardiomyocytes and mouse hearts submitted to DOX effect via tumor suppressor p53 stimulation worsened cardiac and mitochondrial phenotype (Hoshino et al., 2013). Moreover, the authors did not observe changes in mitochondrial dynamics. These studies pointed out conflicting tendencies regarding the relevance of the activation levels of mitochondrial quality control mechanisms in the context of DOX-induced cardiotoxicity. Interestingly, no studies were found concerning possible alterations in brain mitochondrial dynamics, autophagy neither mitophagy with DOX.

Results here presented in studies 2 and 4 (Chapter 3.3 and 3.5) showed that sub-chronic DOX treatment induced increased cardiac, brain cortex and cerebellum mitochondrial fragmentation, as seen by an inhibition of fusion-related proteins (Mfn1/2 and OPA1) and increased DRP1 levels. These alterations on mitochondrial dynamic signaling were accompanied by an increase in autophagy signaling, as well as PINK levels in the heart and brain cortex and Parkin in brain cortex. Generally, there were no differences between the

studied tissues in response to DOX-induced alterations on mitochondrial quality control signaling. Since most parameters of mitochondrial function were disarranged by DOX, including respiration, $\Delta\psi$ and oxidative stress, it is likely that the activation of mitochondrial fragmentation, mitophagy and apoptotic cell death signaling pathways are simultaneously activated. Moreover, Samant et al. (2014) reported that SIRT3 promotes mitochondrial function not only by regulating activity of metabolic enzymes, as previously reported, but also by regulating mitochondrial dynamics by targeting OPA1, since this fusion-related protein is hyperacetylated in hearts under pathological stress and the mitochondrial deacetylase SIRT3 was capable of deacetylating OPA1 and elevating its GTPase activity. The same authors reported that SIRT3-dependent activation of OPA1 contributed to the preservation of mitochondrial networking and protection of cardiomyocytes from DOX-mediated cell death, which might possibly agree with our results (Chapter 3.2 and 3.5). Further studies are needed to better comprehend the time-course, dose and experimental model responses to DOX and other harmful conditions such as aging, regarding the synergistic, complementary or dependent activation of mitochondrial quality control signaling pathways.

Reinforcing the interplay between important cellular mechanisms in the control of adaptive and survival paths, and in this case the role of dyacethylation-dependent fusion against injury, SIRT3-dependent activation of OPA1 seems to contribute to the preservation of mitochondrial network and protection of cardiomyocytes from DOX-mediated cell death (Samant et al., 2014). Accordingly, our findings suggest that both SIRT3 and OPA (not in cerebellum) depletion might contribute to DOX toxicity. Moreover, the relationship between a fragmented mitochondrial profile and increased apoptotic signaling following DOX treatment formerly reported was also found in the present studies (Chapter 3.2, 3.3 and 3.5).

It has been recently suggested that exercise training stimulates not only the biogenesis of mitochondria but also the removal of old and unhealthy mitochondria through mitochondrial

dynamics and autophagy (Yan et al., 2012). However, the relationship between these processes of mitochondrial quality control and exercise are only starting to emerge.

Mitochondrial network dynamics seem to be sensitive to exercise, still to our knowledge, this topic as been mainly addressed in skeletal muscle following acute exercise showing increased Mfn1 and 2 levels in rats and human (Ding et al., 2010; Perry et al., 2010). Moreover, OPA1 appears necessary for the normal adaptive response and mitochondrial biogenesis of skeletal muscle to training (Caffin et al., 2013) and PGC-1α may play an important function in regulating exercise-induced expression of mitochondrial fusion machinery in skeletal muscle. However, the effects of chronic endurance exercise on mitochondrial dynamic mechanism are still scarce.

Recent studies demonstrate that autophagy is induced by acute exercise in skeletal muscle (Grumati et al., 2011; He et al., 2012a), heart (Ogura et al., 2011) and cerebral cortex (He et al., 2012b) Moreover, cellular autophagic responses to physical exercise in skeletal muscle appear to be varied in different exercise protocols (Tam and Siu, 2014) and whether long-term exercise training affects autophagy in multiple organs remain to be ascertained. In the present dissertation, there were no important differences between different exercise models on the effects in autophagy signaling markers. Similar to previous findings on heart and aorta (Sun et al., 2013b; Sun et al., 2013a), the mitochondrial beneficial effects of chronic exercise on heart, brain cortex and cerebellum in our studies were associated with the induction of mitochondrial biogenesis and fusion signaling. Cardiac autophagy signaling, were also reported to be increased with 8 weeks of treadmill exercise and 4 weeks of voluntary exercise (Bhuiyan et al., 2013; Sun et al., 2013a); however, in studies 2 and 3 (Chapter 3.3 and 3.4), autophagy signaling markers only increased in brain cortex and cerebellum (and not so evident in heart). This might be the major difference found between the studied tissues in the present dissertation. Additionally, Lira et al. (2013) reported that autophagy and mitophagy are required for long-term voluntary exercise-induced skeletal muscle adaptation and improvement of physical performance. Jamart et al. (2013) reported that a single bout of

