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Physical exercise as non-pharmacological tool to counteract drug-induced liver mitochondrial injury: effects on mitochondrial bioenergetics, oxidative stress, apoptosis, dynamics and auto(mito)phagy signalling markers

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This dissertation is presented by Estela Alves, graduated in Biochemistry, to obtain the doctoral degree in Biomedicine at the University of Barcelona.

The current thesis work, entitled "Physical exercise as a non-pharmacological tool to counteract drug-induced liver mitochondrial injury: Effects on mitochondrial bioenergetics, oxidative stress, dynamics and auto(mito)phagy signaling" was conducted in the group of Exercise Physiology and Hypoxia of the department of "Biologia Cel·lular, Fisiologia i Immunologia" of the Faculty of Biology, Universitat de Barcelona and in the Laboratory of Metabolism and Exercise of the Research Centre in Physical Activity, Health and Leisure, Faculty of Sport, University of Porto under the supervision of Dr. António Alexandre Moreira Ribeiro de Ascensão and Dr. Joan Ramon Torrella Guio.

Dr. António Ancensão Dr. Joan Ramon Torrella Estela Alves

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All true multicelular life on earth is made up of eukaryotic cells – cells with nucleus. The evolution of these complex cells is shrouded in mistery and may have been one of the most unlikely events in the entire history of life. The critical moment was not the formation of a nucleous, but rather the union of two cells, in which one cell physically engulfed another, giving rise to a chimeric cell containing mitochondria.

- *Power, Sex, Suicide: Mitochondria and the meaning of life*. **Nick Lane**

Dedicado à minha mãe, sem ela não estaria aqui hoje. Obrigada!

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Index

Abbreviations

4´-OH-DF	4´-hydroxydiclofenac
5-OH-DF	5-hydroxydiclofenac
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
CYP450	Cytochrome P450 family
Cyt c	Cytochrome c
DILI	Drug-induced liver injuries
DOX	Doxorubicin
DRP1	GTPase dynamin-related protein 1
ET	Endurance training
ETC	Electron transport chain
Fis1	Mitochondrial fission 1 protein
FW	Freewheel
GPx1	glutathione peroxidase-1
GSH	Glutathione reduced
HCR	High capacity endurance running
IMM	Inner mitochondrial membrane
LCR	Low capacity endurance running
MDA	Malondialdehyde
MFN1/2	Mitofusins 1 and 2
MPTP	Mitochondrial permeability transitory pore
NADH	Nicotinamide adenine nucleotide reduced
NAFLD	Non-alcoholic fatty liver diseases
NSAIDs	Nonsteroidal anti-inflammatory drugs
OMM	Outer mitochondrial membrane

OXPHOS	Oxidative phosphorylation system
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
RCR	Respiratory control ratio
ROS	Reactive oxygen species
-SH	Sulfhydryl groups
SOD	Superoxide dismutase
TCA	Tricarboxylic acid cycle
TFAM	Mitochondrial transcription factor A
VPA	Voluntary physical activity

Abstract

Liver diseases resulting from the toxicity induced by frequent pharmacological drug consumption are among the main health problems of modern western societies. On the other hand, healthy life-style-based behaviors including physical exercise are critical for counteracting, by preventing and/or treating, the drug-associated deleterious consequences for the hepatic tissue. The present thesis aimed to study, in a rat model, the effects of two chronic physical exercise regimens on liver morphological, biochemical and functional features centered on mitochondria, as these subcellular network compartments are known as dynamic structures closely involved in important mechanisms related to both the physiopathology of the disease and the beneficial adaptations of tissues afforded by exercise. Functional alterations in liver mitochondria were measured in *in vitro*: respiratory-driven endpoints, susceptibility to permeability transition pore opening. Additionally, enzymatic activities and the expression of proteins involved in redox response, apoptotic cell death, mitochondrial biogenesis, dynamic and autophagic markers were analyzed throughout the experimental work comprised in this thesis. Basal mitochondrial responses to toxic drugs exposure, both after *in vitro* (diclofenac) and *in vivo* (doxorubicin) stimulation were determined.

It was overall concluded that chronic physical exercise induced liver mitochondrial alterations suggestive of positive remodeling, which were

translated in a resultant more resistant phenotype against the *in vitro* toxicity of diclofenac and the *in vivo* harmful effects of doxorubicin. The observed mitigation effects were associated with favorable modifications in functional endpoints of mitochondrial respiration and in key signaling proteins related to oxidative stress and damage, apoptosis, mitochondrial biogenesis and dynamics, and auto(mito)phagy-related quality control mechanisms.

1.

General Introduction

1.1 Background

The liver has an essential role in catabolic and anabolic metabolism and it is an important contributor to metabolic homeostasis under different pathological and non-pathological conditions. Thus, the liver has a high metabolic activity associated with cholesterol and fatty acid synthesis and to the storage of glucose as glycogen and fatty acids as triglycerides (Chiang, 2014; Sanders and Griffin, 2016). Its capacity to store energetic substrates places the liver in the middle of many essential metabolic pathways, supplying nutrients and energetic substrates to the whole body. For example, it has an important role in maintaining plasmatic glucose levels through the catabolism of stored triglycerides and glycogen, thus providing energy to other glucose-dependent tissues and cells, such as the brain or the erythrocytes (Chiang, 2014). Moreover, the liver has also an important role in the metabolism and detoxification of drugs, xenobiotics and metabolites. Therefore, liver diseases can be devastating and result in a multiple organ dysfunction syndrome, as no other tissue in the body can compensate these functions.

This intensive metabolism exerts a high energetic demand, which is mostly supplied by mitochondria, the main energy producer of the cells. Thus, the above-mentioned hepatic functions (and others) are highly dependent on the mitochondrial performance. In consequence, morphofunctional alterations in this organelle network, as those induced by uncompensated diets, diseases,

drugs and xenobiotics, can affect its structure and energy production capacity, compromising the liver homeostasis and consequently the function of other tissues dependent on the hepatic metabolism.

One of the main non-pharmacological strategies proposed to counteract, either by preventing and/or treating, liver dysfunction induced by the referred putative harmful stimuli is the prescription of physical activity and exercise. Several reviews and studies carried out by our group have provided evidence that physical exercise or increased physical activity resulted in liver protection against stimuli-induced dysfunction, being the mitochondria the target of the more resistant phenotypes (as a consequence of their role in both the pathogenesis of diseases and in the adaptations provided by exercise) (Ascensão et al., 2013a; Beleza et al., 2018; Gonçalves et al., 2013, 2017).

The present doctoral thesis focuses on the crosstalk between drug-induced mitochondrial toxicity (namely doxorubicin and diclofenac) and physical exercise as a non-pharmacological intervention in the hepatic tissue, exploring mitochondrial-mediated adaptations as the center of the more resistant phenotype afforded by exercise against drug-induced liver injury (DILI) and dysfunction.

1.2 Hepatotoxicity

Despite the undeniable economical and health-related benefits associated with the development of modern and industrial societies, mankind is being

increasingly exposed to larger quantities of harmful xenobiotics (e.g. chemical drugs, environmental pollutants, pesticides, food additives, etc.), which can be the source of many pathological conditions, such as obesity, diabetes, cancer, cardiovascular, cognitive and hepatic diseases (Mostafalou and Abdollahi, 2013; Ogu and Maxa, 2000; Simmons et al., 2014).

One of the oldest drugs used in our society is alcohol. Indeed, alcohol history is also civilization history, since the first evidence of alcohol production through fruit fermentation dates back to 7000 BC in China (McGovern et al., 2004). Abusive alcohol consumption can lead to steatosis, hepatitis, steatohepatitis, cirrhosis and even hepatocellular carcinoma (Sakhuja, 2014). DILI is associated with elevated circulating levels of hepatic enzymes, such as alanine and aspartate transaminases (ALT and AST) (Contreras-Zentella and Hernández-Muñoz, 2016; Yu et al., 2017). These enzymes are massively released into the bloodstream when hepatocytes are damaged and thus can be used as health sensitive biomarkers (Yu et al., 2017).

Due to its elevated metabolic rate, liver is one of the principal organs involved in drug metabolization and cleaning. The hepatic biotransformation of drugs and xenobiotics involves large and complex enzymatic systems such as the cytochrome P450 family (CYP450), an enzymatic complex that, among other functions, increases the xenobiotic solubility and clearance using O_2 to obtain hydroxylated products (Chiang, 2014; Jančová and Šiller, 2012; Ogu and Maxa, 2000). Unfortunately, drug metabolization can result in the formation of

reactive electrophilic metabolites, which have a high affinity to hepatic proteins, lipids and DNA, leading to the production of reactive oxygen species (ROS), mitochondrial dysfunction and hepatic damage (Begriche et al., 2011). For example, a study of chronic ethanol consumption in rats conducted by García-Ruiz et al. (1995) showed cytosolic and mitochondrial glutathione depletion, as well as decreased mitochondrial membrane potential, respiratory control ratio (RCR) and adenosine triphosphate (ATP) levels. These alterations were reduced by the administration of S-adenosyl-methione (a glutathione precursor), demonstrating the importance of the antioxidant pool in hepatic mitochondrial protection against drug toxicity.

1.3 Drug-induced hepatotoxicity

1.3.1 Doxorubicin

Doxorubicin (DOX, also known by Adriamycin) is an anthracycline that was isolated for the first time from *Streptomyces peucetius* in the 1960s (Arcamone et al., 1969). It has been used as a highly effective chemotherapeutic agent for the treatment of solid tumors, such as malignant sarcoma, adenocarcinoma and neuroblastoma, and for hematologic malignancies like lymphomas or leukemia (Licata et al., 2000).

DOX anticarcinogenic effect has been associated with several mechanisms, such as inhibition of DNA and RNA synthesis by topoisomerase II poisoning (Edwardson et al., 2015), formation of anthracyclines-DNA adducts by

formaldehyde action (Cutts et al., 2005; Swift et al., 2006) and exacerbated formation of oxidative species (Davies and Doroshow, 1986; Edwardson et al., 2015). Despite the success of DOX as a chemotherapeutic drug, its use is limited by the acute and chronic tissue toxicity, mainly observed in cardiac tissue (Bartlett et al., 2017; Harake et al., 2012). Acute DOX toxicity appears immediately after initiation of the treatment and consists of mild arrhythmias or hypotensive episodes, while chronic DOX toxicity develops after the conclusion of cumulative treatments and is associated with congestive heart failure (Minotti et al., 2004). In some cases, DOX toxicity can appear many years after the drug treatment; for example, Kumar et al. (2012) reported the case of an active, healthy 57-years-old breast cancer survivor who, 17 years after treatment, exhibited symptoms of DOX-induced heart failure, showing the toxic delay associated with DOX treatment.

Although the heart has been referred as the organ where DOX results in higher toxicity levels, other organs such as the brain, the kidney and the liver also suffer from side-effects from DOX treatment schedules (Lahoti et al., 2012; Tangpong et al., 2011; Wu et al., 2018). Regarding the liver, several studies indicate that *in vivo* and *in vitro* DOX treatments increase biomarkers of toxicity and stress, thus disrupting hepatic tissue homeostasis (Wu et al., 2018). Those include histopathological features, increased oxidative stress and damage, apoptotic cell death, mitochondrial dysfunction as well as alterations in

mitochondrial dynamics and auto(mito)phagy signaling proteins (Dirks-Naylor et al., 2014).

Indeed, to attenuate the prevalence of DOX-associated toxicity, the World Health Organization recommends a maximum cumulative dose of 300-500 mg/m^2 in adult humans (WHO Drug Information, 2011).

1.3.1.1 Mechanisms associated with DOX toxicity

Several mechanisms have been described to explain the cellular damage associated with DOX administration, such as iron and calcium metabolism dysregulation, oxidative stress and mitochondrial dysfunction, suggesting that DOX toxicity is a complex and multifactorial process. As mentioned, although DOX-toxicity is frequently associated with heart dysfunction and cardiotoxicity (Carvalho et al., 2014), other tissues have been reported to be affected by DOX treatment. For example, in a clinical study conducted by Superfin et al. (2007) it was observed an increase in circulating levels of transaminases and bilirubin associated with DOX-administration, suggesting that the liver function was compromised. However, the effects of DOX-induced toxicity in tissues other than heart have been largely ignored and the literature is scarce.

The increase of oxidative stress associated with DOX metabolization seems to be one of the mechanisms responsible for hepatic damage. DOX metabolization leads to the formation of several compounds (such as DOX-semiquinone and doxorubicinol) that are extremely reactive (Edwardson et al.,

2015). The accumulation of these compounds increases ROS production, which leads to lipid, protein and DNA damage (Edwardson et al., 2015). Indeed, studies in cardiac tissue demonstrated that DOX-semiquinone is a potent acceptor of electrons that induces a redox futile cycle with mitochondrial nicotinamide adenine nucleotide (NADH) dehydrogenase, compromising the mitochondrial function (Davies and Doroshow, 1986; Oliveira et al., 2000). Furthermore, this toxicity is exacerbated by the high affinity of these compounds to cardiolipin – a tetra fatty acid-containing phospholipid only found in the mitochondrion – which increases their lifetime within the cell (Aryal and Rao, 2016; Nicolay and de Kruijff, 1987; Oliveira et al., 2000).

Additionally, DOX promotes a depletion of endogenous antioxidant molecules and enzymes, such as glutathione (GSH), superoxide dismutase (SOD) or catalase, further contributing to the hepatic oxidative damage (Kaplowitz, 1981; Schaupp et al., 2015; Zhou et al., 2001). Furthermore, hepatic GSH has an additional role on xenobiotic metabolization, as it is used by glutathione-s-transferase in conjugation reactions (Kaplowitz, 1981). Therefore, GSH depletion can result in compromised hepatic drug clearance. Some studies showed that antioxidant depletion is maintained for a long time after DOX treatment, which could partially explain the observed chronic toxicity (Chennuru and Saleem, 2013; Zhou et al., 2001). Thus, the lower or higher content of these enzymes seems to be associated with tissue toxicity susceptibility (Carvalho et al., 2014; Pereira et al., 2012; Zhou et al., 2001).

Indeed, some studies with antioxidant administration in rats showed a decrease of DOX hepatotoxicity, which is associated with up-regulated and strengthened antioxidant defense systems (El-Moselhy and El-Sheikh, 2014; Injac et al., 2008). Although promising experimental results have shown that antioxidants may be effective in protecting against DOX toxicity (Diamanti et al., 2014), the use of antioxidant supplementation during the chemotherapy is a controversial topic mainly due to the antioxidant potential for reducing cytotoxic efficacy against cancer cells (Cappetta et al., 2017; Vincent et al., 2013).

1.3.2 Diclofenac

Another widely used drug in our society is diclofenac, a nonsteroidal anti-inflammatory drug (NSAID) that was developed for the first time in 1970 (Gómez-Lechón et al., 2003). It was originally indicated for osteoarthritis, rheumatoid arthritis and mild-to-moderate acute pain and nowadays its use is popularized for the treatment of minor musculoskeletal disorders, becoming one of the most prescribed NSAID worldwide (Syed et al., 2016). Pharmacological advances allowed to develop several formulations with different absorption times and pharmacological kinetics. For example, diclofenac sodium salt or Voltaren® (commercial name) was designed to release the active compound at higher pH and over a prolonged time. In contrast, diclofenac potassium or Cataflam® was designed for an immediate-release and absorption (Gan, 2010).

Similarly to the mechanisms of action of other NSAIDs, the anti-inflammatory, anti-pyretic and analgesic properties of diclofenac emerge from its capacity to inhibit the synthesis of pro-inflammatory and nociceptive prostaglandin and thromboxane by inhibition of cyclooxygenase 1 and 2 (Kirchheiner et al., 2003).

1.3.2.1 Mechanisms associated with diclofenac toxicity

Diclofenac is well tolerated after treatment, though several studies have shown that chronical administration of NSAIDs, and particularly diclofenac, have been associated with hepatic disorders (Björnsson, 2016; Boelsterli, 2003; Vuda and Kamath, 2016). For example, some clinical studies from the 80´s and 90´s, when diclofenac was frequently used as a chronic treatment, reported jaundice and hepatitis cases (Breen et al., 1986; Helfgott et al., 1990; Scully et al., 1993).

One of the hypothesis to explain diclofenac toxicity is the formation of secondary reactive metabolites. Secondary diclofenac metabolites can be found in different concentrations in patient's urine after diclofenac administration, being 4´-hydroxydiclofenac (4´-OH-DF) and 5-hydroxydiclofenac (5-OH-DF) the two major metabolites. Toxicological studies performed in rat and human hepatocytes showed an increase in ROS, mitochondrial membrane depolarization, ATP synthesis inhibition, apoptotic signaling activation and increased susceptibility to mitochondrial permeability transitory pore (MPTP) induction and consequently apoptosis signaling activation associated with diclofenac incubation as well as to its reactive metabolites (4´-OH-DF and 5-

OH-DF) (Gómez-Lechón et al., 2003; Inoue et al., 2004; Lim et al., 2006; Syed et al., 2016). These secondary metabolites can be oxidized to p-benzoquinone imines and generate covalent bonding with non-protein or protein sulfhydryl groups (-SH). These imines can lead to redox-cycling formation, which depletes the antioxidant defenses and compromise the normal cellular and mitochondrial function (den Braver et al., 2016; Syed et al., 2016). *In vitro* studies where hepatocytes were incubated with antioxidants, showed a prevention of caspase 3, 8 and 9 activation, suggesting an antioxidant-induced protection against diclofenac-derived apoptotic signaling (Gómez-Lechón et al., 2003).

1.4 Role of mitochondria in drug-induced hepatotoxicity

Albert von Kölliker, a Swiss histologist, observed mitochondria for the first time in 1856. These structures were later called "bioblasts" in 1890 by Richard Altmann (Ernster and Schatz, 1981). This nomenclature was maintained until 1899, when Carl Benda coined the term *mitochondrion*. The term derived from the Greek words *mitos* (thread) and *chondros* (granule), because of the long chains that these organelles formed (Ernster and Schatz, 1981). Beyond their interesting structure (Figure 1), mitochondria have an important role in the cellular energy metabolism, reactive oxygen species production, apoptotic signaling and calcium homeostasis (Moreira et al., 2011). These characteristics

make the mitochondria a potential drug target and an important biosensor for drug-induced toxicity.

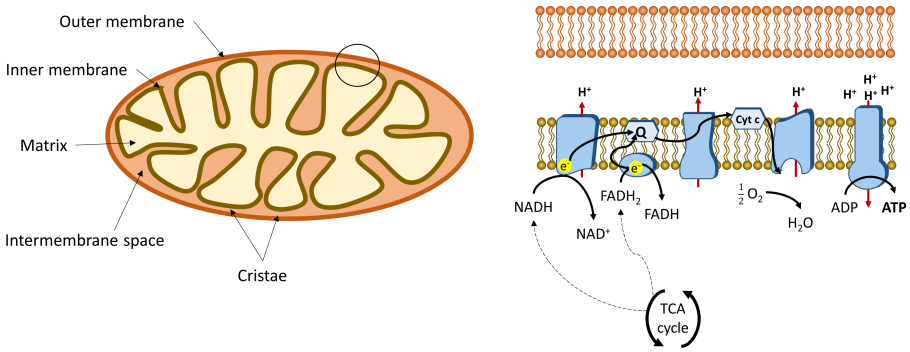

Figure 1. Mitochondria are the principal energy producers in the cell, consuming approximately 90% of inspired oxygen and producing 80-90% of the cellular ATP. Their specific and unique structure allow mitochondria to maintain an electrochemical gradient between their inner (IMM) and outer mitochondrial membrane (OMM), which is essential for the tricarboxylic acid cycle (TCA) cycle substrates oxidation and ATP production (Moreira et al., 2011). Mitochondrial electron transport chain (ETC) is constituted by four enzymatic complexes (I-IV) that are localized in the IMM. Briefly, the complex I and II receive electrons from reducing substrates, such as NADH and $FADH_2$. These electrons are transported to the complexes III and IV, through the oxidation and reduction reactions, until the molecular oxygen electron acceptor (Cecchini, 2003; Jastroch et al., 2010; Sazanov, 2015; Solmaz and Hunte, 2008). This electron transport through complexes I, III and IV is coupled to the transport of protons from the matrix to the intermembrane space, forming an electrochemical gradient that is posteriorly used by the ATP synthase to generate ATP (Jonckheere et al., 2012).

1.4.1 Drug-induced liver mitochondrial toxicity

Mitochondrial dysfunction seems to be one of the major mechanisms underlying DILI. Perturbations on mitochondrial energy production and release of apoptotic proteins can put in serious risk the hepatocytes viability. Drug-

induced mitochondrial dysfunction can also result in hepatic and extrahepatic mitochondrial and/or metabolic abnormalities, such as the formation of megamitochondria or severe lipid accumulation (Vuda and Kamath, 2016). Drugs can induce mitochondrial dysfunction through several mechanisms, such as: 1) oxidative phosphorylation system (OXPHOS) uncoupling; 2) ATP synthesis inhibition; 3) oxidative stress; 4) induction of mitochondrial permeability transition pore; 5) impairment of mitochondrial fatty acid oxidation and 6) mtDNA damage (Vuda and Kamath, 2016).

Oxidative stress is one of the described mechanisms associated with drug-induced mitochondrial dysfunction. Several studies showed that DOX treatment is accompanied by an increase of malondialdehyde (MDA) levels and 8-hydroxydesoxyguanosine, products of lipid and DNA oxidation, respectively (Patel et al., 2010; Serrano et al., 1999). Moreover, Zhou et al. (2001) showed an antioxidant depletion during and after DOX administration as a result of higher oxidative stress environment.

Furthermore, other mechanisms can be associated with DOX-induced mitochondrial dysfunction. For example, Pointon et al. (2010) showed that DOX can directly affect the expression and translation of OXPHOS genes, resulting in compromised ATP synthesis and caspase-3 activation. Some NSAIDs, such as diclofenac, seem to be powerful ATP synthase inhibitors and MPTP inducers. In an *in vitro* study conducted by Syed et al. (2016), a dependent time and concentration inhibition of ATP synthase, when liver mitochondria or

hepatocytes cells were incubated with diclofenac and its metabolites, was observed. These authors hypothesized that ATP depletion led to mitochondrial membrane depolarization, releasing pro-apoptotic molecules, such as cytochrome c and Bax, and inducing MPTP. Animal studies showed that diclofenac promotes severe mitochondrial morphological alterations, namely enlarged size and ruptured mitochondrial membranes (Moorthy et al., 2008). These ultrastructural alterations are usually related to perturbation in mitochondrial dynamics, namely a deregulated fusion and fission balance (Liu and Hajnóczky, 2011).

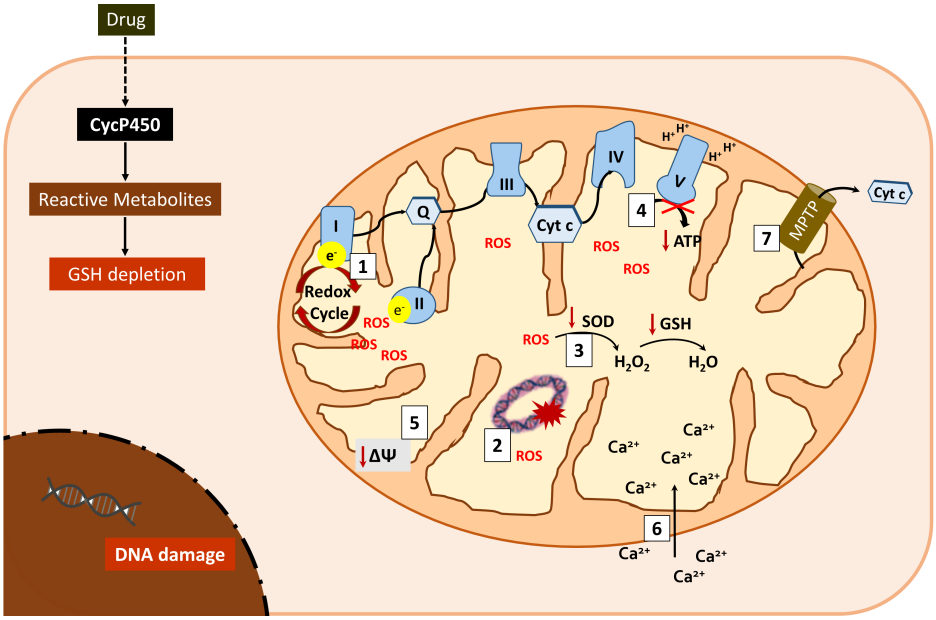

Figure 2. Schematic representation of the mechanisms underlying drug or/and reactive metabolites-induced hepatic mitochondrial dysfunction. Some drugs, such as DOX or diclofenac 1) induce the formation of quinone reactive metabolites that can lead to a futil redox cycle, exacerbating the production of ROS. This oxidative environment results in 2) a depletion of the antioxidant enzymatic system. 3) Both ROS and drug molecules can directly

damage nuclear and mitochondrial DNA, compromising the synthesis of essential proteins such as ETC subunits. 4) Therefore, ATP synthesis can be compromised, leading to energetic stress and 5) membrane potential disruption. Moreover, 6) calcium homeostasis dysregulation induced by drugs stimulates mitochondrial calcium over-accumulation which 7) leads to MPTP induction, cytochrome c (Cyt c) release and apoptotic signaling activation.

1.5 Exercise as a non-pharmacological tool against hepatotoxicity

Several approaches have been suggested to counteract DILI such as drug withdrawal, pharmacotherapy and hepatic transplant (Yu et al., 2017). In addition to these, growing evidences suggest that physical exercise could be a potential non-pharmacological strategy against DILI (Berzigotti et al., 2016; Morris et al., 2016).

Exercise has been shown to be an important non-pharmacological strategy against many chronic and metabolic diseases, such as obesity, diabetes and insulin resistance (Gonçalves et al., 2014a). Furthermore, strong evidence demonstrates that physical exercise is also an important tool in tissue protection against toxicity induced by xenobiotics (Ascensão et al., 2012; Magalhães et al., 2017; Marques-Aleixo et al., 2015a, 2016, 2017). During exercise, several cytokines are produced by the skeletal muscle, the adipose tissue and the brain and are released into the circulation. Despite being beneficial to the tissue of origin through paracrine and autocrine mechanisms, these molecules might also produce endocrine-like effects targeting distant organs and promoting positive adaptations (Beleza et al., 2018). Some of these cytokines stimulate reaction cascades of signaling pathways involved in the

positive remodeling of many tissues including liver (Berzigotti et al., 2016). These effects may be achieved through the biosynthesis of proteins associated with the increased resistance of cellular and subcellular structures, providing increased resistance of hepatocytes to deleterious stimuli, including high fat diets, anti-inflammatory and anti-cancer drugs.

Aside with voluntary physical activity (VPA), one of the most studied exercise models in health promotion is endurance training (ET). ET is characterized as a moderate-high aerobic exercise, such as swimming or running. Despite the differences between ET protocols on the intensity, frequency and duration of the training, scientific literature shows that ET can promote positive hepatic adaptations besides the well-known muscular and cardiac ones (Fletcher et al., 2014; Zacarias et al., 2017).

Although the liver is not a contractile organ, its metabolic role has an important function on energy supply during and after ET. Concomitantly, the hepatic (mitochondrial) function is also modulated by physical exercise. For example, Haase et al., (2011) showed that ET promotes a hepatic increase in peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) expression, a master regulator of oxidative metabolism and mitochondrial homeostasis. Indeed, Sun et al., (2010) reported increased mitochondrial ETC activity and GSH levels in trained rats. Data revealed that increased aerobic fitness and physical exercise represent valuable mitigating strategies against liver toxicity (Ascensão et al., 2013; Gonçalves et al., 2013; Rector and Thyfault,

2011). Accumulating evidence agrees that increased VPA and ET are able to improve hepatic tissue and mitochondrial phenotypes. Accordingly, several works from our lab demonstrated that mitochondrial features and endpoints from wild-type animals and rats exposed to high-fat diet induced non-alcoholic steatohepatitis (Gonçalves et al., 2014b, 2014a, 2016) or *in vitro* salicylate (Ascensão et al., 2012) were positively modulated by increasing physical activity levels and/or ET. Among the biomarkers and endpoints evaluated, one can highlight histopathological hallmarks, mitochondrial phospholipidic profile, mitochondrial respiratory capacity, membrane potential, susceptibility to calcium-induced MPTP and apoptotic signalling, oxidative stress and damage markers (Gonçalves et al., 2017).

Despite the abovementioned deleterious effects of ROS in liver tissue homogenates and mitochondria, several authors demonstrated that their production during and after exercise is one of the main mechanisms responsible for the observed benefits in hepatic function (Hoene and Weigert, 2010; Powers and Jackson, 2008). This hypothesis is supported by the concept of hormesis, which proposes that regular, controlled sublethal levels of a stressful stimulus can induce beneficial cellular and metabolic adaptations (Ascensão et al., 2013; Radak et al., 2017). Indeed, to further support this theory, studies with excessive antioxidant supplementation showed a prevention and decrease of the metabolic and mitochondrial exercise-induced adaptations (Gomez-Cabrera et al., 2008; Ristow et al., 2009).

To further support the notion that ET can effectively modulate the hepatic function, studies with selectively bred rats with low capacity endurance running (LCR) fed with a high-fat diet showed lower rates of hepatic fatty acid oxidation and increased susceptibility to develop nonalcoholic fatty liver disease than selectively bred rats with higher capacity endurance running (HCR). Furthermore, LCR exhibited decreased TCA genes expression compared to HCR (Morris et al., 2016; Thyfault et al., 2009). Thus, HCR had healthier and more resilient livers than LCR, indicating a clear relationship between exercise and hepatic metabolism.

Taking into account the described cross-talk between exercise, mitochondria and hepatic function, ET and increased physical activity levels emerge as a potential protective tool against liver diseases. The experimental work comprised in the present thesis has the goal of elucidating the role of physical exercise against hepatic mitochondrial dysfunction induced by diclofenac and DOX, two widely used drugs in the pharmacology with distinct purposes. Specifically, we aim to analyze liver mitochondrial function, oxidative damage biomarkers, mitochondrial dynamics comprising biogenesis, fusion and fission, as well as alterations in auto(mito)phagy signaling molecules, in order to evaluate quality control mechanisms involved in mitochondrial and tissue remodelling when facing deleterious *in vitro* and *in vivo* stimuli. Finally, a comprehensive review paper integrating some of the potential mechanisms by with physical activity and exercise afford protection against DOX-induced tissue

toxicity and injury is also presented it this thesis. The focus is centered on mitochondrial adaptations, including those of liver, in an attempt of better elucidate how this reticular network influences exercise-induced cross tolerance against DOX.

2.

Objectives

General Objective:

To analyze two different chronic physical exercise regimens, namely forced endurance training and voluntary physical activity, on the mitochondrial function, morphology and biochemistry of the rat hepatic tissue. Additionally, to ascertain whether these alterations translate into increased tolerance of liver mitochondria to the *in vitro* deleterious consequences of diclofenac and to the *in vivo* consequences of DOX administration.

Specific objectives and studied endpoints were achieved through the determination of several features and the establishment of models as follows:

1. To establish two chronic physical exercise regimens: forced endurance training and voluntary physical activity in a free wheel in the course of 12 weeks.
2. To analyze the effect of endurance training and voluntary physical activity in the hepatic mitochondrial function.
3. To analyze the hepatic mitochondrial function in an *in vitro* diclofenac-induced toxicity model and in an animal model of subchronic administration of DOX.
4. To analyze if physical exercise can mitigate the mitochondrial permeability transition pore opening induced by high calcium and diclofenac concentrations.

5. To confirm the hepatic damage induced by the subchronic DOX treatment and analyze if physical exercise can revert or mitigate the oxidative damage induced by subchronic DOX treatment in liver.
6. To analyze if physical exercise can revert or mitigate the alterations on the expression of proteins related with mitochondrial biogenesis, dynamic and auto(mito)phagic induced by subchronic DOX treatment in liver.
7. To evaluate if physical exercise can modulate or mitigate the hepatic mitochondrial ultrastructure alterations induced by subchronic DOX treatment.
8. To critically analyze the potential mechanisms by which physical exercise modulates tissues like heart, skeletal muscle, brain and liver in response to the toxicity caused by DOX. This analysis comprises an integrated view of studies in the field, including several performed by our group.

3.

Director´s report

As supervisors of the present Doctoral Thesis, Doctor Joan Ramon Torrella Guio and Doctor Antonio Ascensão hereby state that the student Estela Filipa dos Santos Alves has actively participated in the overall process conducting to a considerable amount of work done in the context of exercise-drug toxicity crosstalk. This include the peer-reviewed papers that conform this thesis, as reflected by their author order and composition. Estela had an essential role in the development and execution of the experimental design, data acquisition and treatment, and in the diffusion and publication of the main results and conclusions.

What follows below is a concise overview of the peer-reviewed papers included in the current Doctoral Thesis:

Paper I

Title: Exercise modulates liver cellular and mitochondrial proteins related to quality control signaling

Authors: Santos-Alves E., Marques-Aleixo I., Rizo-Roca D., Torrella J.R., Oliveira P.J., Magalhães J., Ascensão A.

Journal: Life Sciences

Year of publication: 2015 Volume: 125 Pages: 124-130

DOI: 10.1016/j.lfs.2015.06.007

JCR Impact Factor (2015): 2.685

JCR 5 Years Impact Factor: 2.627

Doctoral student participation: Animal care, including application of the training protocols, tissue sampling, liver mitochondrial isolation and functional evaluation. Sample preparation and analysis through different techniques, such as Western Blot and electronic microscopy. Statistical analysis, graphic treatment and interpretation of the obtained data. Contributed to the conception and design of the study. Drafting of the manuscript.

Paper II

Title: Exercise mitigates diclofenac-induced liver mitochondrial dysfunction

Authors: Santos-Alves E., Marques-Aleixo I., Coxito P., Balça M.M., Rizo-Roca D., Rocha-Rodrigues S., Martins S., Torrella J.R., Oliveira P.J., Moreno A.J., Magalhães J., Ascensão A.

Journal: European Journal of Clinical Investigation

Year of publication: 2014 Volume: 44 Number: 7 Pages: 668-677

DOI: 10.1111/eci.12285

JCR Impact Factor (Year): 2.734

JCR 5 Years Impact Factor: 2.741

Doctoral student participation: Animal care, including application of the training protocols, tissue sampling, liver mitochondrial isolation and functional evaluation. Sample preparation and analysis through different techniques, such as Western Blot, enzymatic and colorimetric assays and electronic microscopy. Statistical analysis, graphic treatment and interpretation of the obtained data.

Contributed to the conception and design of the study. Drafting of the manuscript.

Paper III

Title: Physical exercise positively modulates DOX-induced hepatic oxidative stress, mitochondrial dysfunction and quality control signaling

Authors: Santos-Alves E., Rizo-Roca D., Marques-Aleixo I., Martins S., Guimarães J.T., Oliveira P.J., Torrella J.R., Magalhães J., Ascensão A.

Journal: Mitochondrion (under review)

Doctoral student participation: Animal care, including application of the training protocols and drug administration, tissue sampling, liver mitochondrial isolation and functional evaluation. Sample preparation and analysis through different techniques, such as Western Blot, enzymatic and colorimetric assays and electronic microscopy. Statistical analysis, graphic treatment and interpretation of the obtained data. Contributed to the conception and design of the study. Drafting of the manuscript.

Paper IV

Title: The beneficial role of exercise in mitigating doxorubicin-induced Mitochondrionopathy

Authors: Marques-Aleixo I., Santos-Alves E., Moreira P.I., Oliveira P.J., Magalhães J., Ascensão A.

Journal: Biochimica et Biophysica Acta – Reviews on Cancer

Year of publication: 2018 Volume: 1869 Number: 2 Pages: 189-199

DOI: 10.1016/j.bbcan.2018.01.002

JCR Impact Factor (Year): 8.220

JCR 5 Years Impact Factor: 8.901

Doctoral student participation: Along the process and as a result of her committed involvement in the study of the cross-tolerance effects of physical exercise against the toxicity associated with drugs and/or diseased conditions, Estela actively participated in the definition and development of the outline, in the selection and critical analysis of the literature as well as in the drafting of the manuscript.

4.

Publications

Paper I

Exercise modulates liver cellular and mitochondrial proteins related to quality control signaling (2015). Santos-Alves E.[1], Marques-Aleixo I.[1], Rizo-Roca D.[2], Torrella J.R.[2], Oliveira P.J.[3], Magalhães J.[1], Ascensão A.[1] Life Sci. 15:124-30. DOI: 10.1016/j.lfs.2015.06.007

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Exercise modulates liver cellular and mitochondrial proteins related to quality control signaling

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ABSTRACT

Aims: The effects of exercise on cardiac and skeletal muscle, including the increase on mitochondrial function, dynamics, biogenesis and autophagy signaling are well described. However, these same effects on liver mitochondria, important in the context of hepatocyte ability to mitigate drug-induced injury and obesity-related disorders, are not fully understood. Therefore, the effects of two distinct chronic exercise models (endurance training—ET and voluntary physical activity—VPA) on liver cellular and mitochondrial quality control were analyzed.
Main methods: Eighteen male-adult Sprague–Dawley rats were divided into sedentary (SED), ET (12-week treadmill) and VPA (12-week voluntary free wheel). Liver mitochondrial alterations were evaluated by semi-quantification of proteins involved in oxidative stress (SIRT3, p66shc, p66(Ser36)), biogenesis (citrate synthase, PGC-1α and mtTFA), dynamics (MFN1, OPA1 and DRP1) and auto(mito)phagy (Beclin-1, Bcl-2, LC3II/LC3I, p62, Parkin and PINK) signaling. Liver ultrastructural alterations were also evaluated.
Key findings: Both exercise models induced beneficial alterations on liver mitochondrial morphology and increased mitochondrial biogenesis (PGC-1α and mtTFA), autophagy-related proteins (Beclin-1, LC3-II, LC3II/LC3I), and DRP1 and SIRT3 proteins. Increased citrate synthase activity and OPA1, p62 and Parkin content as well as decreased PINK protein levels were only observed after ET. VPA decreased OPA1, Beclin-1/Bcl-2, Parkin and p66(Ser36). Mitochondrial density and circularity increased in both exercised groups.
Significance: Both chronic exercise models increased proteins related with mitochondrial biogenesis and alteration proteins involved in mitochondrial dynamics and autophagy signaling, suggesting that exercise can induce liver mitochondrial adaptive remodeling and hepatocyte renewal.

1. Introduction

The mechanisms by which physical exercise modulates the liver mitochondrial phenotype, including alterations in the phosphorylative system and its components, substrate oxidation capacity, mitochondrial biogenesis, oxidative damage and antioxidants, as well as the induction of permeability transition and apoptotic signaling have been described [1,2]. Moreover, as it has recently been demonstrated by our group [3], exercise-induced adaptations result in improved liver mitochondrial function, increasing the resistance to deleterious conditions such as drug- and obesity-related disorders [2,4]. However, emerging importance has been attributed to the interplay between mitochondrial biogenesis, dynamics and auto(mito)phagy in exercise-induced regulation of cellular adaptation to stress, although this was never explored in the context of the hepatic tissue. Therefore, in complement to the previously referred and published paper [3], the present work aimed to analyze the effects of two distinct physical exercise modalities (ET and VPA), which respectively intend to mimic systematic training programs and voluntary daily physical activity, on markers of mitochondrial biogenesis, fusion and fission, and autophagy. As autophagy and mitochondrial dynamics deregulation have been implicated in the pathogenesis of common liver diseases, since inadequate activation of mitochondrial repair processes may contribute to accumulation of mitochondrial damage [5], it is likely that the manipulation of these interrelated processes through exercise may hold potential therapeutic value. Our current work hypothesis is that physical exercise increases protein markers related to the maintenance of mitochondrial quality control contributing, at least in part, to a more fitness and resistance phenotype.

2. Materials and methods

2.1. Animals

All experimental procedures were conducted in accordance with the Directive 2010/63/EU of the European Parliament and were approved

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0024-3205/

by the local board. Eighteen male Sprague–Dawley rats (aged 21 days old in the beginning of the protocol) were housed in collective cages (two rats per cage) in 12 h light/dark cycles with free access to food and water and were randomly divided into three groups (n = 6 per group): sedentary (SED), endurance training (ET) and voluntary physical activity (VPA).

2.2. Endurance training

The animals from the ET group 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 $m \cdot min^{-1}$, with daily increases of 5–10 min until 30 min was achieved. Habituation was followed by one consecutive week of continuous running (30 $min \cdot day^{-1}$) at 15 $m \cdot min^{-1}$ and was gradually increased until 60 $min \cdot day^{-1}$ on the second week [6].

2.3. Voluntary physical activity

The animals from the VPA group were housed in a polyethylene cage equipped with a running wheel [perimeter = 10.5 cm, Type 304 Stainless steel (2154F0106-1284L0106) Technicplast, Casale Litta, Italy]. The rats were allowed to exercise voluntarily with unlimited access to the running wheel 24 h/day. Running distance was recorded using a digital counter (ECO 701 Hengstler, Lancashire, UK).

2.4. Animal sacrifice and liver extraction

Forty-eight hours after the last exercise bout, non-fasted rats were euthanized by cervical dislocation between 9:00 and 10:00 AM to eliminate possible effects due to diurnal variation. After expedite opening of the abdominal cavity, rat livers and hearts were rapidly excised, rinsed, carefully dried and weighed.