low-intensity endurance running increases skeletal-muscle autophagy and mitophagy markers in the fasted state (not fed). These results were accompanied by an increased in mitochondrial fission DRP1$^{Ser616}$ and no alterations on Mfn1 and Mfn2 by exercise were observed independently of the nutritional *status*. In the context of the chronic exercise models used in the present dissertation, an increase in mitophagy-related protein PINK1 only in brain cortex were shown in studies 2 and 3. This possibly suggests that the effects of exercise on mitophagy signaling might be tissue dependent. Interestingly, in parallel to the elevation of PINK1 levels, an increase in mitochondrial fusion signaling of brain cortex mitochondria was observed. If this phenotype could be considered beneficial against many brain insults that result in mitochondrial fragmentation, it is not consistent with some reports indicating that autophagy and fragmentation are complementary processes (Chen and Chan, 2009; Lee et al., 2011; Twig et al., 2008).

On the basis of the above-referred mitochondrial-related remodeling features caused by chronic exercise, one may expect that exercise would alter mitochondrial architecture and quality control signaling, being these mechanisms important contributors for mitigating DOX toxicity. On the other hand, another rational could be that exercise induced a variety of improvements in cellular and mitochondrial defense systems that avoid such an intense activation of autophagic forms of tissue renewal/clearance. In this regard, and as far as we know, it may well be the first time that mitochondrial quality control (particularly auto and mitophagy) was analyzed in the context of exercise performed during sub-chronic DOX treatment.

The studies comprising the present dissertation showed that chronic exercise performed before and during the course of sub-chronic DOX treatment interferes with mitochondrial dynamic protein profiles by mitigating mitochondrial fragmentation found in DOX-treated animals, suggesting that these adaptations may contribute to prevent mitochondrial bioenergetics collapse characterizing hearts and brain of rats undergoing DOX treatment (Chapter 3.3 and 3.5). Similarly, 8 wks treadmill exercise increased the expression of cardiac

Mn-SOD, mitochondrial electron transport chain complexes, and Mfn1 and 2 in mice sub-chronically treated with DOX (Dolinsky et al., 2013).

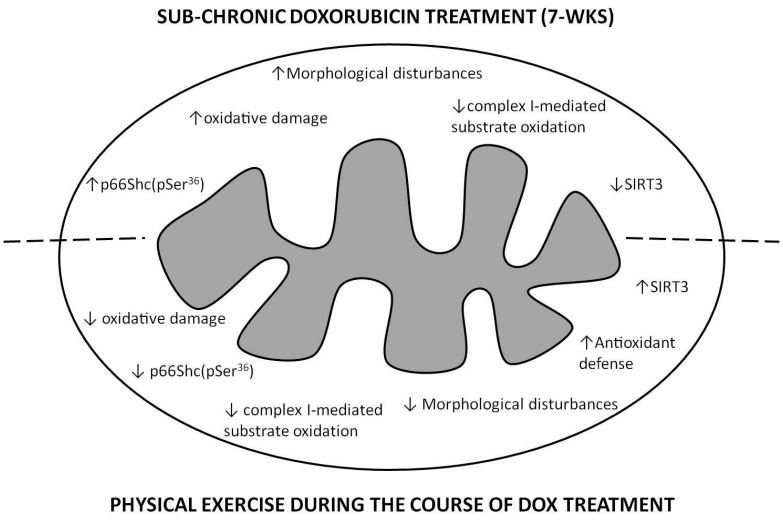
The modulation of autophagy following exercise in DOX treated animals is currently limited to two studies in skeletal muscle and cardiac tissue. Smuder et al. (2011, 2013) reported that 5 days of treadmill exercise blunted the global induction of autophagy markers in the *soleus* muscles and hearts of rats treated with a single DOX dose, being these alterations markedly mitigated by prior short-term exercise training. Similar results were found in our experiments, suggesting that both models of chronic exercise performed prior and during the course of sub-chronic DOX treatment administration might had attenuated many of the DOX-induced changes to autophagy markers in heart as well in brain and cerebellum tissue. Interestingly, the above mentions authors also reported that exercise did not result in skeletal and cardiac muscles autophagy modulation in non–DOX-treated animals, reinforcing that these signaling mechanisms depend on the severity of the stimulus.