A liver homogenate was obtained in a RIPA buffer (#20-188) with protease and phosphate inhibitor cocktail, in a ratio of 100 $mg \cdot mL^{-1}$ using a Teflon pestle on a motor driven Potter-Elvehjem glass homogenizer at 0–4 °C three to five times for 5 s at low speed setting. Homogenates were centrifuged at 12 000 × *g* for 10 min at 4 °C and resulting supernatants were prepared and stored at −80 °C for later semi-quantification of protein expression by Western blotting. Protein content was spectrophotometrically determined by the Bradford method using bovine serum albumin (BSA) as standard [7].

2.5. Isolation of liver mitochondria

Liver mitochondria were daily isolated using conventional methods of differential centrifugation as described previously [8]. The final concentration of mitochondrial protein was spectrophotometrically determined using the Biuret method with BSA as standard [9]. Mitochondrial fractions were separated and prepared for later semi-quantification of proteins by Western blotting as detailed below [8].

2.6. Liver mitochondrial citrate synthase activity

Liver mitochondrial citrate synthase activity was measured using the method proposed by Coore et al. [10]. The colorimetric assay was performed against a blank test. Total citrate synthase activity was expressed in nanomoles per milligrams of mitochondrial protein ($\varepsilon_{412} = 13.6\ mM^{-1} \cdot cm^{-1}$).

2.7. Immunoblotting detection of liver tissue and mitochondrial proteins

Equivalent amounts of liver tissue or mitochondria (1 $mg \cdot mL^{-1}$) were denatured in sample loading buffer and separated by dodecyl sulfate–polyacrylamide gel electrophoresis SDS/PAGE (12% gels) as described by Laemmli [11], followed by blotting on PVDF membranes according to the method of Locke et al. [12]. The mitochondrial content of the outer mitochondrial translocator TOM20 or β-actin was used as a protein loading control for mitochondrial and liver tissue-quantified proteins, respectively. In addition, membranes were stained with Ponceau-S to verify the efficiency of transfer and equal protein loading. After blotting, membranes were blocked for 2 h with 5% (*w*/v) non-fat dry milk powder in T-TBS (Tris-buffered saline with 0.1% Tween 20) to decrease non-specific binding.

Membranes were incubated with anti-PGC-1α (3 $\mu g \cdot mL^{-1}$; ab106814 goat monoclonal IgG, Cambridge, UK), anti-p62 (2 $\mu g \cdot mL^{-1}$; ab56416 mouse monoclonal IgG, Cambridge, UK), anti-OPA1 (1 $\mu g \cdot mL^{-1}$; ab119685 mouse monoclonal IgG, Cambridge, UK), anti-PINK (1:1000; ab23707 rabbit polyclonal IgG, Cambridge, UK), anti-Parkin (1:500; #4211 mouse monoclonal IgG, Danvers, MA, USA), anti-DRP1 (1:1000; #8570 rabbit monoclonal IgG, Danvers, MA, USA), anti-Bcl-2 (1:1000; #2870 rabbit monoclonal IgG, Danvers, MA, USA) anti-SIRT3 (1:1000; #2627 rabbit monoclonal IgG, Danvers, MA, USA), anti-shc [p66] (1:500; #2432 rabbit polyclonal IgG, Danvers, MA, USA), anti-shc [p66 (pSer36)] (1:1000; 6E10 mouse monoclonal IgG, Merck Millipore, Darmstadt, Germany), anti-mtTFA (1:1000; sc-23588; goat polyclonal IgG, Dallas, TX, USA), anti-MFN1 (1:1000; sc-50330 rabbit polyclonal IgG, Dallas, TX, USA), anti-β-actin (sc-1616; goat polyclonal, Dallas, TX, USA) and anti-TOM20 (1:1000; sc-11415 rabbit polyclonal IgG, Dallas, TX, USA). Primary antibodies were diluted in TBS-T containing 2% of non-fat dried milk or BSA for 8 h at 4 °C. Following primary antibody incubation, membranes were washed and incubated with secondary horseradish-peroxidase-conjugated anti-mouse (1:10 000; sc-2005, Dallas, TX, USA), anti-goat (1:10 000; sc-2354, Dallas, TX, USA) or anti-rabbit (1:10 000; sc-4004, Dallas, TX, USA) antibodies 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® Plus™, visualized with the ChemiDoc XRS + system (Bio-Rad Laboratories, Amadora, Portugal) and analyzed with the 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 variation of the SED control group.

2.8. Transmission electron microscopy (TEM)

The collected liver tissues were first fixed in 2.5% of glutaraldehyde solution, subsequently washed in phosphate buffer and post-fixed in 1% osmium tetroxide. The samples were then dehydrated in graded ethanol (95–100%) with 100% of propylene oxide as transitional solvent, and embedded in Epon resin blocks. Ultrathin sections (50–60 nm) were collected on copper grids, stained with uranyl acetate and lead citrate and finally examined under a transmission electron microscope (JEM1400, USA). Alterations in mitochondrial morphology were evaluated using the following parameters as described [13]: area of mitochondria (A_m), perimeter (P_m, length of the mitochondrial outline), circularity (a value of 1 indicates a perfect circle and values approaching 0 indicate an increasingly elongated shape; $4 \cdot \pi \cdot \text{area} / \sqrt{P_m}$), aspect ratio (AR, ratio between the major and minor axes of the ellipse equivalent to the mitochondria; max diameter / min diameter) and the percentage of the picture area occupied by mitochondria. Images were analyzed with ImageJ (Version 1.49b).

Table 1
Animal data.

	SED	ET	VPA
Initial body weight (g)	207.70 ± 3.41	206.50 ± 5.31	213.75 ± 2.59
Final body weight (g)	603.60 ± 13.81	539.33 ± 13.21*	497.31 ± 11.13*
Distance ($m \cdot day^{-1}$)	–	1437 ± 65	3920 ± 163*
Liver weight (g)	13.14 ± 0.56	12.47 ± 0.67	10.46 ± 0.32*
Heart weight (g)	1.39 ± 0.04	1.92 ± 0.08*	1.85 ± 0.08*
Liver weight/body weight ($mg \cdot g^{-1}$)	21.74 ± 0.61	23.57 ± 1.08	20.04 ± 0.851&
Heart weight/body weight ($mg \cdot g^{-1}$)	2.32 ± 0.09	3.87 ± 0.15*	2.99 ± 0.23*&

Values (mean ± SEM).
* vs. SED, significantly different ($p \leq 0.05$).
& vs. ET, significantly different ($p \leq 0.05$).

2.9. Statistical analysis

All data are expressed as the mean ± SEM (Standard Error of the Mean). Statistical analyses were performed using the GraphPad Prism 6.0 software (GraphPad software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to examine the possible effect of exercise. To determine specific group differences, one-way ANOVA was followed by the Bonferroni post-hoc test. In all cases, the significance level was set at 5%.

3. Results

3.1. Animals

There were no differences between groups regarding the initial body weight and liver–body weight ratio. ET and VPA increased the heart weight and heart–body weight ratio, and decreased the final body weight (Table 1). The animals from the VPA group increased their activity from the beginning of the protocol until the 7th week, with a subsequent decrease until the end (data not shown).

Semi-quantitative analysis of electron micrographs of liver tissue from sedentary and exercised groups indicated that physical exercise induced morphological alterations suggestive of adaptive remodeling. As seen in Fig. 1, an apparent increase in the mitochondrial area was observed. In addition, increased organelle circularity and estimated aspect ratio were noted in both exercised groups.

After the qualitative analysis of liver morphology suggested a mitochondrial adaptive remodeling, we next determined whether physical exercise altered the expression of proteins associated with liver mitochondrial biogenesis, as well as citrate synthase activity, which can be considered a marker of mitochondrial mass. As shown in Fig. 2, ET and VPA induced increases in citrate synthase activity (only significant in the VPA group), as well as in PGC-1α and mtTFA protein contents.

Additionally, exercise-induced alterations in proteins related to mitochondrial dynamics including fusion and fission were obtained (Fig. 3). Despite no changes between groups were observed regarding

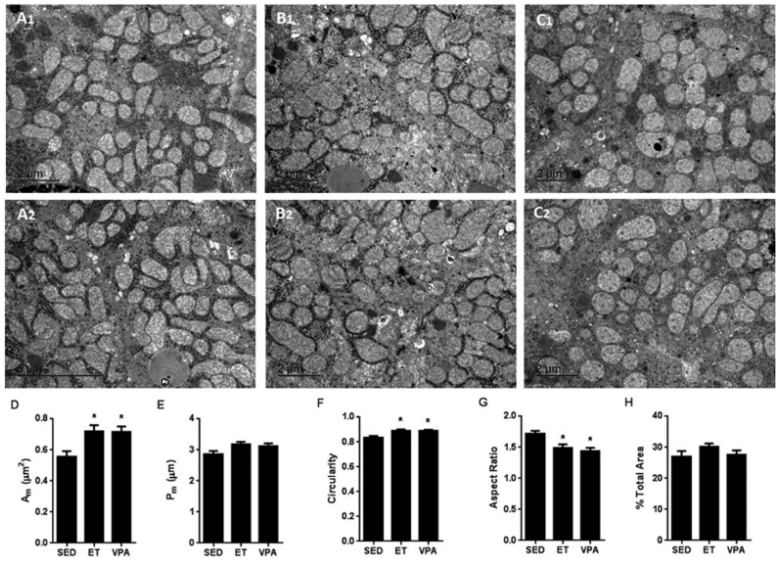

Fig. 1. Representative electron micrographs (A–C) and end-points for liver mitochondrial morphology (D–H) in all groups: (A_1; A_2) SED; (B_1; B_2) ET; (C_1; C_2) VPA (magnification: ×15 000). Results were expressed as means ± SEM. (*) vs. SED is significantly different ($p \leq 0.05$).

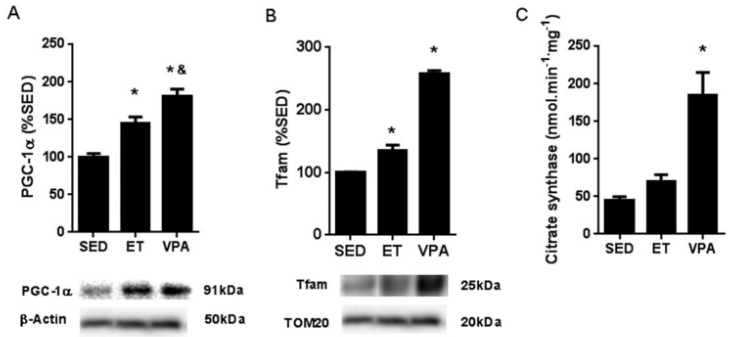

Fig. 2. Effect of ET and VPA on liver tissue PGC-1α (A), mitochondrial mtTFA (B) and citrate synthase activity (C). Typical immunoblots determined by Western blotting are presented below the histograms; TOM20 or β-actin was used as a control for protein loading in the gel for proteins measured in isolated mitochondrial fraction and whole tissue homogenate, respectively, and the results were expressed as percentage of SED group. Data are means ± SEM for liver mitochondria or liver tissue obtained from different preparations for each group. (*) vs. SED and (&) vs. ET are significantly different ($p \leq 0.05$).

MFN1 protein content, ET increased, and VPA decreased OPA1 protein content. Both ET and VPA increased DRP1 protein content in whole liver tissue.

We also measured alterations in proteins related with liver auto(mito)phagy signaling in order to determine possible exercise-related effects on cellular clearance and renewal signaling mechanisms (Fig. 4). As seen, ET increased the protein content of Beclin-1 and p62 possibly suggesting increased autophagosome formation. Curiously, VPA decreased Beclin-1 content as well as the Beclin-1/Bcl-2 ratio. Both exercise modalities increased LC3II content and the LC3I/LC3II ratio. ET increased Parkin and decreased PINK protein content, while VPA decreased Parkin content but did not alter PINK protein levels.

As cellular adaptations to different stimuli are thought to be, at least partially, regulated by oxidized/reduced alterations [14], two important redox modulators (SIRT3 and p66shc) were also measured in the present work (Fig. 5). As shown, exercise increased the protein content of mitochondrial SIRT3 but no alterations in tissue p66shc(ser36) and p66shc(ser36)/p66shc ratio were observed between groups. Also, VPA decreased p66shc(ser36) content.

4. Discussion

In a complementary paper recently published by our group, we showed that chronic exercise induces adaptations in molecular and functional parameters associated with mitochondrial functionality, including respiratory fitness, permeability transition pore resistance, metabolic enzymes, oxidative stress and apoptotic signaling [3]. However, given the relevant interplay between mitochondrial dynamics and autophagic mechanisms in cellular and mitochondrial quality control and metabolism, the present work aimed at determining, for the first time, the effect of two distinct chronic exercise modalities on quality control-related liver protein signaling markers. We considered these exercise modalities, as they are different regarding intensity and duration. ET imposes a forced pace running that is typical of a systematic exercise

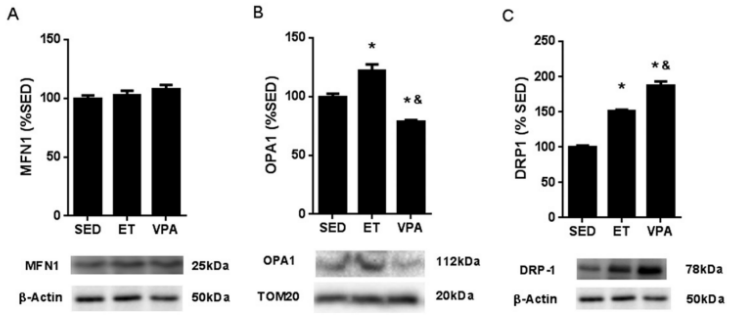

Fig. 3. Effect of ET and VPA on liver mitochondrial dynamics signaling proteins MFN1 (A), OPA1 (B) and DRP1 (C). Typical immunoblots determined by Western blotting are presented below the histograms; TOM20 or β-actin was used as control for protein loading in the gel for proteins measured in isolated mitochondrial fraction and whole tissue homogenate, respectively, and the results were expressed as percentage of SED group. Data are means ± SEM for liver mitochondria or liver tissue obtained from different preparations for each group. (*) vs. SED and (&) vs. ET are significantly different ($p \leq 0.05$).

training program and, on the other hand VPA, which mimics daily life voluntary physical activity, depends on animal motivation and is self-paced.

Complementarily to previously published data [3], a novel finding of the present work is that signaling markers of mitochondrial dynamics and autophagy are increased by both chronic exercise modalities, which suggest that these quality control-related processes are, at least in part, involved in liver plasticity and in the physiological adaptations that result in a more "energetically fit" and robust hepatic phenotype against the deleterious consequences of aging, metabolic diseases and drug-induced liver injury [1–3,8,15]. In the present study we did not test whether these particular exercise-related liver mitochondrial adaptations are also reproducible in models of obesity or other stressful pathological conditions to the liver. However, the obtained results

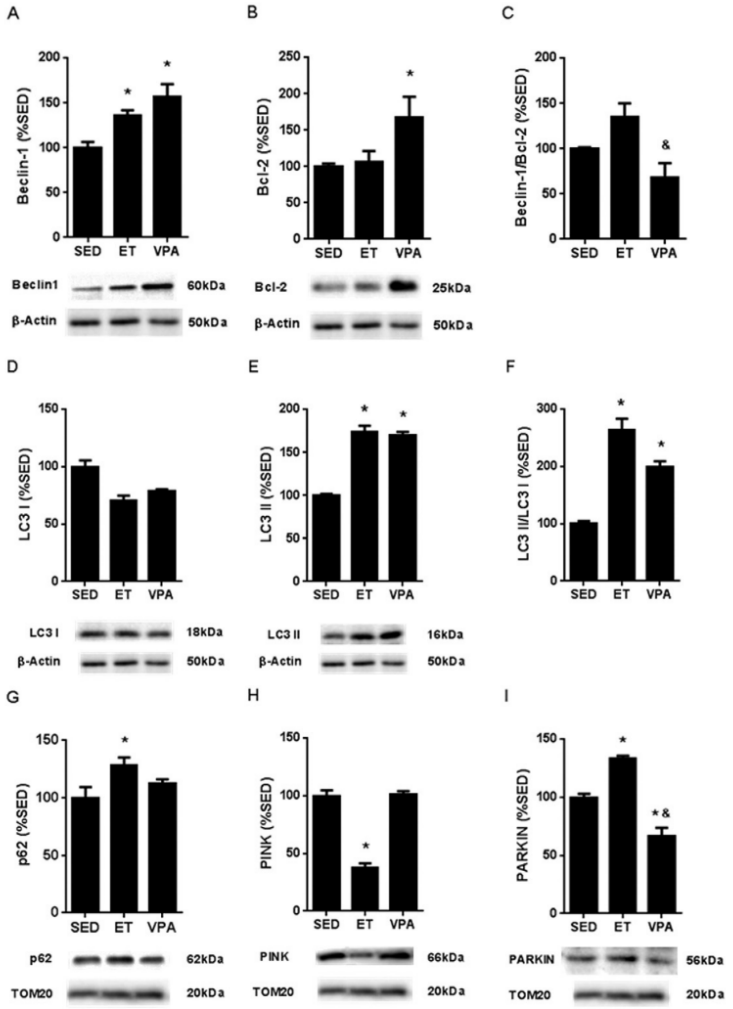

Fig. 4. Effect of ET and VPA on liver markers of auto(mito)phagy signaling Beclin-1 (A), Bcl-2 (B), Beclin-1/Bcl-1 (C), LC3I (D), LC3II (E), LC3II/LC3I (F), p62 (G), PINK (H) and Parkin (I). Typical immunoblots determined by Western blotting are presented below the histograms; TOM20 or β-actin was used as control for protein loading in the gel for proteins measured in isolated mitochondrial fraction and whole tissue homogenate, respectively, and the results were expressed as percentage of SED group. Data are means ± SEM for liver mitochondria or liver tissue obtained from different preparations for each group. (*) vs. SED and (&) vs. ET are significantly different ($p \leq 0.05$).

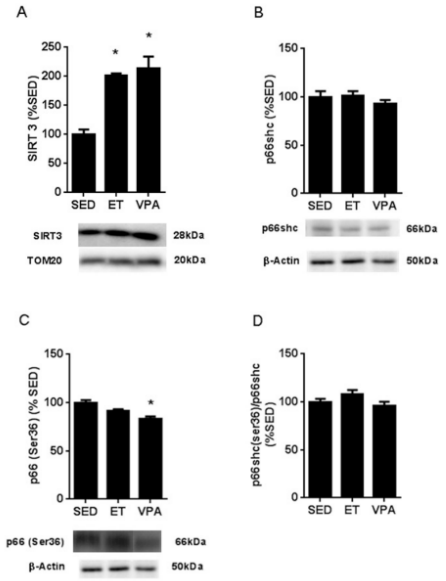

Fig. 5. Effect of ET and VPA on mitochondrial SIRT3 (A), liver p66shc (B), p66shc(ser36) (C) and p66shc(ser36)/p66shc (D) content. Typical immunoblots determined by Western blotting are presented below the histograms; TOM20 or β-actin was used as control for protein loading for proteins measured in isolated mitochondrial fraction and whole tissue homogenate, respectively, and the results were expressed as percentage of SED group. Data are means ± SEM for liver mitochondria or liver tissue obtained from different preparations for each group. (*) vs. SED is significantly different ($p \leq 0.05$).

may possibly anticipate that these quality control-related mechanisms are possibly involved in the protective phenotypes resulting from chronic exercise under the referred harmful conditions.

One possible contributor mechanism of the observed phenotypic improvement is mitochondrial biogenesis, of which PGC-1α is one of the master regulators. Alterations in the protein content of this transcription factor have been specifically associated with exercise training-induced improvements in oxidative capacity of several tissues, including the liver [16]. In the present study, the content of both PGC-1α and mtTFA in the liver as well as citrate synthase activity were augmented by chronic exercise (particularly higher in the VPA group, Fig. 2), suggesting increased liver mitochondrial biogenesis, which is also suggested from electron micrograph qualitative analysis. Despite both exercise modalities increased this mitochondrial biogenesis signaling response, it seems that VPA activity is more effective than ET in the induction of the mentioned adaptation.

Mitochondria are highly dynamic organelles and their morphology, size and distribution are precisely controlled in order to meet cellular requirements [17]. Moreover, to maintain an adequate mitochondrial network within the tissue, mitochondria undergo constant continuous shape cycles of fission and fusion, promoting the redistribution of refurbished contents throughout the system [17,18]. Molecular members of the machinery regulating mitochondrial fusion and fission have been identified; mitochondrial fission depends on DRP1, a cytoplasmic dynamin GTPase that participates in the fragmentation of mitochondria. Outer mitochondrial membrane fusion requires two mitofusins (MFN1 and 2) with apparent distinct importance. Fusion can also be triggered by inner membrane MFN1-dependent OPA1 [17]. Despite no alterations were observed in the content of MFN1 after exercise, our results indicate that both chronic exercise modalities interfere with proteins related to mitochondrial dynamic signaling and that this (particularly in fusion) is exercise type-dependent, as observed in OPA1 content (Fig. 3). Moreover, the increased DRP1 content observed after both exercise types suggests an increased activation of fission signaling, which is in accordance with the observed increase in morphological circularity.

The interplay between distinct quality control-related mechanisms is critical for mitochondrial homeostasis, being fusion, fission and mitochondrial autophagy coordinately controlled to determine the fate of an individual mitochondrion within the network, and ultimately regulating cellular quality control. Actually, these events determine the architecture of the entire mitochondrial population, influencing important mitochondrial features such as oxidative phosphorylation, calcium buffering capacity, and apoptosis [19]. Although the classical view proposes that the activation of fission facilitates and precedes mitochondrial autophagy [17], both fusion and mitophagy could also be complementary processes [19]. The selective fusion of healthy mitochondrial cohorts and the segregation of damaged mitochondria from the fused population not only prevent the migration of damaged components into fitter mitochondria, but also signal dysfunctional components for autophagy [19].

Despite differences between exercise modalities in the autophagosome formation-related protein Beclin-1 and in the Beclin-1/Bcl-2 ratio as well as in the PINK and Parkin contents, our results generally suggest that both exercise models promote increased liver autophagic signaling, as seen by the increased LC3II content and LC3I/LC3II ratio, which were accompanied by increased DRP1 (Figs. 3 and 4). It is however worthy to note that, the observed variations in liver mitochondrial Parkin expression in response to both chronic stimuli are exercise type-dependent as it increased with ET and decreased with VPA (Fig. 4). Moreover, liver mitochondrial PINK expression decreased after ET but not after VPA (Fig. 4). Further studies are needed to better understand the effects of different exercise modalities on the regulation and signaling of these autophagy-related adaptive processes. Despite the observed distinct responses, our results generally suggest that exercise may stimulate increased segregation of superfluous, damaged or less functional organelles for selective autophagy, possibly increasing the "healthy" mitochondrial network of the cells.

As the analyzed fusion-related proteins MFN1 and OPA1 did not follow the same pattern in response to the distinct exercise models used, the straight relationship between fusion signaling and other related processes is not clear. Further studies are in fact needed to better comprehend the influence of exercise on liver mitochondrial dynamics and quality control mechanisms.

Although the mechanisms by which physical exercise coordinates these quality control events in the liver are still unclear, possible hypotheses may be related to alterations in the content and/or activity of proteins that are essential in the control of post-translational modifications of other important proteins, thus influencing activity, trafficking or translocation processes through (de)phosphorylation, (de)methylation and (de)acetylation. One example is SIRT3, a mitochondrial deacetylase involved in the control of multiple metabolic processes that was increased by both ET and VPA (Fig. 5). Accordingly, Samant et al. [20] reported that SIRT3 promotes mitochondrial function not only by regulating the activity of metabolic enzymes, but also by regulating mitochondrial dynamics by targeting OPA1, since this fusion-related protein is hyperacetylated under pathological stress and the mitochondrial deacetylase SIRT3 was capable of deacetylating OPA1 and elevating its GTPase activity. Moreover, recent data from our laboratory suggest that chronic exercise-induced increased hepatic mitochondrial SIRT3 was accompanied by improved mitochondrial function and increased resistant to non-steroid anti-inflammatory drug-induced injury [8,21].

Despite some studies suggesting that p66shc promotes mitochondrial and intracellular redox (in)balance and activates apoptosis [22], no alterations in liver p66shc(ser36) and p66shc(ser36)/p66shc ratio were observed between groups. Also, VPA decreased p66shc(ser36) content. Further studies measuring the content of these proteins in sub-cellular fractions are needed in order to better understand its influence on redox-mediated adaptations induced by physical exercise in the liver.

In summary, these results complement our previous data [3] and suggest that chronic exercise alters liver mitochondrial dynamic and autophagy signaling hypothetically turning the mitochondrial phenotype to be more resistant to deleterious stimuli such as obesity-related disorders and drug-induced damage.

5. Conclusion

Both chronic exercise modalities augmented the expression of proteins related to mitochondrial biogenesis, and induced alterations in proteins involved in mitochondrial dynamics and autophagy signaling. This suggests that exercise can induce liver mitochondrial adaptive remodeling and hepatocyte renewal.

Acknowledgments

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Paper II

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ORIGINAL ARTICLE

Exercise mitigates diclofenac-induced liver mitochondrial dysfunction

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ABSTRACT

Background Several strategies have been developed to counteract liver injury as a consequence of nonsteroid anti-inflammatory drugs toxicity. Here, we aimed to determine whether physical exercise results in liver mitochondrial protection against *in vitro* diclofenac toxicity.

Material and methods Male adult Sprague-Dawley rats were divided into sedentary, 12-week endurance training (ET) and voluntary activity (VPA). *In vitro* liver mitochondrial function as assessed by oxygen consumption, transmembrane electric potential ($\Delta\Psi$) and susceptibility to the mitochondrial permeability transition pore (MPTP) was evaluated in the absence and presence of diclofenac. Mitochondrial oxidative stress markers [MnSOD, aconitase, -SH and MDA, SIRT3, p66shc(Ser36)/p66shc ratio] and apoptotic signalling (caspases 3, 8 and 9, Bax, Bcl-2 and CypD) were assessed. Content of OXPHOS components and qualitative liver morphological evaluation were assessed.

Results Despite no effects of ET and VPA on basal liver mitochondrial oxygen consumption or $\Delta\Psi$ endpoints, exercised animals showed lower susceptibility to MPTP. Diclofenac-induced decrease in $\Delta\Psi$, increased state 4 respiration and susceptibility to MPTP opening were all prevented by exercise. Under untreated conditions, VPA group showed higher aconitase activity, while ET decreased MDA and increased Bax content. VPA decreased p66shc(Ser36), complex III and V OXPHOS subunits. Both ET and VPA increased complex IV OXPHOS subunit, and SIRT3 and Bcl-2 content and decreased caspase 9 activity. Unexpectedly, ET and VPA decreased ANT.

Conclusions Both chronic physical exercise models augmented the resistance to *in vitro* diclofenac-induced mitochondrial alterations, including increased MPTP susceptibility, possibly by modulating oxidative stress and MPTP regulators.

Keywords Apoptotic signalling, bioenergetics, exercise, liver toxicity, nonsteroid anti-inflammatory drugs, oxidative stress.

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Introduction

The prescription and use of pharmacological agents, including nonsteroid anti-inflammatory drugs (NSAIDs), has been increasing worldwide. Diclofenac is one of the most consumed NSAID, used as an anti-inflammatory and analgesic in different clinical settings. Mitochondrial damage and oxidative stress are major endpoints of diclofenac toxicity contributing to drug-induced liver injury [1]. It is described that diclofenac disturbs *in vitro* liver mitochondrial function at multiple levels, including the phosphorylating system and mitochondrial permeability transition pore (MPTP), being associated with increased oxidative stress and apoptotic signalling [2–7].

Several nonpharmacological interventions antagonize metabolic and cardiovascular diseases [8,9] as well as drug-induced liver dysfunction [10]. Physical exercise is a recognized and effective nonpharmacological strategy [11–13]. Exercise afforded liver protection against deleterious conditions [11,13,14]. Our group recently showed that endurance training (ET) attenuates some of the adverse effects associated with

the NSAID salicylate treatment on liver mitochondria [12]. However, whether exercise provides liver mitochondrial protection against diclofenac toxicity is still unknown. The hypothesis of present work is that exercise prevents the *in vitro* toxicity of diclofenac on isolated hepatic mitochondria, being relevant as the knowledge of the mechanisms by which exercise modulates mitochondrial bioenergetics may facilitate the understanding of how these interventions can impact drug-induced organ injury.

Methods

Animals and exercise

Eighteen Sprague-Dawley male rats (aged 6 weeks) were randomly divided into three groups (n = 6 per group): sedentary (SED), endurance training (ET) and free wheel voluntary physical activity (VPA).

The animals from the ET group were exercised 5 days per week (Monday–Friday) in the morning (between 10:00 AM. and 12:00 PM), for 12 weeks on a LE8700 motor-driven treadmill (Panlab Harvard Apparatus, Holliston, MA, USA) [15].

The animals from the VPA group were housed in a polyethylene cage equipped with a running wheel [perimeter = 10·5 cm, Type 304 stainless steel (2154F0106-1284L0106) Technicplast, Casale Litta, Italy]. The rats were allowed to exercise voluntarily with unlimited access to the running wheel 24 h/day. Running distance was recorded using a digital counter (ECO 701 Hengstler, Lancashire, UK).

Animal sacrifice, blood, liver, soleus extraction and isolation of liver mitochondria

Forty-eight hours after the last exercise bout, nonfasted rats were euthanized by cervical dislocation. Approximately 3–5 mL of blood was collected, and plasma was separated and stored at −80 °C, and later on, alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured using conventional methods with an Beckman–Coulter AU5400 automated clinical chemistry analyser. After quickly opening the abdominal cavity, rat livers were then rapidly excised, rinsed, weighed and separated in portions of approximately 50 mg for later semi-quantification of protein expression by Western blotting. Right lobe of the liver was homogenized, and isolated mitochondria were obtained using differential centrifugation [12].

The right *soleus* muscle was also extracted, weight and stored for later determination of citrate synthase activity [16].

Mitochondrial respiratory activity and transmembrane electric potential

Respiratory function was polarographically measured using a Clark-type oxygen electrode (YSI), and transmembrane electric potential ($\Delta\psi$) was indirectly monitored based on the activity of the lipophilic cation tetraphenylphosphonium (TPP^+) using a TPP^+-selective electrode [17].

Reactions were conducted at 30 °C in a magnetically stirred glass chamber containing 1 mg/mL of mitochondrial protein in a respiration buffer (130 mM sucrose, 50 mM KCl, 2·5 mM $MgCl_2$, 2·5 mM KH_2PO_4, 5 mM HEPES, 0·1 mM EGTA, pH 7·4). After 1-min equilibration period, mitochondrial respiration was initiated by adding glutamate/malate (G/M, 5 and 2.5 mM, respectively) and 3 μM TPP^+. Phosphorylation cycle and state 3 respiration were obtained after adding ADP (150 nmol), and state 4 was measured as the rate of oxygen consumption after ADP phosphorylation. The RCR (state3/state 4) and the ADP/O (the number of nmol ADP phosphorylated by natomsO consumed during state 3), were calculated [18].

Oxygen consumption and $\Delta\psi$ endpoints were also determined in the presence of diclofenac (15 and 25 μM) incubated in the respiratory medium before ADP addition. The used concentrations of diclofenac were in the range of 0·01–0·2 mM, which according to previous studies led to uncoupling and are close to the pharmacological doses [6].

Mitochondrial permeability transition pore

Mitochondrial osmotic volume changes were followed by monitoring the absorbance decrease at 540 nm with a Jasco V-630 spectrophotometer. The average rate of swelling progression after calcium addition was defined as a MPTP susceptibility index. A negative control was performed with 1 μM of cyclosporin A, a MPTP desensitizer [19].

Mitochondrial oxidative damage and antioxidants

Before analysis, membranes of isolated mitochondrial fractions were disrupted by a combination of freeze–thawing cycles. Malondialdehyde content was measured by colorimetric assay [20], according to a modified procedure [21]. Oxidative modified -SH groups, including GSH and other SH-containing proteins, were quantified [22]. Aconitase activity was measured by monitoring the formation of cis-aconitate from isocitrate [15], and superoxide dismutase (MnSOD) activity was measured using a commercially available kit (RANSOD; Random Labs, Crumlin, UK) according to manufacturer instructions.

Liver caspase and soleus citrate synthase activity

Caspases-like activities were determined by following the detection of the chromophore pNA after cleavage from the labelled substrate Ac-LEHD-p-nitroanilide at 405 nm [23]. *Soleus* citrate synthase activity was measured using the method proposed by Coore *et al.* [16].

Immunoblotting for detection of liver mitochondrial proteins

Equivalent amounts of liver mitochondria (1 mg/mL) were denatured in sample loading buffer and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS/PAGE) (12% gels) [24], followed by blotting on PVDF membranes [25]. Mitochondrial content of the outer mitochondrial membrane translocator TOM20 was used as control for protein loading.

Membranes incubated with anti-Bcl-2 (1 : 1000; #2870 rabbit monoclonal IgG), anti-Bax (1 : 1000; #2772 rabbit polyclonal IgG), anti-CypD (1 : 1000; ab110324 mouse monoclonal IgG), anti-ANT 1/2/3 (Q-18) (1 : 500, sc-9300 goat polyclonal IgG), anti-Sirt3 (1 : 1000, #2627 rabbit monoclonal IgG), anti-shc (p66; 1 : 500, # 2432 rabbit polyclonal IgG), anti-shc [p66 (pSer36)] (1 : 1000, 6E10 mouse monoclonal IgG) and anti-TOM20 (1 : 1000; sc-11415 rabbit polyclonal IgG). In addition, mitochondrial proteins blotted onto membranes were incubated with anti-OXPHOS subunits (1 : 1000 ab110413 mouse monoclonal IgG).

Protein bands were visualized by treating the immunoblots with ECL® PlusTM, visualized with the ChemiDoc XRS+ system (Bio-Rad Laboratories, Amadora, Portugal) and analysed with the image analysis program Image Lab software (Bio-Rad Laboratories). The results were expressed as percentage variation of SED control group.

Transmission electron microscopy

To observe exercise-induced alterations in liver mitochondrial morphology, transmission electron microscopy (TEM) was performed. The images obtained were collected at original magnifications of 15 000×, digitalized and stored in a tagged image file format in the microscope image analysis software (Quartz PCI, Scientific Image Management System, v 5.1; Quartz Image Corp., Vancouver, Canada).

Statistical analysis

Data are expressed as the mean ± SEM. Two-way ANOVA was used to examine possible effect of treatment and/or exercise. To determine specific group differences, two-way ANOVA followed by Bonferroni *post hoc* test was used. The significance level was set at 5%.

Results

Animal characterization

As seen in Table 1, no differences between groups were observed regarding initial body weight and liver body weight ratio. Both ET and VPA decreased heart and heart body weight ratio, while only VPA decreased final body and liver weights. ALT and AST in VPA were higher than SED and ET groups. Although unexpected, this slight increase can hypothetically be related to the fact that animals from VPA group had free access to running wheels until the time before sacrifice. Therefore, the consequent possible increased running activity of these animals in the period preceding sacrifice and blood collection could justify this possible transient alteration [26]. However, it conflicts with studies reporting no changes of these markers after chronic exercise [27].

During the 12 weeks of the protocol, the animals from VPA group increased their activity from the beginning of the protocol until the seventh week, which is followed by a decrease until the end of protocol (data not shown).

Table 1 Animal data

	SED	ET	VPA
Initial body weight (g)	207·4 ± 3·91	214·0 ± 3·76	211·5 ± 1·55
Final body weight (g)	598·5 ± 10·58	522·3 ± 9·87[c]	498·3 ± 5·98[c]
Distance (m/day)	–	1437 ± 65	3920 ± 163[d]
Liver weight (g)	13·14 ± 0·56	12·47 ± 0·67	10·46 ± 0·32
Liver weight/body weight (mg/g)	0·022 ± 0·001	0·023 ± 0·001	0·020 ± 0·001
Heart weight/body weight (mg/g)	2·32 ± 0·09	3·87 ± 0·15[c]	2·99 ± 0·23[c]
Femur length/body weight (mm/g)	0·08 ± 0·00	0·08 ± 0·00	0·09 ± 0·00
Soleus citrate synthase activity (nmol/min/mg)	10·94 ± 2·07	23·34 ± 1·79[c]	11·22 ± 2·65
Alanine aminotrasferase (ALT) (U/L)	189 ± 14·07	201 ± 16·54	233·6 ± 6·16[c,d]
Aspartate aminotransaminase (AST) (U/L)	41 ± 2·55	40 ± 2·95	53·33 ± 3·11[c,d]

Values (mean ± SEM). (c) vs. sedentary, (d) vs. treadmill are significantly different ($P \leq 0.05$).

Respiratory activity, electric transmembrane potential and transmission electron microscopy

Figure 1 (a–f) shows the effects of ET and VPA on liver mitochondrial oxygen consumption and ΔΨ endpoints in the presence of the two concentrations of diclofenac. In the SED group, state 4 increased with *in vitro* diclofenac incubation, while no drug-related effect was observed in state 3. Diclofenac significantly compromised the respiratory control ratio (RCR) in all groups. However, the uncoupling effect caused by diclofenac in liver mitochondria from SED animals as suggested by state 4 increase was significantly attenuated by chronic exercise (ET and VPA). No ET- and VPA-related effects on RCR were

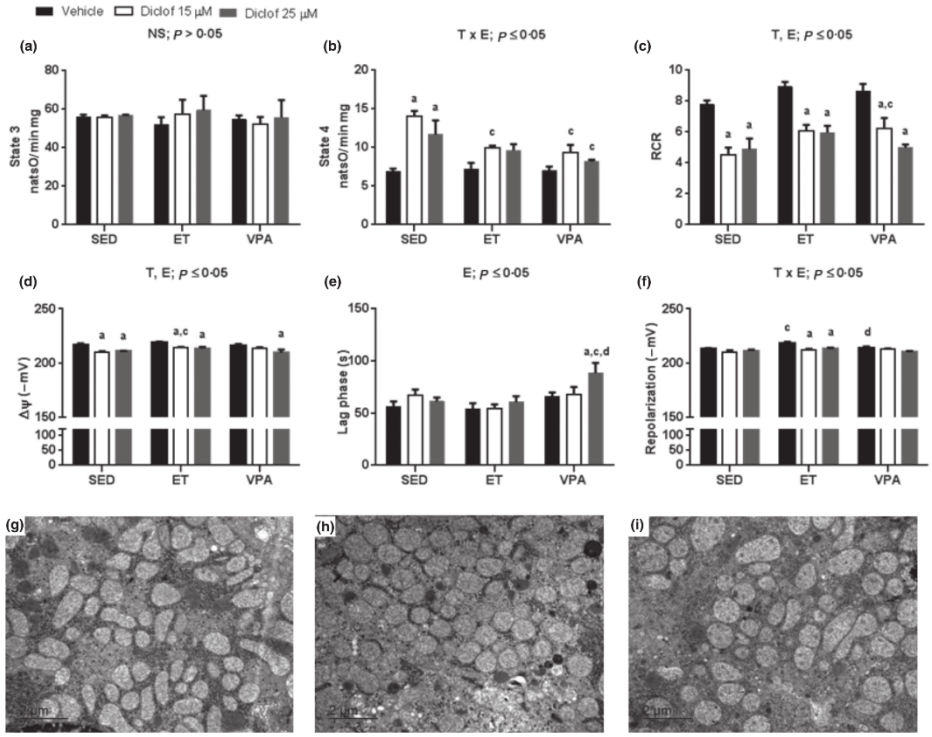

Figure 1 Effects of ET and VPA on liver mitochondrial oxygen consumption (a–c), Δψ fluctuations (d–f) in untreated vehicle conditions and in the presence of diclofenac and qualitative analysis of the ET and VPA effects on liver tissue morphology, particularly on mitochondrial alterations (g–i). Figure shows mitochondrial respiration, Δψ with glutamate (5 mM) plus malate (2·5 mM) as substrates in a total volume of 1·1 mL and representative electron micrographs of liver tissue from (a) SED, (b) ET and (c) VPA groups. (Magnification: 15 000×). Data are means ± SEM for liver mitochondria (1 mg/mL protein) obtained from different mitochondrial preparations for each experimental group ($n = 6$). (a) vs. vehicle; (c) vs. sedentary; and (d) vs. treadmill are significantly different ($P \leq 0{\cdot}05$). Effects of exercise (E), treatment (T) or their interaction (E × T) are shown; nonsignificant (NS, $P > 0{\cdot}05$).

observed with the exception of 15 μM diclofenac incubated with the VPA group, in which the RCR was higher in VPA than in SED group.

Simultaneously, with oxygen consumption, fluctuations in ΔΨ were also measured under vehicle and drug conditions (Fig. 1 d–f). *In vitro* diclofenac treatment caused a significant decrease in liver mitochondrial maximal ΔΨ in the SED group. However, ET and VPA attenuated the ΔΨ-related uncoupling effects of diclofenac but only for the 15 μM concentration. No differences in ADP phosphorylation lag phase resulted from exercise alone or after treatment with diclofenac, with the exception of an unexpected increase when mitochondria from VPA group were incubated with 25 μM diclofenac.

The effect of exercise on liver ultrastructure, in particular on liver mitochondria, was qualitatively described (Fig. 1 g–i). From the qualitative analysis of the electron micrographs, one can suspect that ET group presented signs of mitochondrial remodelling, including variations in size and shape. As can be observed in the representative micrograph (panel h), liver mitochondria from ET group appear to have an increased mitochondria density, a more round-like shape as well as an apparent cristae density when compared to SED and VPA groups.