# 5.

# CONCLUSIONS

Based on the experimental results of the different original studies comprising the present thesis, the following conclusions can be taken:

- Sub-chronic DOX administration resulted in: (i) altered heart mitochondrial morphology, (ii) altered cognitive performance, (iii) compromised heart and brain mitochondrial activity, (iv) decreased heart and brain OXPHOS complex activity, and heart OXPHOS subunit content and organization, (v) lower heart and brain mitochondrial biogenesis signaling (vi) increased heart and brain oxidative stress, (vii) increased heart and brain mPTP susceptibility, (viii) augmented heart and brain apoptotic signaling (ix) increased fission and decreased fusion markers, thus possibly impairing mitochondrial dynamics and (x) increased markers of autophagy and mitophagy signaling.
- Cardiac mitochondrial structural alterations, bioenergetics dysfunction, biogenesis and oxidative damage characterizing sub-chronic DOX treatment were mitigated by both models of exercise, when performed before and during the course of DOX administration (Figure 1; Study 1 - Chapter 3.2).




**Chapter 5 - Figure 1.** Scheme summarizing the described adaptations induced by chronic physical exercise performed before and during sub-chronic DOX treatment against heart mitochondrial dysfunction and increased oxidative stress induced by sub-chronic DOX treatment. Upper panel shows the alterations result from DOX treatment. Lower panel depicts the counteracting effects elicited by physical exercise.

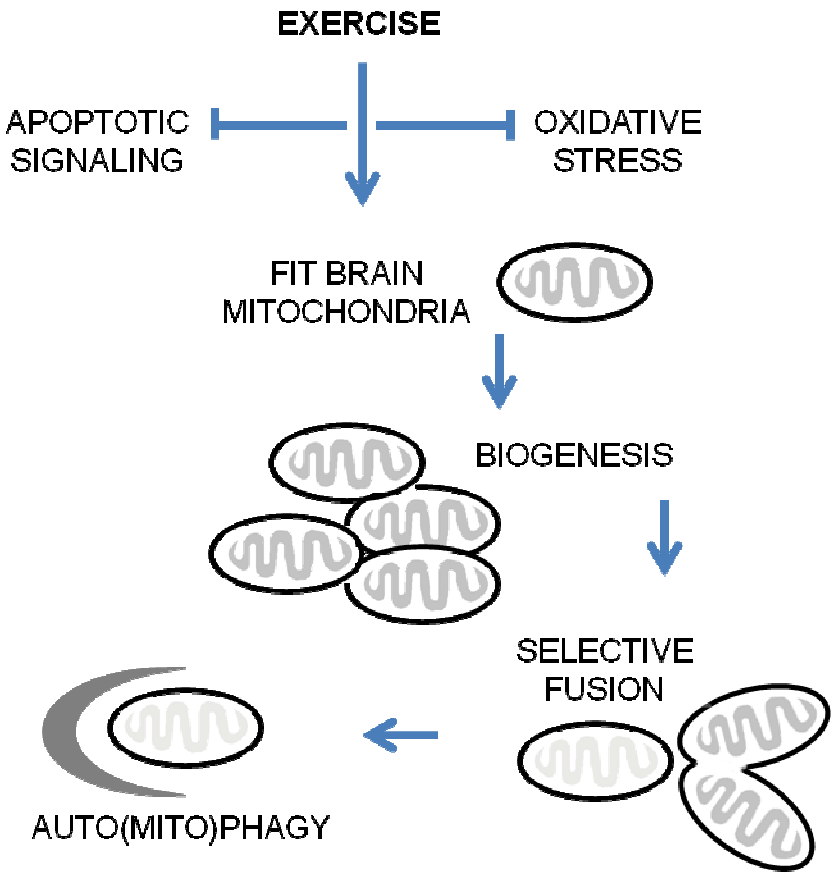
- Physical exercise performed before and during the course of a sub-chronic, clinical relevant DOX treatment, decreases the threshold for mPTP induction, markers of apoptotic signaling and modulates mitochondrial dynamics and quality control signaling in cardiac tissue (Table 1; Study 2 - Chapter 3.3).