OXPHOS subunits were also semi-quantified by Western blotting (Fig. 2). As shown, both ET and VPA *per se* increased liver mitochondrial complex IV OXPHOS subunit and VPA decreased complex III and V OXPHOS subunits. No alterations were detected in complex I and II subunits.

Mitochondrial oxidative stress and damage

We next analysed whether alterations mitochondrial markers of oxidative stress and damage induced by exercise could, in part, contribute to explain the obtained functional respiratory and ΔΨ-related measurements. Mitochondrial SOD and aconitase activities, as well as MDA and -SH contents, were quantified under basal untreated conditions (Fig. 3 a–d). VPA induced an increase in aconitase activity (only a nonsignificant increase was observed in the ET group) and both chronic exercise models decreased liver mitochondrial MDA content. No significant differences were observed regarding MnSOD activity and -SH groups.

Alterations in liver mitochondrial content in SIRT3, p66shc and p66shc(Ser36), emerging proteins involved in the control of mitochondrial metabolism and redox state, were also measured by Western blotting (Fig. 3 e–h). As shown, VPA decreased p66shc(Ser36) and both chronic exercise models increased liver SIRT3 content. No significant difference between groups was observed regarding p66shc expression and p66shc(Ser36)/p66shc ratio.

MPTP induction, regulation and apoptotic signalling

Another sensor of mitochondrial toxicity induced by diclofenac is the increased susceptibility to MPTP. The osmotic average rates of absorbance decrease after calcium addition were evaluated during swelling experiments under basal vehicle conditions and in the presence of both diclofenac concentrations

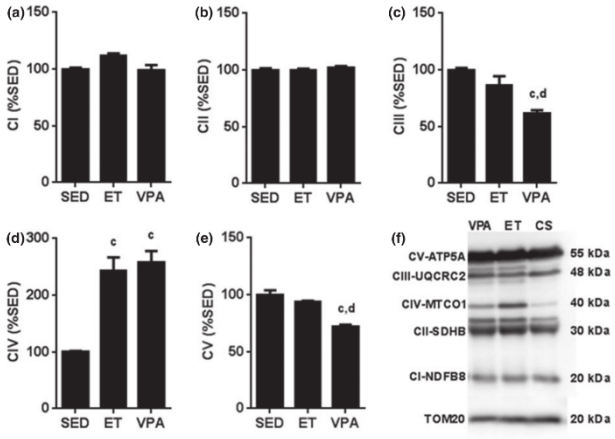

Figure 2 Effect of ET and VPA on liver mitochondrial OXPHOS complex subunits. Complex I NDUFB8-20 kDa (a), complex II SDHB-30 kDa (b), complex III Core protein 2-48 kDa (c), complex IV subunit I-40 kDa (d) and ATP synthase alpha subunit-55 kDa (e). Typical immunoblots of respective proteins are presented (f). TOM20 was used as control for protein loading, and the results were expressed as percentage of SAL+SED group. Data are means ± SEM for liver mitochondria obtained from different preparations for each animal and group ($n = 4$). (c) vs. sedentary and (d) vs. treadmill are significantly different ($P \leq 0{\cdot}05$).

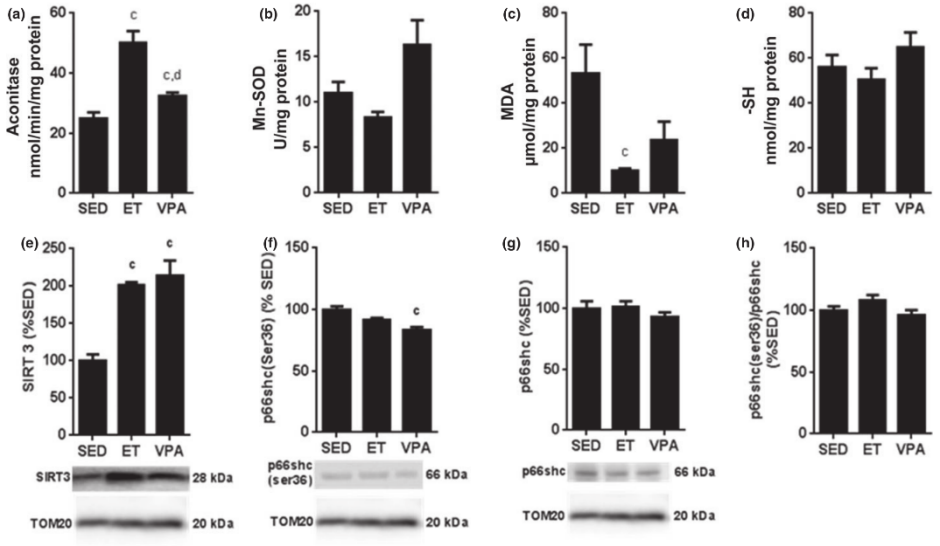

Figure 3 Effect of ET and VPA on mitochondrial markers of oxidative stress and damage measured under untreated control conditions. Typical immunoblots of markers determined by Western blotting are presented below the histograms; TOM20 was used as control for protein loading, and the results were expressed as percentage of SAL+SED group; data are means ± SEM for liver mitochondria or liver tissue obtained from different preparations for each animal and group (n = 4). (c) vs. sedentary; (d) vs. treadmill. $P \leq 0{\cdot}05$.

(Fig. 4 a–b). 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 may act as a potent pore sensitizer independently of other regulators such as the redox state of pyridine nucleotides, $\Delta\Psi$, pH and ROS production [28]. Hence, we decided to perform all studies involving MPTP induction with succinate as the substrate.

As seen, both chronic exercise types induced a significant decrease in susceptibility of hepatic mitochondria to undergo permeability transition pore opening. This protection was observed under control (vehicle) conditions and in the presence of the two used diclofenac concentrations.

The next step was to evaluate the content of proteins or enzymes known to be involved in the regulation and/or sensitization of the MPTP as well as in apoptotic signalling under untreated conditions. These include the mitochondrial content of cyclophilin D, ANT, Bax and Bcl-2 as well as tissue caspases 3, 8 and 9 activities (Fig. 5 a–h). As depicted, both ET and VPA increased mitochondrial Bcl-2 and decreased the Bax/Bcl-2 ratio and caspase 9 activity (Fig. 5 b, c and h). The expression of mitochondrial Bax was only increased in ET group (Fig. 5 a). Unexpectedly, both exercise types induced a decrease in ANT content (Fig. 5 f), while CypD was unaltered. Neither ET nor VPA induced significantly alterations in the activities of liver tissue caspase 3 and 8.

Discussion

The interest on the effects of physical exercise in the context of hepatic alterations in many diseased states has emerged. In fact, studies showed that an inactive life style lacking daily VPA or a low level of aerobic capacity translates into a liver phenotype more susceptible to the deleterious consequences of

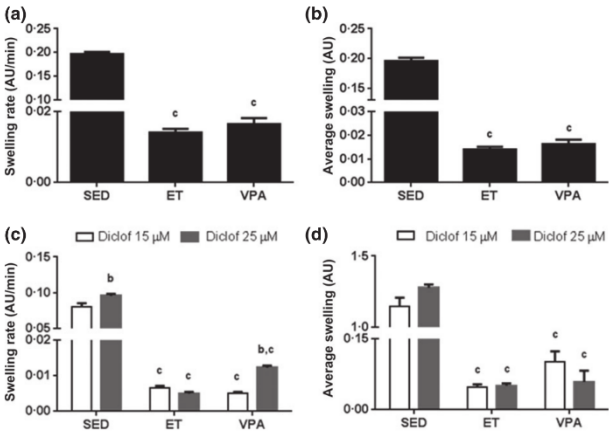

Figure 4 Effect of ET and VPA on diclofenac-induced MPTP opening susceptibility in succinate-energized liver mitochondria. Calcium-induced MPTP was followed under untreated conditions (panels a and b) and in the presence of diclofenac (panels c and d). (b) vs. [15 μM] diclofenac; (c) vs. sedentary; (d) vs. treadmill. $P \leq 0{\cdot}05$. The absorbance of mitochondrial suspension was followed at 540 nm. Data are means ± SEM for liver mitochondria obtained from different preparations for each animal and group ($n = 6$).

metabolic-related complications have been published [11,13,14]. However, the role of exercise in hepatic deleterious alterations caused by the consumption of pharmacological agents has received less attention. In the present study, we used isolated liver mitochondria as an *in vitro* toxicological model to test the hypothesis that both ET and VPA improve hepatic harmful consequences of diclofenac, a widely used NSAID. The results showed for the first time that both models of chronic exercise attenuated liver mitochondrial functional impairments induced by diclofenac, namely state 4 and MPTP induction. These functional improvements were accompanied by favourable alterations in basal oxidative stress markers as well as in some proteins associated with apoptotic signalling and MPTP regulation.

Even though the underlying mechanisms are still unknown, the redox challenge caused by exercise, where systemic signalling (such as inflammatory mediators or hormones) has an important role, is thought to lead to liver tissue remodelling. Furthermore, chronic exercise exerts a whole-body beneficial effect that exceeds striated muscle adaptation, likely through mechanosensitive afferent nerves and β-endorphin release into body fluids that promote mitochondrial remodelling in distant organs, including the liver [29,30].

The protective liver mitochondrial phenotype against diclofenac side effects resulting from ET and VPA is in accordance with recent data from our group, in which salicylate-induced liver mitochondrial dysfunction was antagonized by five weeks of ET [12].

We here showed that although the models of chronic exercise used did not improve the impairments in respiratory activity caused by diclofenac, namely the decreased coupling between oxygen consumption and ADP phosphorylation (Fig. 1), decreased stimulation of state 4 (Fig. 1) and increased resistance against calcium-induced MPTP were observed both in the presence and absence of diclofenac (Fig. 4). These responses observed against diclofenac-induced liver bioenergetics disruption suggest that physical exercise may have distinct liver mitochondrial targets, resulting in different adaptive alterations.

Curiously, the respiratory activity was accompanied by decreased content in ETC complex III and V subunits in VPA group and an increased complex IV subunit in both ET and VPA (Fig. 2), alterations that did not seem to parallel with variable responses in respiratory activity both under basal untreated conditions and in the presence of diclofenac.

One important liver mitochondrial frailty caused by diclofenac is sensitization to MPTP [2], a critical sensor of cellular toxicity, which is activated under conditions of increased oxidative stress and calcium overload [31,32]. As mentioned previously, both ET and VPA augmented the resistance of liver mitochondria against diclofenac-induced MPTP opening (Fig. 4). The decreased oxidative stress and mediated alterations observed in exercised groups seen by the increased aconitase activity and by decreased lipid peroxidation may be possible explanations for the observed protection. Moreover, ET and VPA resulted in increased content of liver

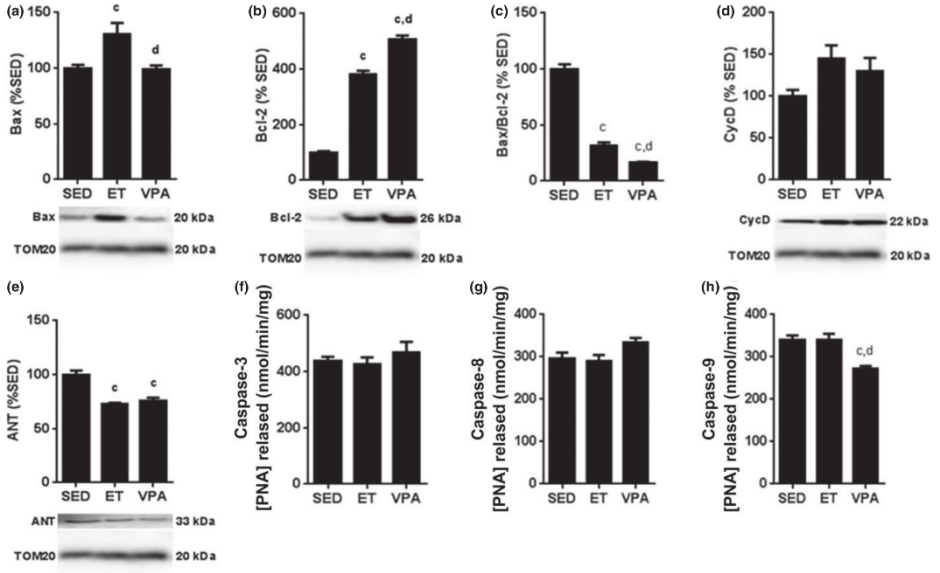

Figure 5 Effect of ET and VPA on MPTP regulators and apoptotic signalling markers measured under untreated control conditions. Typical immunoblots of markers determined by Western blotting are presented below the histograms; TOM20 was used as control for protein loading, and the results were expressed as percentage of SAL+SED group; data are means ± SEM for liver mitochondria or liver tissue obtained from different preparations for each animal and group (n = 4). (c) vs. sedentary; (d) vs. treadmill. $P \leq 0{\cdot}05$.

mitochondrial SIRT3 (Fig. 3e), which may eventually contribute to the protection conferred against *in vitro* diclofenac effects. SIRT3 has a role in the deacetylation of innumerous mitochondrial proteins, which are prone to hyperacetylation in response to metabolic stress, including complexes I–V and citric acid cycle enzymes, as well as antioxidant enzymes such as MnSOD [33,34]. Of note is the fact that our results regarding SIRT3 did not completely parallel with variations in liver MnSOD and OXPHOS subunits observed under basal conditions after exercise.

Considering that VPA significantly decreased mitochondrial p66shc(Ser36), it is possible that exercise may influence signalling pathways involving p66shc and its regulation of ROS production. In fact, p66shc activation involves its phosphorylation-mediated basal oxidative stress, allowing its translocation to mitochondria [35], where it catalyses ROS production possibly via cytochome c oxidation [36]. Further studies are needed to ascertain whether alterations in mitochondrial redox state regulators through exercise translate into distinct functional mitochondrial activities in response to NSAID treatment.

We also determined the effect of both models of chronic exercise in the relative content of proteins involved in apoptotic signalling and/or in the regulation of the multi-protein complex MPTP, including Bax, Bcl-2, CypD, ANT (Fig. 5) as well as the above-referred SIRT3 (Fig. 3). Despite no detected variations in CypD content were observed, it is possible that the observed decrease in mitochondrial Bax-to-Bcl-2 ratio, associated with the up-regulation of SIRT3 induced by exercise may be relevant in the increased resistance of liver mitochondria to the MPTP opening and consequent downstream apoptotic activation. Bax, and perhaps its homologs Bak and Bid, may regulate MPTP opening, being these effects antagonized by the anti-apoptotic Bcl-2 family members [37]. In addition, SIRT3 prevents apoptosis by decreasing ROS production by

mitochondria, leading to MPTP inhibition by CypD deacetylation [38]. Therefore, mitochondrial deficits associated with pathophysiological conditions that include NSAID treatment can be slowed or even prevented by SIRT3 activation [39].

In conclusion, both physical exercise models studied augmented the hepatic mitochondrial resistance to increased MPTP opening sensitization and against the uncoupling effect caused by *in vitro* diclofenac treatment. It is possible that the modulator effect of chronic exercise on oxidative stress and MPTP regulators, including on SIRT3 and Bcl-2, may lead to protection against drug-induced liver injury.

Our results agree with previous work stressing the importance of considering ET and VPA as practical nonpharmacological strategies to be used in subjects with risk factors for the toxicity of several clinically used drugs and other pathological liver conditions such as NASH [40,41]. Albeit isolated mitochondrial fractions are proved to be an established model to investigate drug-induced safety/toxicity [42], further *in vivo* studies using acute and chronic diclofenac treatment schedules are warranted to comprehend if the same outcomes here obtained can be translated into to a more complex biological system. Questions to be answered in future *in vivo* studies may include the following: (i) Is the protective role of physical exercise dependent on the dosage of administrated drug? (ii) Is there a host-dependent effect on the ET/VPA protective priming effects? and Does other hepatic mitochondrial-related mechanisms such as mitophagy and mitochondrial dynamics are implicated in the referred protective phenotype?

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Paper III

Physical exercise positively modulates DOX-induced hepatic oxidative stress, mitochondrial dysfunction and quality control signaling (In review). Santos-Alves E[1,2,3], Rizo-Roca D[1,2], Marques-Aleixo I[1,2,4], Martins S[5,6,7], Guimarães JT[5,6,7], Oliveira PJ[8], Torrella JR[3], Magalhães J[1,2], Ascensão A[1,2]

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Abstract

Doxorubicin (DOX), a widely used and efficient antineoplastic agent, is mainly limited by cardiotoxicity, although other tissues including liver are also affected. The effects of exercise to cope with DOX side-effects has already been studied in the heart and brain, demonstrating successful results. However, the benefits of this non-pharmacological strategy have not been so extensively checked in the liver. We here aimed to ascertain whether exercise could mitigate DOX-induced liver harmful effects using mitochondria as a model for evaluating toxicity.

Twenty-four male rats were divided into four groups: SED+SAL (sedentary with saline administration), SED+DOX (sedentary with DOX administration), ET+DOX (endurance-trained with DOX administration) and VPA+DOX (voluntary physical activity with DOX administration). Isolated liver mitochondria were obtained for evaluation of their respiratory activity and transmembrane electrical potential endpoints. Molecular markers of oxidative damage (carbonyls, MDA, aconitase, MnSOD), mitochondrial dynamics (PGC-1α, TFAM, OPA1, DRP1, MNF1) and auto(mito)phagy signaling (p62, LC3, Beclin1, Bcl-2, PINK, Parkin) were measured. Transmission electron microscopy evaluation was used to analyze mitochondrial morphological alterations.

When compared to SED+SAL, respiratory function of SED+DOX was compromised. Also decreased SOD and aconitase activities and increased MDA content, decreases in PGC-1α, TFAM, OPA1 and MNF1 expressions, and increases in DRP1 and LC3II/LC3I ratio were observed after DOX administration. However, these alterations were reverted or mitigated in ET+DOX group. Semi-quantitative and qualitative analyzes from microphotographs showed that liver mitochondria of SED+DOX animals were more circular and had lower density, whereas the animals with exercise showed a tendency to revert this phenotype and increase the mitochondrial density.

Taken together, our results suggest that physical exercise, particularly ET, positively reversed deleterious effects caused by DOX administration, such as oxidative damage, mitochondrial dysfunction, and altered mitochondrial dynamics toward fission, thus contributing to increase liver resistance against DOX administration.

Keywords: Exercise, doxorubicin, hepatotoxicity, mitochondrial dysfunction

1. Introduction

Doxorubicin (DOX), also known as Adriamycin, is an effective anticancer agent against several types of malignancy, although limited by a dose-dependent cardiotoxicity (Carvalho et al., 2014; Šimůnek et al., 2009). DOX-induced cardiotoxicity is mainly associated with increased oxidative stress and mitochondrial dysfunction, which leads to disturbances in energy metabolism (Kalender et al., 2005; Nagai et al., 2016). Although cardiovascular toxicity is the most relevant of DOX side-effects, others tissues, such as the liver, are also affected by DOX-related although its mechanisms have not been elucidated yet (Damodar et al., 2014; Nagai et al., 2016). Hepatic alterations caused by DOX include increased oxidative stress as well as the activation of apoptotic and autophagy signalling (Dirks-Naylor et al., 2014; El-Moselhy and El-Sheikh, 2014; Nagai et al., 2016). Moreover, perturbations in mitochondrial bioenergetics occur in livers from DOX-treated animals (Patel et al., 2010; Pereira et al., 2012; Santos et al., 2002), reinforcing the role of these organelles as therapeutic targets to reduce DOX-induced off-target toxicity (Oliveira, 2011; Wallace, 2008).

Several non-pharmacological strategies have been used to mitigate DOX toxicity, being physical exercise a strategy that positively modulates cardiac function at several levels of cellular organization, including mitochondria, contributing to decrease DOX cardiotoxicity (Ascensão et al., 2011a, 2011b; Magalhães et al., 2017; Marques-Aleixo et al., 2015a, 2017). However, data on the cross-tolerance mechanisms of exercise against hepatic DOX-related effects are scarce. Considering the role of liver in drug detoxification, the preservation of liver structure and function is essential in DOX-related metabolism and therapeutics. Moreover, being physical exercise a potential strategy in the establishment of an hepatic mitochondrial phenotype more resistant to deleterious conditions caused by drug- and diet-induced liver injury (Ascensão et al., 2013; Gonçalves et al., 2013, 2016; Santos-Alves et al., 2014). Therefore, we here hypothesized that physical exercise performed during the course of DOX-treatment could improve liver mitochondrial defenses, thus possibly contributing to increase the hepatic resistance against drug-related side-effects. Although several studies showed that exercise can improve mitochondrial function in numerous metabolic diseases, such as non-alcoholic steatohepatitis (NASH) or diabetes type II (Gonçalves et al., 2014, 2016; Rector et al., 2008), and our laboratory has already demonstrated that 12 weeks of exercise improved mitochondrial and liver functionality in lean rats, no data is available regarding DOX hepatic toxicity (Santos-Alves et al., 2015). To test whether exercise induces a protective mitochondrial phenotype against DOX-induced hepatotoxicity, we investigated liver mitochondrial functional endpoints, including

oxygen consumption and transmembrane electric potential, oxidative stress markers and autophagy signaling, as well as mitochondrial biogenesis and dynamics.

2 Methods

2.1 Chemicals

Adenosine 5´diphosphate sodium salt (ADP; A2754), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; C2920), Glutamic acid (G1626), Glutaraldehyde solution (G5882), Malic acid (M7397), Oligomycin (O4876), Osmium tetroxide (201030), Propylene oxide (110205), Sodium cacodylate trihydrate (C0250), Tetraphenylphosphonium chloride (TPP^+; 218790) were purchased at Sigma Aldrich (Darmstadt, Germany)

2.2 Animals

All experimental procedures were conducted in accordance to the Directive 2010/63/EU of the European Parliament and were approved by the Ethics Committee of the Research Centre in Physical Activity, Health and Leisure (Faculty of Sport, University of Porto). Twenty-four male Sprague-Dawley rats (aged 21 days old at the beginning of the protocol) were housed in temperature and light-controlled conditions with free access to food (Scientific Animal Food and Engineering, A04, Perotech, Toronto, Canada) and water. Animals were randomly divided into four groups (n=6 per group): sedentary animals + saline administration (SED+SAL), sedentary animals + DOX administration (SED+DOX), endurance trained animals + DOX administration (ET+DOX) and animals that performed voluntary physical activity + DOX administration (VPA+DOX).

2.3 Physical exercise protocols and doxorubicin treatment

Endurance training (ET) was performed 5 days/week, during 12 weeks on a LE8700 motor driven treadmill (Panlab, Harvard, USA). The protocol included 5 days of habituation (week 0) followed by continuous running (60 min/day) with a gradually increase in velocity from 18 m/min to 27 m/min for 12 weeks. After the first DOX administration, velocity was gradually adjusted to 20 m/min. Animals from the VPA+DOX group were housed in polyethylene cages equipped with a running wheel [perimeter 105 cm, Type 304 Stainless steel (2154F0106-1284L0106) Tecniplast, Casale Litta, Italy)]. The rats had unlimited access to the wheels 24 h/day and the running distance was recorded using ECO 701 (Hengstler, Lancashire, UK).

Sub-chronic DOX treatment started after the 5^{th} week of the beginning of these protocols and consisted in a weekly intraperitoneal (i.p) administration of DOX (2 mg/kg, Ferrer

Farma, Barcelona, Spain) for seven weeks. Furthermore, an equivalent volume of vehicle solution (NaCl, 0.9%) was administered as placebo, in the same conditions to SED+SAL animals.

2.4 Animal euthanasia, blood and isolation of liver mitochondria

Forty-eight hours after the last ET session, non-fasted rats were injected i.p. with Imalgene® 1000 (Ketamine, 90 mg/kg) and Rompum™ (Xylazine, 10 mg/kg) purchased in Propecuária, (Batalha, Portugal). Upon absence of eye-blink, toe-pinch and righting reflexes, blood was collected for determination of alanine transaminase (ALT) and aspartate aminotransferase (AST) by conventional methods using a Beckman-Coulter AU5400® automated clinical chemistry analyzer (Brea, CA, USA). Thereafter, the livers and hearts were immediately excised, washed and weighed. The livers were sliced and one fraction was frozen and stored at -80°C while the other one was used for mitochondrial isolation. Liver mitochondrial isolation was performed using conventional methods of differential centrifugation as previously described (Ascensão et al., 2012).

2.5 Mitochondrial respiratory activity and transmembrane electric potential

Mitochondrial respiratory function was performed in liver mitochondria fraction and measured polarographically at 30°C using a Clark-type oxygen electrode (YSI) connected to a paper chart recorder (Linseis L200E, Linseis Inc, USA). The assay was initiated by adding glutamate/malate (G/M, 10 and 5 mM, respectively) obtaining a basal rate (state 2); state 3 respiration was determined after adding ADP (125 nmol); state 4 was measured as the rate of oxygen consumption after ADP phosphorylation. The respiratory control ratio (RCR, state 3 over state 4 respiration) and the ADP/O ratio (amount of ADP added over oxygen consumed during state 3 respiration) were posteriorly calculated (Estabrook, 1967). Additionally, oligomycin and FCCP (1.5 µmol/mL and 2 µmol/mL, respectively) were added after state 4 measurements to evaluate the coupling between the maximal respiratory rate and the synthesis of ATP (Pereira et al., 2007).

Mitochondrial transmembrane electrical potential ($\Delta\psi$) was indirectly monitored using the lipophilic cation tetraphenylphosphonium (TPP^+, 3 µM) at 30°C, according to Kamo et al. (1979). Energization was carried out with G/M (10 and 5 mM, respectively) and ADP (125 nmol) was used to induce a phosphorylation cycle. The time between the depolarization

induced by ADP phosphorylation and repolarization (lag phase) was also measured. No passive binding of TPP^+ to mitochondrial membranes was calculated.

2.6 Enzymatic colorimetric assays

The extent of lipid peroxidation was determined by measuring malondialdehyde (MDA) content in liver tissue through a colorimetric assay (Ascensão et al., 2005; Ohkawa et al., 1979). Aconitase activity was measured in liver mitochondrial fractions by monitoring the formation of cis-aconitase from isocitrate as previously described (Ascensão et al., 2005). Mitochondrial antioxidant manganese-dependent superoxide dismutase (MnSOD) activity was measured in liver mitochondrial fractions using a commercial kit (RANSOD, Randox Labs, UK), according to the manufacturer's instructions.

2.7 Western Blotting analysis

Equivalent amounts of liver mitochondria or whole liver tissue were separated by SDS/PAGE (12% gels) followed by blotting on PVDF membranes (Millipore, Massachusetts, USA). The membranes was blocked with 5% of nonfat dry milk (Bio-Rad Laboratories, Amadora, Portugal) and incubated with: anti-PGC-1α (1:500; ab106814 goat monoclonal IgG), anti-OPA1 (1:1,000; ab119685 mouse monoclonal IgG), anti-PINK (1:500; ab23707 rabbit polyclonal IgG), anti-p62 (1:1,000; ab56416 mouse monoclonal IgG) from from Abcam (Cambridge, UK), anti- whereas DRP1(1:1,000; #8570 rabbit monoclonal IgG), anti-Beclin1 (1:1,000, D40C5 #3495 rabbit monoclonal IgG), anti-Bcl-2 (1:1,000; #2870 rabbit monoclonal IgG) from Cell Signaling technology® (Danvers, USA), anti-MFN1 (1:1,000; sc-50330 rabbit polyclonal IgG) from Santa Cruz® biotechnology, Inc. (Dallas, Texas, USA), anti-LC3 (1:1,000; PD014 rabbit polyclonal IgG) and anti-DNP (D9656) from Sigma-Aldrich (Saint Louis, MO, USA), whether PVDF membrane was loaded with liver tissue homogenate. Anti-PARKIN (1:500, # 4211 mouse monoclonal IgG) from Cell Signaling technology® (Danvers, USA) and anti-TFAM (sc-23588; goat polyclonal IgG) from Santa Cruz® biotechnology, Inc. (Dallas, Texas, USA) was used in PVDF membranes loaded with mitochondrial faction. After incubation with the corresponding secondary horseradish peroxidase-conjugated anti-mouse (1:10,000; sc-2005), anti-rabbit (1:10,000; sc-2004), or anti-goat (1:10,000; sc-2354) antibodies (Santa Cruz® biotechnology, Dallas, Texas, USA), protein bands were imaged with ChemiDoc XRS^+ system (Bio-Rad Laboratories, Amadora, Portugal). Data were observed as band intensity of immunostaining values (arbitrary units), and the results were normalized to

Ponceau staining (Romero-Calvo et al., 2010) and expressed relative to the SAL+SED group.

2.8 Transmission electron microscopy

The collected liver tissues were sliced with approximately 1 mm of thickness and fixed in a 2.5% glutaraldehyde solution in 0.2 M sodium cacodylate buffer (pH 7.4). The samples were then postfixed in 2% osmium tetroxide for 2 hours and dehydrated in sequential steps of ethanol (75%, 95%, and 100% twice) and propylene oxide as transitional solvent. Propylene oxide with increasing concentrations of Epon (25, 50, 75 and 100%) (TAAB, Aldermaston, UK) was used to impregnate the samples. Ultrathin sections (50–60 nm) were collected on copper grids and stained for contrast with 0.5% uranyl acetate and 0.2% lead citrate. The grids were examined under a transmission electron microscope (JEM-1400, JEOL, Peabody, USA) and the acquired images were blindly analyzed by a single researcher using ImageJ 1.48v software (Wayne Rasband National Institutes of Health, USA). Images were obtained at x12,000 magnification and the following parameters were measured or calculated: 1) mitochondrial area (μm^2); 2) mitochondrial density (number of mitochondria per μm^2); 3) circularity ($4 \cdot \pi \cdot area/perimeter^2$), which ranges from 0 to 1, with 1 being a perfect circle; 4) Aspect Ratio (AR), calculated as major axis/minor axis; and 5) the percentage of area occupied by mitochondria.

2.9 Statistical analysis

All data are expressed as the mean±SD. Statistical analyzes were performed using GraphPad Prism 7.0 software (GraphPad software, San Diego, CA, USA). The t-Student test was used to evaluate statistical differences of DOX effect between sedentary groups (SED+SAL and SED+DOX). A one-way ANOVA followed by Tuckey post-hoc tests were used to assess the statistical differences among the different physical exercise protocols in animals treated with DOX (SED+DOX, VPA+DOX and ET+DOX). The significance level was set at $p < 0.05$.

3 Results

3.1 Body, heart, and liver weight and liver serum markers

As seen in Table 1, DOX induced a decrease in heart and body weight and increased AST and ALT levels in the serum of SED+DOX animals. Exercised groups (ET+DOX and VPA+DOX) showed an increase in heart weight and decreased ALT and AST levels when compared to SED+DOX rats. However, the body weight loss was not reverted by neither

ET nor VPA. No statistical significant differences were found between groups in HM:BM, liver mass or LM:BM.

Table 1. Animal and tissue characterization and hepatic plasma markers

	Initial body mass (g)		Final body mass (g)		Heart mass (g)		HM:BM (mg/g)		Liver mass (g)		LM:BM (mg/g)		AST (U/L)		ALT (U/L)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
SED+SAL	207.4	3.9	598.5	10.6	1.4	0.1	2.3	0.2	13.1	0.6	21.7	1.2	189.0	14.1	41.0	2.6
SED+DOX	208.8	7.9	438.0	6.2*	1.1	0.03*	2.6	0.4	10.5	2.9	21.8	4.2	315.2	22.6*	62.9	3.7*
ET+DOX	207.6	11.2	426.0	10.8	1.5	0.1#	3.3	0.4	10.4	0.9	24.8	2.2	242.9	13.9#	49.4	1.2#
VPA+DOX	208.0	6.4	428.5	16.9	1.6	0.1#	3.1	0.6	11.5	1.4	23.2	1.8	243.7	15.6#	43.0	1.1#§

Data are expressed as means±SD (n = 6). Significance ($p < 0.05$): (*) vs. SED+SAL; (#) vs. SED+DOX; (§) vs. ET+DOX. SED+SAL, sedentary animals with saline administration; SED+DOX, sedentary animals with DOX administration; ET+DOX, endurance trained animals with DOX administration; VPA+DOX, voluntary physical activity animals with DOX administration. HM:BM, ratio between heart and final body mass; LM:BM, ratio between liver and final body mass; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

3.2 Liver mitochondrial bioenergetics

3.2.1 Oxygen consumption

The analysis of functional end points of isolated liver mitochondria showed no statistically significant changes in state 3 respiration between any of the four experimental groups (Figure 1A). However, DOX induced a significant increase in state 4 (Figure 1B) and a compromised RCR and ADP/O ratios in SED+DOX animals (Figures 1C and D) compared to control. None of the exercise regimens (ET+DOX and VPA+DOX) reverted DOX effects on mitochondrial respiration (Figure 1B-D).

Furthermore, DOX administration induced an increase in oligomycin-insensitive state 4 respiration (Figure 1E), but not in FCCP-induced uncoupled respiration (data not shown), thus decreasing the FCCP/oligomycin ratio (Figure 1F). When compared to SED+DOX, ET+DOX animals showed a decrease in oligomycin-insensitive state 4 respiration, but no significant alterations were observed in uncoupled respiration or in the FCCP/oligomycin ratio. The VPA regimen did not induce significant alterations in these evaluated parameters.

3.2.2 Mitochondrial transmembrane electric potential ($\Delta\psi$)

No significant alterations were observed in the maximal $\Delta\psi$ between the experimental groups (Figure 1G). DOX sub-chronic administration significantly increased the ADP-phosphorylation lag phase in sedentary animals. Both exercise regimens were able to restore DOX-induced increased lag phase (VPA+DOX and ET+DOX) (Figure 1H).

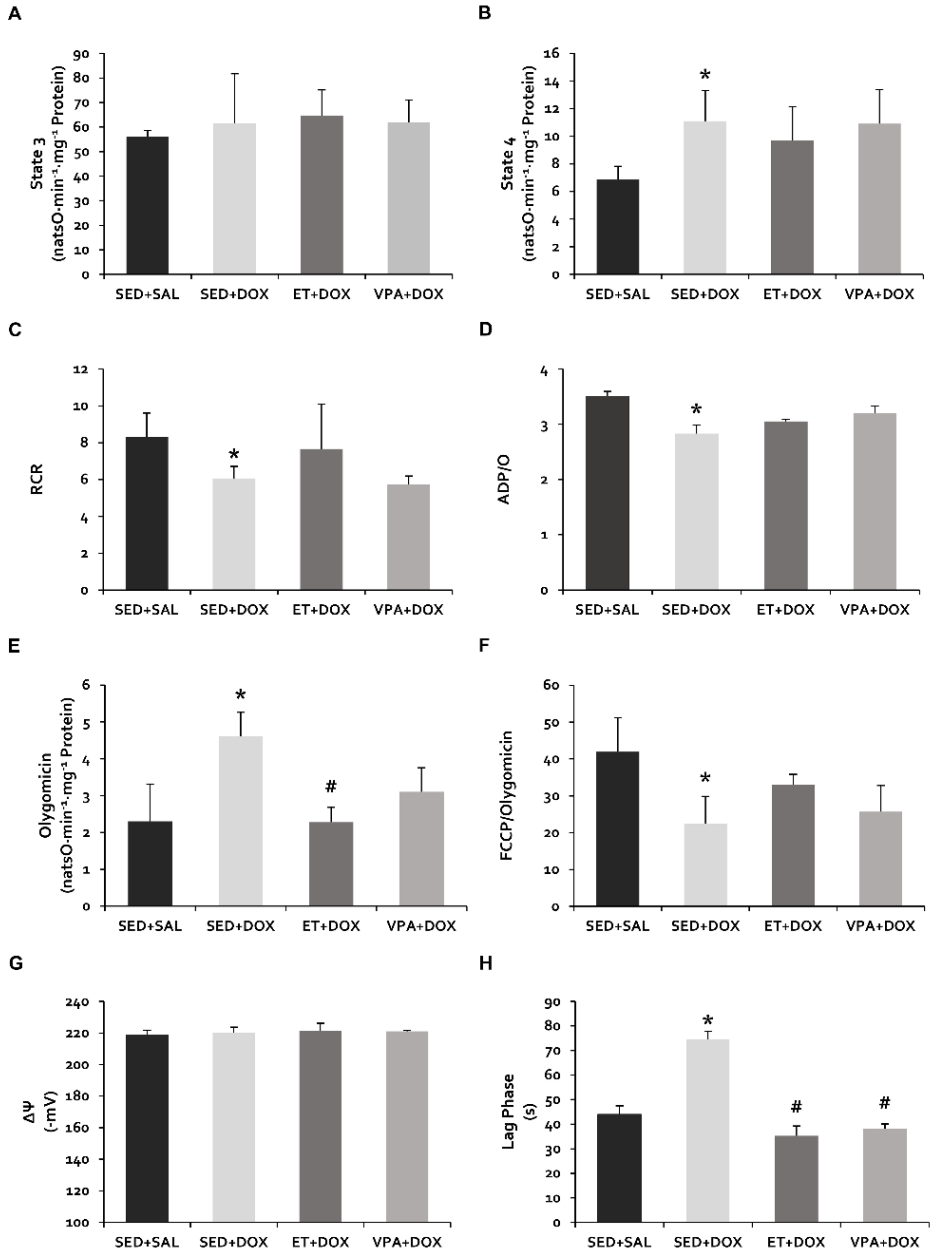

Figure 1. Effects of sub-chronic DOX treatment and physical exercise on liver mitochondrial assays. Mitochondrial transmembrane electrical potential and oxygen consumption were assayed with glutamate (10 mM) plus malate (5 mM) as substrates and tetraphenylphosphonium ion (TPP^+, 3 μM, for ∆ψ assays) in a total volume of 1 mL. Oligomycin (1.5 μmol/mL) and FCCP (2 μmol/mL) were added after state 4 to inhibit the ATP synthase and to uncouple respiration, respectively. (A) State 3, (B) state 4, (C) Respiratory control ratio (RCR), (D) ADP/O, oligomycin-insensitive respiration (E), FCCP (F), (G) maximal ∆ψ and (H) lag phase. Data are expressed as means±SD (n=6). Significance ($p < 0.05$): (*) vs. SED+SAL; (#) vs. SED+DOX. SED+SAL, sedentary animals with saline administration; SED+DOX, sedentary animals with DOX administration; ET+DOX, endurance trained animals with DOX administration; VPA+DOX, voluntary physical activity animals with DOX administration.

3.3 Oxidative damage and mitochondrial enzyme activities

Table 2 results show that DOX treatment induced a decrease in the mitochondrial enzymatic activities of MnSOD and aconitase, while an increase in MDA content was observed in sedentary animals. Both exercise models (ET and VPA) counteracted the increase in MDA, but did not reverse the DOX effect in aconitase and MnSOD activities, although a non-significant trend was observed. A decrease in carbonylated proteins content in VPA+DOX group was also measured. This decrease was not observed in ET+DOX group probably due to a high data dispersion.

Table 2. Effects of sub-chronic DOX treatment on liver malondialdehyde (MDA) and carbonylated proteins content and mitochondrial antioxidant manganese superoxide dismutase (MnSOD) and aconitase activities in sedentary and exercised animals (ET and VPA).

	SED+SAL		SED+DOX		ET+DOX		VPA+DOX	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
MDA (nmol/mg)	46.7	9.4	145.2	25.0*	77.5	15.2#	76.9	4.2#
Carbonylated proteins (% SED+SAL)	100	43.8	164.1	74.3	81.2	25.6	44.1	6.7#
Aconitase (nmol/min/mg)	20.2	1.7	7.7	3.5*	10.9	4.8	12.2	4.3
MnSOD (U/mg)	10.6	2.1	2.9	1.7*	5.2	1.5	4.6	3.4

Data are expressed as means±SD (n=6). Significance ($p < 0.05$): (*) vs. SED+SAL; (#) vs. SED+DOX; (§) vs. ET+DOX. SED+SAL, sedentary animals with saline administration; SED+DOX, sedentary animals with DOX administration; ET+DOX, endurance trained animals with DOX administration; VPA+DOX, voluntary physical activity animals with DOX administration.

3.4 Protein markers of mitochondrial dynamics, biogenesis and auto(mito)phagy signaling

Mitochondrial dynamics was evaluated by the determination of fusion (OPA1 and MFN1) and fission-related proteins (DRP-1) content in homogenized tissue. The results showed that DOX induced a decrease of OPA1 and MFN1 (Figure 2A and B) and an increase of DRP1 (Figure 2C) protein content in SED+DOX group compared to the control group (SED+SAL). When compared to SED+DOX animals, ET reversed OPA1 and MFN1 content, while VPA+DOX animals did not show significant alterations in these proteins (Figure 2A-C).