**Chapter 5 - Table 1.** Table summarizing the described adaptations induced by chronic physical exercise *per se* and against heart mitochondrial dysfunction induced by sub-chronic DOX treatment.

| | Compared with Saline Sedentary | | | Compared with DOX Sedentary |
|---|---|---|---|---|
| | 12 wks Treadmill endurance training | 12 wks Voluntary free wheel running | Sub-chronic DOX treatment | Physical exercise before and during DOX treatment |
| mPTP susceptibility | ↔ | ↓ | ↑ | ↓ |
| Apoptotic signaling | ↓ | ↓ | ↑ | ↓ |
| Mitochondrial fusion | ↑ | ↑ | ↓ | ↑ |
| Mitochondrial fission | ↔ | ↔ | ↑ | ↓ |
| Quality control mechanisms | ↔ | ↔ | ↑ | ↓ |

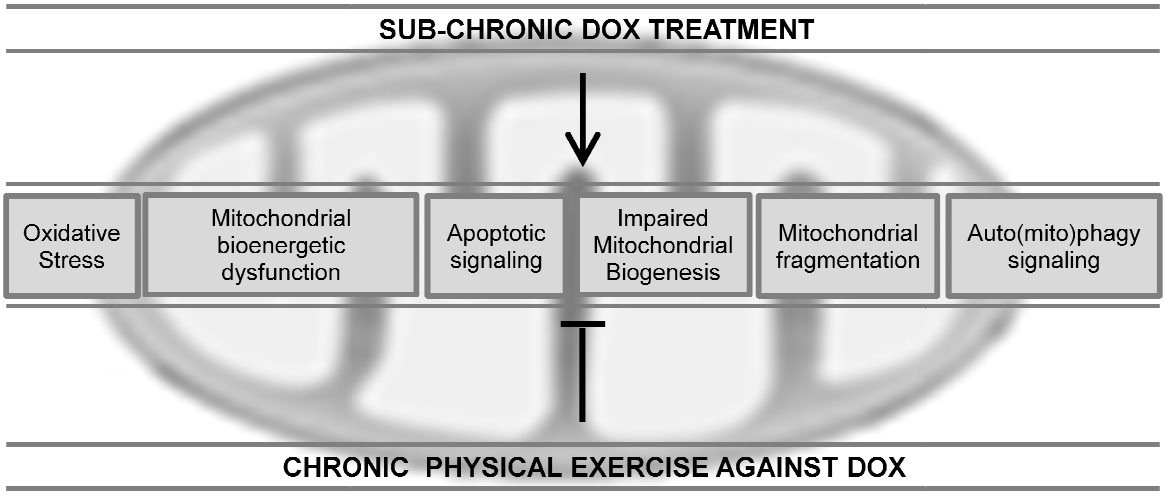
↓increase ↑decrease or ↔no alterations

- 12 wk of treadmill exercise training and voluntary physical exercise are able to favourably alter cognition, mitochondrial bioenergetics endpoints, molecular markers of mitochondrial biogenesis and oxidative damage. It also ameliorated mPTP susceptibility, decreased apoptotic signaling and modulated mitochondrial dynamics and auto(mito)phagy signaling markers in brain cortex and cerebellum (Figure 2; Study 3 - Chapter 3.4).




**Chapter 5 - Figure 2.** Scheme summarizing the described adaptations induced by chronic physical exercise on brain mitochondria. Physical exercise positively modulated mitochondrial respiratory activity, decreases oxidative damage and apoptotic signaling. The improvements in mitochondrial function were accompanied by increased biogenesis, an increase in brain cortex and cerebellum selective healthy mitochondrial fusion and segregation of damaged mitochondria removed by auto(mito)phagy.

- Physical exercise performed before and during the course of sub-chronic DOX treatment prevented behavioral alterations and the compromised mitochondrial bioenergetic, biogenesis, oxidative damage, mPTP opening susceptibility, apoptotic signaling and modulated mitochondrial dynamics and auto(mito)phagy in brain cortex and cerebellum (Figure 3; Study 4 - Chapter 3.5).




**Chapter 5 - Figure 3.** Scheme summarizing the described adaptations induced by chronic physical exercise against brain cortex and cerebellum mitochondrial dysfunction and related impairments induced by DOX treatment.

In summary, these findings suggest that exercise is likely to contribute to a protective phenotype against the cardiac and brain side-effects characterizing DOX treatment. Provided data increase the knowledge of mechanisms associated with the protective and/or therapeutic role of physical exercise modalities in the set of physiological cardiovascular and nervous tissue remodeling. Moreover, as high energy-dependent tissues, heart and brain are organs that importantly benefit of fit and plastic mitochondria for better providing energy and regulating several mechanisms for cell survival and adaptation, highly dependent on

mitochondrial functionality. These include the redox modulation and apoptotic cell death regulation. Moreover, our results provide evidence, for the first time, that the improvements in mitochondrial functionality are associated with modifications in molecular markers of biogenesis, dynamics and autophagy, which possibly contribute in a synergistic and/or dependent manner to the observed phenotypes more resistant to the deleterious effects of DOX treatment.

Be brainy and exercise your heart!

# 6.

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