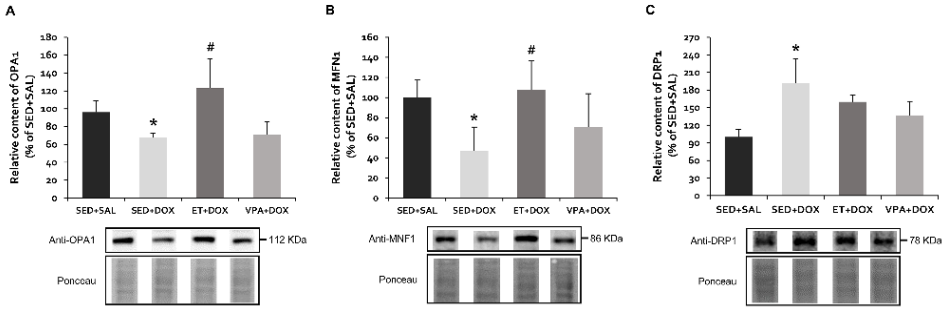

Figure 2. Effects of sub-chronic DOX treatment and physical exercise on the expression of mitochondrial dynamic signaling markers in liver tissue homogenate. (A) Optic atrophy type 1 (OPA1); (B) mitofusin-1 (MFN1); (C) dynamin-related protein (DRP-1). Data are expressed as means±SD (n=4) and in percentage of SED+SAL group. Bands were normalized to Ponceau staining. Significance ($p < 0.05$): (*) vs. SED+SAL; (#) vs. SED+DOX; (§) vs. ET+DOX. SED+SAL, sedentary animals with saline administration; SED+DOX, sedentary animals with DOX administration; ET+DOX, endurance trained animals with DOX administration; VPA+DOX, voluntary physical activity animals with DOX administration.

DOX administration also induced a significant decrease in the content of the mitochondrial biogenesis-related proteins PGC-1α and TFAM, which was completely reverted by ET, but not by VPA (Figure 3).

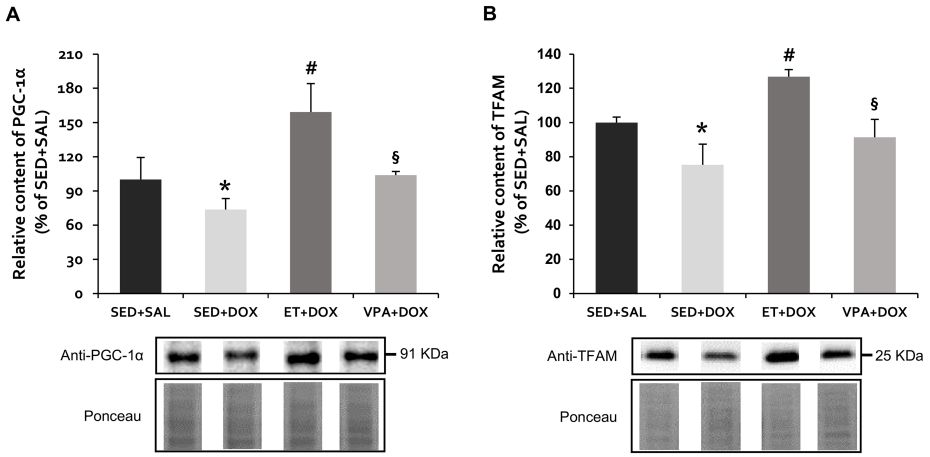

Figure 3. Effects of sub-chronic DOX treatment and physical exercise on the expression of mitochondrial biogenesis signaling markers in liver tissue homogenate. (A) Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and (B) mitochondrial transcription factor A (TFAM). Data are expressed as means±SD (n=4) and in percentage of SED+SAL group. Bands were normalized to Ponceau staining. Significance ($p < 0.05$): (*) vs. SED+SAL; (#) vs. SED+DOX; (§) vs. ET+DOX. SED+SAL, sedentary animals with saline administration; SED+DOX, sedentary animals with DOX administration; ET+DOX, endurance trained animals with DOX administration; VPA+DOX, voluntary physical activity animals with DOX administration.

The evaluation of auto(mito)phagy biomarkers revealed an increase of Bcl-2, LC3II content and LC3II/LC3I ratio in the SED+DOX group compared to SED+SAL, without alterations in other related proteins (Figure 4B, E and F). The increased observed in Bcl-2, LC3II content and LC3II/I ratio for SED+DOX group was decrease in the VPA+DOX. Although ET+DOX showed a similar tendency for a decrease in Bcl-2 and LC3 II contents, these decreases

were not significant (Figure 4B and E) due to the large standard deviation. The ET+DOX group also showed an increase in Parkin and PINK content (Figure 4H and I), which were not observed in VPA+DOX group. No significant alterations were observed in the others evaluated markers of autophagy.

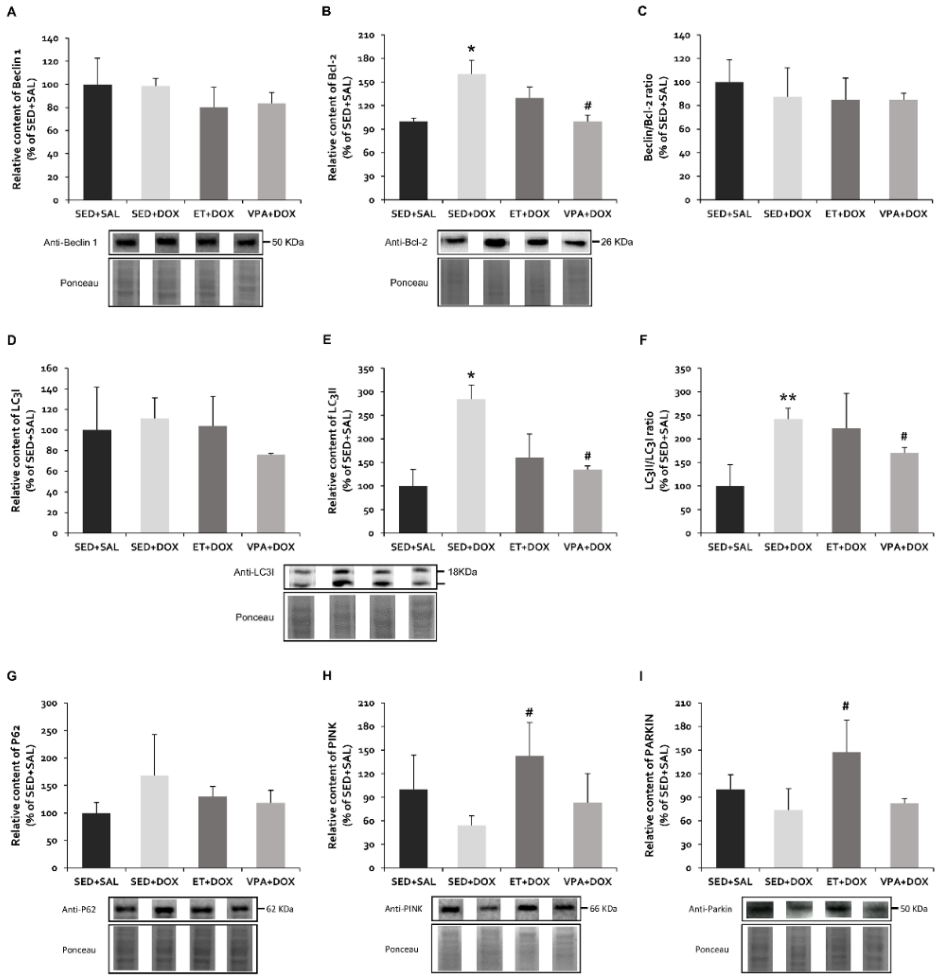

Figure 4. Effects of sub-chronic DOX treatment and physical exercise on the expression of auto(mito)phagy signaling markers in liver tissue homogenate and mitochondrial fraction. (A) Beclin 1; (B) Bcl-2; (C) Beclin 1/Bcl-2 ratio; (D) LC3I; (E) LC3II; (F) LC3II/LC3I ratio and (G) p62; and mitophagy markers; (H) PTEN-induced putative kinase 1 (PINK); and (I) Parkin. Data are expressed as means±SD (n=4) and in percentage to SED+SAL group. The bands were normalized to Ponceau staining. Significance ($p < 0.05$): (*) vs. SED+SAL; (#) vs. SED+DOX; (§) vs. ET+DOX. SED+SAL, sedentary animals with saline administration; SED+DOX, sedentary animals with DOX administration; ET+DOX, endurance trained animals with DOX administration; VPA+DOX, voluntary physical activity animals with DOX administration.

3.5 Mitochondrial morphology

Liver tissue sections were analyzed with transmission electron microscopy (TEM) to evaluate alterations in mitochondrial morphology. Figures 5A to D show an example of TEM images for each experimental group. DOX-treated groups showed an increase in the mitochondrial area and circularity (Figures 5E and H), and a decrease in AR and mitochondrial density (Figure 5G and I) compared to SED+SAL. Both exercise models (ET and VPA) reverted the density and size alterations observed in SED+DOX animals (Figures 5E and I), although in a non-statistically significant manner (p value of 0.07 for both groups). Moreover, the VPA+DOX group showed a decrease of circularity and an increase of AR (Figures 5G and H).

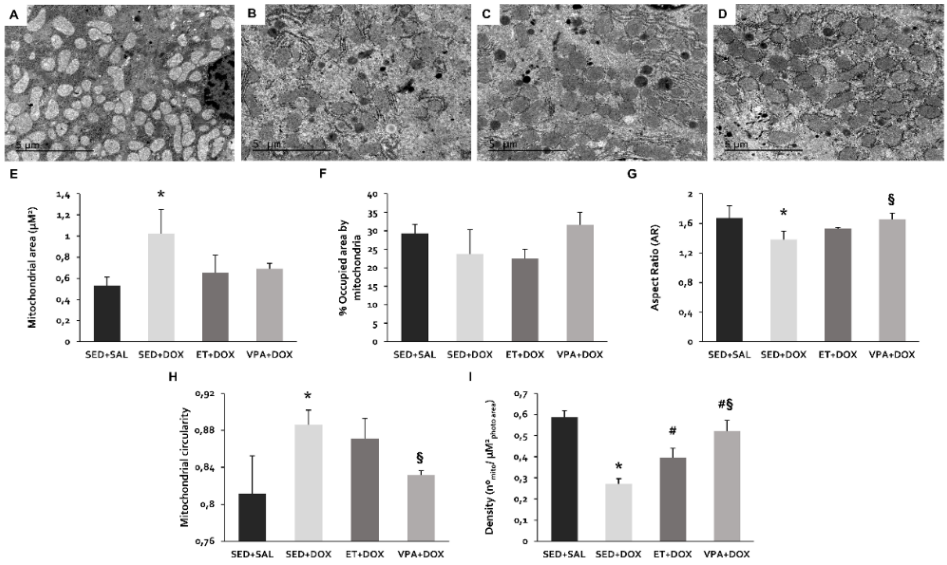

Figure 5. Effects of sub-chronic DOX treatment and physical exercise on mitochondrial morphology assessed by semi-quantitative and qualitative analysis of electron microphotographs. A) SED+SAL; B) SED+DOX; C) ET+DOX; D) VPA+DOX; E) mitochondrial area (μm^2); F) percentage of area occupied by mitochondria; G) Aspect Ratio (AR); D) circularity; and H) mitochondrial density (number of mitochondria per μm^2). Data are expressed as means±SD (n=4). Significance ($p < 0.05$): (*) vs. SED+SAL; (#) vs. SED+DOX; (§) vs. ET+DOX. SED+SAL, sedentary animals with saline administration; SED+DOX, sedentary animals with DOX administration; ET+DOX, endurance trained animals with DOX administration; VPA+DOX, voluntary physical activity animals with DOX administration.

4 Discussion

DOX-induced cardiotoxicity is a pathological condition mainly associated with increased oxidative stress and mitochondrial dysfunction, which lead to disturbances in energy metabolism (Kalender et al., 2005; Nagai et al., 2016). Although DOX-induced hepatotoxicity has been reported previously, its mechanisms have not yet been elucidated (Damodar et al., 2014; Nagai et al., 2016). Due to the liver relevance in drug detoxification, the preservation of liver structure and function is essential in DOX-related therapeutics to

eliminate the reactive metabolites and free radicals produced during the course and after DOX-treatment. Thus, we aimed to determine whether physical exercise could protect the rat liver tissue and mitochondria from the potential deleterious effects of a sub-chronic DOX administration.

4.1 Overview of findings

Our results demonstrated that sub-chronic DOX treatment induced hepatic damage, as reflected by the increase of circulating transaminases AST and ALT, the decrease in some liver mitochondrial respiratory parameters, the up-regulation of mitochondrial fission markers and the down-regulation of fusion and biogenesis proteins. These findings are associated to a decrease in the antioxidant enzymes levels and a concomitant oxidative damage increase. Our results highlight that ET could be used as a potential strategy against the liver mitochondrial dysfunction caused by sub-chronic DOX treatment. The alterations in mitochondrial dynamics signaling markers observed in SED+DOX rats were mitigated in the ET+DOX group, as suggested by the increase in fusion and biogenesis signaling markers and the reverted alterations in the mitochondrial function and redox status. These results correlated with the decreased levels of circulating ALT and AST, suggesting a preservation of the hepatocyte membrane integrity and function. Most of the positive outcomes induced by physical exercise were observed in the ET+DOX group, but not in VPA+DOX, suggesting that the chronic exercise-induced benefits to counteract the DOX-induced hepatotoxicity are dependent on the intensity and duration of each exercise protocol.

4.2 Animal characterization and serum markers

Consistently with previous results (Ascensão et al., 2011a; Marques-Aleixo et al., 2015; Santos et al., 2002), DOX-treated rats exhibited body weight loss and thoracic and abdominal ascites, regardless of whether they were exercised or not. However, both exercise protocols reverted the heart weight loss associated with DOX administration. Additionally, circulating levels of the hepatotoxicity biomarkers ALT and AST were elevated in SED+DOX animals. Despite some mixed results, other studies have also reported increased transaminases levels after sub-chronic DOX administration (Alshabanah et al., 2010; Koti et al., 2013). In our study, this increase was attenuated in ET+DOX and VPA+DOX groups, indicating that exercise had a protective effect against the DOX-induced hepatocellular damage.

4.3 Oxidative damage and antioxidant markers

Although studies in DOX-induced hepatic toxicity are scarce, it has been hypothesized that damage is associated to oxidative stress (Dirks-Naylor et al., 2014; Nagai et al., 2016; Taskin and Dursun, 2015), as also described in other tissues, such as heart and skeletal muscle (Davies and Doroshow, 1986; Kavazis et al., 2017). Accordingly, SED+DOX animals exhibited an increase of the oxidative damage marker MDA and decreased enzymatic antioxidant activity (aconitase and MnSOD), suggesting an imbalance in the redox status that may result impairment of the mitochondrial function (Marques-Aleixo et al., 2015). Although no protection against the alterations in antioxidant enzymatic activities was found in the exercised animals compared to SED+DOX, VPA induced a significant reduction of MDA and carbonylated proteins, while ET elicited a similar although non-significant response, suggesting that physical exercise modulated ROS production in the liver of DOX-treated rats. Despite being a non-contractile tissue, it has been extensively reported that exercise can protect the liver against oxidative stress (Ascensão et al., 2012; Oh et al., 2013; Santos-Alves et al., 2014).

4.4 Mitochondrial bioenergetics

Similarly to other studies performed in the heart tissue (Marques-Aleixo et al., 2015; Pereira et al., 2012), our results showed that DOX caused an impairment of the mitochondrial oxidative phosphorylation capacity in complex I associated to a decrease in RCR and ADP/O ratios, which were accompanied by an increase of state 4, lag phase and oligomycin-inhibited respiration. Taken together, these results suggest an uncoupling between mitochondrial respiration and proton gradient, leading to impaired mitochondrial function. These alterations were partially attenuated by exercise. In fact, ET reverted the DOX-induced increase of lag phase and oligomycin-resistant respiration and showed a non-significant state 4 decrease and ADP/O increase, denoting a greater membrane integrity stability and a more efficient oxidative phosphorylation, which suggest the preservation of a control mitochondrial phenotype even during DOX treatment.

4.5 Mitochondrial biogenesis and dynamics

DOX treatment induced a decrease in fusion markers (MFN1 and OPA1) and an increase in DRP1, an essential fission protein, compared to SED+SAL. Dynamic processes determine the architecture of the mitochondrial network, influencing mitochondrial function. Thus, the homeostasis between these two processes is extremely important for cell survival and viability (Gomes and Scorrano, 2013; Gomes et al., 2011). In accordance with other studies (Dirks-Naylor et al. 2014), our results showed clear imbalance in mitochondrial dynamics markers towards mitochondrial fission, which could had led to a fragmentation of

the mitochondrial network in DOX-treated animals. On the other hand, the ET+DOX group showed increased content of fusion markers, which may had compensate the increase in DRP1 content.

Furthermore, DOX induced a decrease of TFAM and PGC-1α expression in sedentary animals, which suggests a decline in mitochondrial biogenesis, which may have contributed to the impairment of the mitochondrial turnover. Indeed, ET has been previously reported to be able to prevent the DOX-induced decrease of PGC-1α expression in the rat skeletal muscle and the heart (Kavazis et al., 2014; Marques-Aleixo et al., 2015).

Several studies showed that a predominantly mitochondrial fragmented phenotype is associated with a decrease in the mitochondrial area and with an increase in the mitochondrial number (Ong and Hausenloy, 2010; Ong et al., 2010; Westrate et al., 2014). However, our morphological results showed an increase of abnormally large mitochondria and a decrease in mitochondrial density in SED+DOX animals. The presence of abnormally large mitochondria has been described before in several liver diseases, such as alcoholic liver disease or Wilson´s disease (Bruguera et al., 1977; Mateos et al., 1995; Shawky et al., 2010). It has been hypothesized that this phenotype is associated with disturbances in the water-electrolyte equilibrium between the mitochondria and cytoplasm, probably due to the lack of ATP (Wilson and Leduc, 1963). Indeed, our results showed an impairment in mitochondrial function in SED+DOX group by RCR and ADP/O reduction, probably compromising ATP levels. Moreover, DOX administration altered the mitochondrial shape, as reflected by the increase in circularity and the decrease in AR, giving rise to less elongated and more circular mitochondria. Both exercise protocols reverted the DOX-induced mitochondrial size and density alterations. Furthermore, VPA+DOX animals exhibited a mitochondrial morphology more similar to SED+SAL rats. Thus, these results demonstrate that ET and VPA provide a protective phenotype against the DOX-induced impairment of the mitochondrial turnover, dynamics and morphology.

4.6 Auto(mito)phagy

Previous results seem to indicate that DOX induces a fragmentation of the mitochondrial network, which could increase the mitochondrial susceptibility to mitophagy (Twig et al., 2008). Despite the increased ratio of LC3II/LC3I, which is considered a key signaling parameter for cell autophagy (Gimenez-Xavier et al., 2008), the remaining of the analyzed protein biomarkers related to auto(mito)phagy did not exhibit any alterations when compared to SED+SAL rats. Thus, DOX seems to initiate the autophagic signaling by converting LC3I in LC3II (an essential step for autophagosome formation), but this stimulus was likely not sufficient to increase the expression of other necessary proteins involved in auto(mito)phagy (Giménez-Xavier et al., 2008; Wu et al., 2015), which could eventually led

to the accumulation of damaged mitochondria. Similarly, both physical exercise programs (ET and VPA) induced an increase of LC3II/LC3I ratio without alterations in other autophagy signaling proteins. However, ET+DOX animals showed increased expression of mitophagy biomarkers PINK and Parkin, probably reflecting a more active process of mitochondrial quality control, which would contribute to remove damaged mitochondrial structures resulting from DOX treatment. Accumulation of damaged mitochondria in DOX-treated rats can translate into decreased respiration and lower $\Delta\psi$, as we observed. However, precaution should be taken when interpreting these data, mainly based on variations in the expression of protein signaling biomarkers, as without the use of specific inhibitors, signaling proteins analysis only provides a static picture of an otherwise very dynamic process.

In conclusion, the present data showed for the first time that physical exercise, in particular ET, improves mitochondrial function, reduces oxidative damage and modulate the expression of biomarkers related to mitochondrial dynamics, therefore contributing to increase liver resistance against DOX-induced toxicity.

Author Contributions

ESA, IMA, JM and AA contributed to the conception and design of the study; ESA, DRR, SM and JTG conducted experiments and contributed to the acquisition, analysis and interpretation of data; ESA and DRR, contributed to the drafting of the manuscript; IMA, PJO, JRT, JM and AA reviewed and edited the manuscript; JM. and AA contributed to the final approval of the submitted manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Paper IV

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Review

The beneficial role of exercise in mitigating doxorubicin-induced Mitochondrionopathy

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ABSTRACT

Doxorubicin (DOX) is a widely used antineoplastic agent for a wide range of cancers, including hematological malignancies, soft tissue sarcomas and solid tumors. However, DOX exhibits a dose-related toxicity that results in life-threatening cardiomyopathy. In addition to the heart, there is evidence that DOX toxicity extends to other organs. This general toxicity seems to be related to mitochondrial network structural, molecular and functional impairments. Several countermeasures for these negative effects have been proposed, being physical exercise, not only one of the most effective non-pharmacologic strategy but also widely recommended as booster against cancer-related fatigue.

It is widely accepted that mitochondria are critical sensors of tissue functionality, both modulated by DOX and exercise. Therefore, this review focuses on the current understanding of the mitochondrial-mediated mechanisms underlying the protective effect of exercise against DOX-induced toxicity, not only limited to the cardiac tissue, but also in other tissues such as skeletal muscle, liver and brain. We here analyze recent developments regarding the beneficial effects of exercise targeting mitochondrial responsive phenotypes against redox changes, mitochondrial bioenergetics, apoptotic, dynamics and quality control signalling affected by DOX treatment.

1. Introduction

Doxorubicin (DOX, or 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-known efficacy of this antineoplastic agent, DOX clinical use is limited due to a dose-dependent development of cardiovascular toxicity, which can lead to congestive heart failure and can be fatal [1]. Furthermore, DOX toxicity also affects other organs besides the heart, including skeletal muscle, liver and brain. It has been proposed that multiple sequential exposures to DOX treatment, together with lifestyle changes, including physical inactivity, increase the possibility of cardiovascular disease among breast cancer survivors [2]. Indeed, due to the recent advances in early diagnosis and efficacy cancer therapy, cardiovascular disease is considered the predominant cause of mortality in breast cancer survivors among older women [3].

DOX-induced cellular dysfunction is associated with increased oxidative damage and apoptosis, involving mitochondrial changes in the process [4–7]. Published data also suggest that DOX disturbs the proper balance between mitochondrial fusion and fission mechanisms that are essential for healthy cell and mitochondrial regulation [8]. Moreover, DOX may unbalance cellular and mitochondrial quality control regulation [9,10]. Together, the shifts of these processes from regulatory and adaptive to disruptive are thought to contribute to the cellular 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

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the reduction or elimination of tumor cell growth. Therefore, an ongoing challenge in cancer treatment is to exploit the DOX anti-tumor effects, while minimizing tissue toxicity in general, and particularly, cardiac toxicity and the associated mitochondrial damage. One of the most studied non-pharmacological and promising strategies used to counteract DOX side effects is chronic physical exercise [11,12]. Considering the important role of mitochondrial disturbances in DOX-related toxicity and, accounting for the beneficial role of physical exercise on mitochondrial function in control and DOX-treated animals is enough evidence to justify the critical role of those organelles in the cross-tolerance phenomenon.

The present review briefly highlights some recent published data supporting the role of exercise, particularly chronic interventions, as a strategy to mitigate DOX-induced cardiac toxicity, targeting the mitochondrial-driven mechanisms including antioxidants and apoptotic signalling, mitochondrial dynamics and quality control. The cross-tolerance effect of exercise against the deleterious effects of DOX in other tissues, including skeletal muscle, liver and brain are also briefly addressed.

2. Mechanisms of DOX-induced mitochondrial toxicity

Several mechanisms have been proposed to explain DOX-induced cardiomyopathy, suggesting that this is a multifactorial process. Generally, mitochondria 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 [13–16]. Moreover, the possible existence of a heart specific isoform of the NADH dehydrogenase (mitochondrial complex I) able to initiate DOX redox cycling and, consequently, promoting additional reactive oxygen species (ROS) formation, may be a critical step of DOX-induced deterioration of cardiac function and onset of chronic clinical cardiotoxicity [13,17,18]. The elevated affinity of DOX by cardiolipin, the higher mitochondrial content and the lower antioxidant capacity of cardiac tissue compared to other tissues, justify the decreased heart capability of dealing with the increased oxidative stress induced by DOX [7,19]. Consequently, several authors have already discussed that DOX-induced increased oxidative damage is associated with mitochondrial bioenergetics disruption, interference with calcium homeostasis and enhanced apoptotic signalling through the increased susceptibility to mitochondrial permeability transition pore (mPTP) opening [1,6,7,11,12]. Additionally, it has been proposed that DOX-induced toxicity to cardiomyocytes associated with increased ROS production is accompanied by decreased levels of oxoguanine-DNA glycosylase-1 (OGG1), a major DNA glycosylase that hydrolyzes oxidized-guanine (8-oxo-dG) to guanine, and with increased mtDNA damage [20–22].

Differences in location, biochemical properties, morphology and organization between subsarcolemmal and intermyofibrillar mitochondrial sub-populations [23,24], can lead to subtle differences in their sensitivity and responsiveness to metabolic challenges including exercise [25,26]. Differences in the impact of DOX toxicity between cardiac mitochondrial sub-populations have also been reported. Kavazis [27] and co-workers suggested that subsarcolemmal mitochondria accumulate greater amounts of DOX, while intermyofibrillar mitochondria are more susceptible to apoptotic and autophagic responses following acute DOX treatment.

During stressful conditions, cardiac myocytes respond by triggering a defense mechanism, involving selective sequestration and subsequent degradation of the dysfunctional mitochondria before they cause metabolic rupture of even the activation of cell death [28]. Besides energy production, a critical role of mitochondria to ensure proper cardiac muscle contraction involves the regulation and adaptations in mitochondrial network structure [29]. The mitochondrial plastic features are driven from the dynamic interaction of mitochondrial fusion, fission, auto(mito)phagy and biogenesis, which ensures proper organization of the mitochondrial network [30]. DOX-induced cardiotoxicity has been suggested to be associated with fragmentation of the mitochondrial network [20,31,32], and to the inhibition of mitochondrial fission protects the heart against DOX-induced cardiac injury [33]. Moreover, the loss of mitochondrial connectivity can predispose cardiomyocytes to apoptosis [34,35], and/or mitochondrial division can designate dysfunctional organelles with low membrane potential for mitophagy [36].

The precise mechanisms linking DOX cardiomyopathy to mitochondrial dynamics, apoptosis and auto(mito)phagy are still largely unknown. Opposing results regarding the effects of DOX treatment on auto(mito)phagic flux suggest both the adaptive and maladaptive consequences described next, probably dependent on the severity of the stimulus: DOX treatment (i) attenuates Parkin-mediated mitophagy [37], (ii) causes a shift from autophagy to apoptosis [38], and (iii) induces autophagy, probably acting as an adjuvant mitigating strategy against DOX-induced myocardial damage [38–40]; others associate DOX treatment with elevated autophagic signalling, suggesting that increased autophagy mediates DOX-induced cardiotoxicity [9,10,41–44]. The most common pathways underlying DOX-induced cardiac mitochondrial toxicity are briefly summarized in Fig. 1.

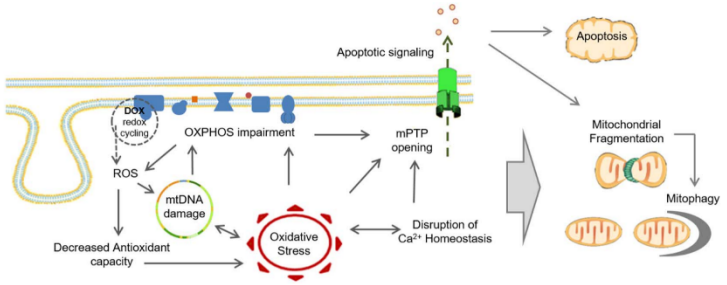

Fig. 1. Mechanisms of DOX-induced cardiac mitochondrial toxicity. DOX is reduced by complex I, forming a highly reactive semiquinone, initiating a redox cycle after reacting with oxygen and releasing ROS in the process. DOX-increased ROS generation compromises antioxidant machinery and damages lipids, proteins and nucleic acids, consequently resulting in an inhibition of oxidative phosphorylation, mitochondrial depolarization, ATP depletion, loss of calcium (Ca^{2+}) loading capacity, increase susceptibility to mPTP opening and release of pro-apoptotic proteins. In addition, mtDNA damage is also evident after DOX exposure. Together, these events may also result in mitochondrial fragmentation and contribute to an increased auto(mito)phagy signalling.

3. Exercise mitigates DOX-induced cardiac mitochondriopathy: modulation of redox state, apoptotic signalling and mitochondrial network

Considering the multiple mechanisms by which DOX-induced mitochondrial impairment can lead to dysfunction or even cardiomyocyte death, many pharmacological and non-pharmacological interventions (*e.g.* physical exercise) have been developed to target mitochondria in order to minimize DOX changes.

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 [12,45]. Those are important metabolic adaptations on the mitochondrial phosphorylative system that can result in improved ability to oxidize substrates [45–50] and could afford an increased tolerance of these organelles to harmful physiopathological conditions associated with mitochondrial dysfunction, including those triggered by DOX treatment.

As previously mentioned, the heart is particularly susceptible to DOX-induced oxidative damage. Therefore, since chronic physical activity is known to interfere with function and plasticity of these organelles, it is expected that exercise positively modulates some important cardiac defense systems to antagonize the toxic effects caused by DOX treatment [11,12].

Chronic physical exercise is widely documented to improve the cellular antioxidant machinery consequently contributing to decrease oxidative stress markers [25,26,45–47,51,52]. This is particularly important, as the heart has lower antioxidant defenses compared with other tissues [46,53], and a common response to DOX treatment is the decrease in the content/activity of antioxidant enzymes and machinery, including reduced glutathione (GSH) [54–56], glutathione peroxidase (GPx) [57–59], total superoxide dismutase (SOD) [56,58], MnSOD [59], catalase (CAT) [56,58]. Importantly, low intensity exercise training may not be sufficient to trigger myocardial antioxidant defenses [60]. Brito and co-workers [61] showed for the first time the protective effect of maternal exercise against DOX toxicity in offspring cardiomyocytes. The authors proposed that this increased intergenerational resistant phenotype of neonatal cardiomyocytes is associated with decreased oxidative stress and increased DNA integrity.

Exercise has also been associated to an augmented capacity of mitochondria to tolerate calcium, with further consequences on the control of mitochondrial-driven apoptotic cell death [26,46,62]. 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 [6,7]. Although the effects of exercise on calcium-induced mPTP opening are not completely understood, it has been reported that acute and chronic exercise prevented increased mPTP opening susceptibility in cardiac mitochondria of DOX-treated animals [46,63–65], producing heart mitochondria with a more resistant phenotype against DOX-induced apoptosis.

Data suggested that chronic exercise-induced cardioprotection may also be associated with an improvement of mitochondrial quality control [66,67]. Exercise increases cardiac mitochondria renewal and remodeling by promoting mitochondrial biogenesis, fusion, and auto (mito)phagy, in order to eliminate damaged mitochondria [67]. It is however noteworthy that the precise mechanisms and the signalling pathways behind exercise-related protective mitochondrial phenotypes are not fully understood so far. A growing number of studies have reported the potential involvement of exercise in cardiac mitochondrial quality control signalling, but these are mainly descriptive [68]. The mitochondrial beneficial effects of chronic exercise on heart seem to be associated with the induction of mitochondrial biogenesis and fusion signalling [65,67,69]. Cardiac autophagy signalling increased after exercise [67,70]; however, there are also reports where cardiac autophagy and mitophagy signalling were not altered after chronic exercise [44,65], suggesting that exercise induced a variety of improvements in cellular and mitochondrial defense/remodeling systems that possible refrain the activation of autophagic forms of renewal/clearance. As detailed in further sections (summarized in Fig. 3), adaptations in the mitochondrial network remodeling, including signalling mechanisms related to redox homeostasis, (de)activation of apoptotic death pathways, mitochondrial dynamics (biogenesis, fusion and fission) and quality control stimulated by chronic physical exercise might represent integrated and interdependent critical processes in the mitigation of DOX-induced cardiac and mitochondrial toxicity [44,65].

3.1. 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 increased ROS production. The mechanism behind the protective phenotype is thought to be through activating antioxidant signalling pathways, together with the improvement of mitochondrial bioenergetics and calcium loading capacity, and inhibition of apoptotic signalling, all of these altered in the course of DOX cardiotoxicity [11,12,71].

Several authors reported that exercise preconditioning (short and long-term exercise training – 5 days to 14 weeks) is an effective strategy to counteract acute toxicity resulting from a single dose of DOX (10 to 20 mg/kg), translated as increased cardiac oxidative damage [46,47,63,72–75], altered mitochondrial bioenergetics [46,47,74], increased apoptotic signalling [46,74], and altered mitochondrial biogenesis [76]. The increased expression of peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) by exercise in cardiac tissue from DOX-treated animals resulted in decreased forkhead-box O1 (FoxO1) mRNA, with subsequent attenuated expression of the muscle ring finger-1 (MuRF-1), a gene involved in muscle catabolism [76]. Generally, these results suggest that an active life style prior to DOX treatment may prevent DOX-associated mitochondrial bioenergetics collapse, and pro-apoptotic and oxidant unbalance. Table 1 details the effects of chronic exercise preconditioning against the changes in markers of mitochondrial dysfunction and associated signalling mechanisms induced by DOX.

3.2. Exercise during the course of DOX treatment

Although 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 remain incompletely understood. The effect of chronic exercise performed during DOX therapy were first examined by Kanter and co-workers [77], 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 sub-chronic DOX treatment resulted in increased antioxidant protection, and inhibition of apoptotic signalling [60]. Accordingly, concurrent moderate aerobic training and DOX treatment increased manganese superoxide dismutase (MnSOD), decreased oxidative damage, and attenuated the decreased activity of mitochondrial ETC complexes I and II in DOX-treated mice [78].

Independently of exercise intensity, voluntary exercise and treadmill running performed before and during sub-chronic DOX treatment counteracted disruptions of mitochondrial morphology (Fig. 2), bioenergetics, complex I activity and content, increased oxidative damage and the decreased activity of antioxidant networks, increased apoptotic signalling, and augmented mPTP opening susceptibility characterizing DOX treatment [65,69].

Table 1
Summary of some described mitochondrial-related changes 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
Mitochondrial morphology			
Abnormal mitochondria	↑ [46,48,69,79–82]	↓ [46,48]	↓ [69]
Density	↓or↑ [8,69]		↑ [69]
Oxidative stress			
Antioxidants	↓or= [46,47,60,69,74,78]	↑ [46,47,63,74]	↑or= [60,69,78]
Oxidative damage	↑or= [47,69,74,78,79,82–84]	↓ [46,47,63,74]	↓or= [60,69,78]
HSPs	↑or= [47,60,63]	↓ [47,63,74]	= [60]
Sirt3	↓ [20,69]		↑ [69]
p66Shc(pSer^{36})/p66Shc	↑ [69]		↓ [69]
Mitochondrial bioenergetics			
State 3	↓ [8,46,47,69,74,81,83,85]	↑or= [46,47];[74]	↑ [69]
RCR	↓or= [8,46,47,69,74,81,84–86]	↑or= [46,47,74]	↑ [69]
Maximal Δψ	↓or= [8,69,86]		↑ [69]
Lagphase	↑or= [69,86]		↓ [69]
Complex I activity and/or content	↓ [69,78,81,85]		↑ [69,78]
Complex II activity or content	↓or= [69,78,85]		↑or= [69,78]
Complex V activity and content	↓or= [69]		↑ [69]
AMPK activation	↓or= [76,78,87,88]	= [76]	= [78]
Apoptosis			
mPTP susceptibility	↑ [8,65,81]	↓ [46]	↓ [65]
Bax/Bcl2	↑ [46,65,82,83]	↓ [46]	↓ [65]
Caspase 3	↑ [38,60,65,83]	↓ [74]	↓ [60,65]
Caspase 8	↑ [65]		↓ [65]
Caspase 9	↑ [46,65]	↓ [46]	↓ [65]
Calpain	↑ [74,84]	↓ [74]	
TUNEL-positive nuclei	↑ [74,89]	↓ [74]	
Mitochondrial biogenesis			
PGC1α	↑or= [8,69]	↑ [76]	= [69]
TFAM	↓ [69,82]		↑ [69]
Mitochondrial dynamics			
Mfn1/2	↓,↑or= [8,65,78]	↑ [76] (Mfn2)	↑ [65,78]
Opa1	↓or↑ [8,65]		↑ [65]
DRP1	↑or= [8,65]		↓ [65]
Auto(mito)phagy signalling			
Beclin	↑ [44,65]	↓or= [44]	↓ [65]
Beclin/Bcl2	↑ [44,65]	↓ [44]	↓ [65]
Atg12	↑or= [44]	↓ [44]	
Atg12-Atg5	↑ [44]	↓ [44]	
Atg4	↑or= [44]	↓or= [44]	
Atg7	↑or= [44]	↓or= [44]	
LC3II/LC3I	↑ [44,65]	= [44]	↓ [65]
Pink	↑ [65]		↓ [65]
Parking	= [65]		= [65]
p62	↑ [65]		= [65]

AMPK – AMP activated protein kinase; 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; Δψ- 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; TUNEL, terminal (TdT)-mediated dUTP-biotin nick end labeling; 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 changes.

3.3. Mitochondrial dynamics and auto(mito)phagy: effects of exercise preconditioning and exercise during the course of DOX treatment

As mitochondria are organelles with multiple interconnected cell functions, published data have demonstrated 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 [44,65,78].

The effect of exercise on the regulation of mitochondrial dynamics is scarcely explored. To our knowledge, only two studies examined the effects of chronic exercise during the course of DOX treatment on modulation of primary regulators of mitochondrial dynamics machinery, including mitofusin (Mfn)1/2 [78], optic atrophy type 1 (OPA1) and dynamin-related protein (DRP)1 [65]. These studies suggested that exercise might have contributed to a proper regulation of mitochondrial dynamics essential for cellular survival.

Additionally, auto(mito)phagy is also tightly associated with the regulation and renewal of the mitochondrial network. It has been suggested that DOX effects on cell death pathways depends 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 [10]. Studies have shown the co-existence of increased apoptosis and auto(mito)phagy in the hearts of DOX-treated rats, both attenuated by voluntary and forced endurance exercise during DOX treatment [65]. Similarly, exercise preconditioning may play a role against DOX-induced activation of cardiac autophagy/lysosomal system pathway [44]. From the

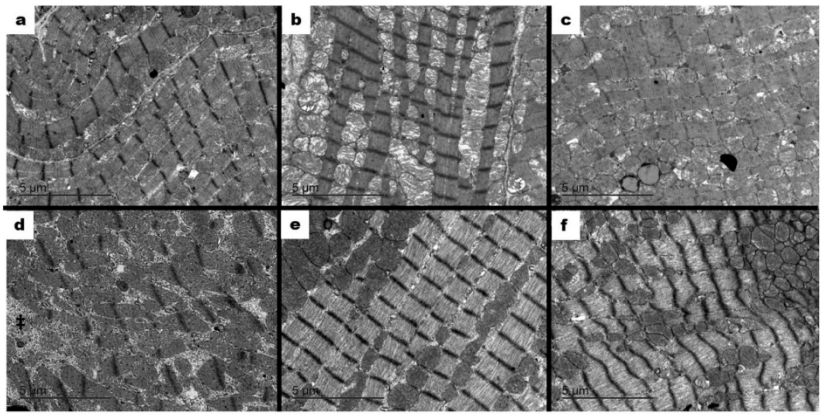

Fig. 2. Representative electron micrographs of cardiac tissue from (a) saline + sedentary group, (b) saline + TM (12-weeks treadmill) group, (c) saline + FW (12-weeks voluntary free-wheel) group, (d) DOX (7-weeks of sub-chronic DOX treatment 2 mg.kg-1 *per* week) + sedentary group (e) DOX + TM group and (f) DOX + FW group (magnification: 12000×). Note the protection afforded by chronic exercise (e and f) against impaired mitochondrial morphological changes characterizing heart tissue from sedentary DOX-treated group (d). From Marques-Aleixo et al. [69].

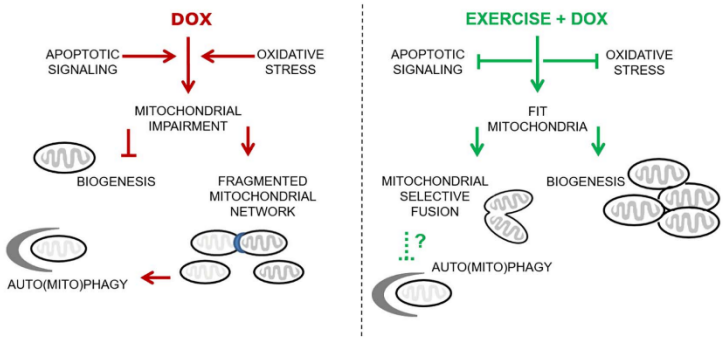

Fig. 3. Scheme summarizing heart mitochondrial dysfunction pathways induced by DOX treatment (on the left panel) and the described adaptations induced by chronic physical exercise against heart mitochondrial dysfunction induced by DOX treatment (on the right panel).

data described so far, 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 (Fig. 3). 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 [28]. These authors argue that under tolerable and mild stressful conditions, mitophagy acts 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 load that autophagosomes are unable to efficiently remove. These structurally and functionally damaged mitochondria increase free radical production with consequent oxidative stress, release of pro-apoptotic proteins from intermembrane space, and increase apoptotic protease activity, resulting in attenuated mitophagic flux and allowing for the execution of cell death [28].

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 performed before (pre-conditioning) and during DOX treatment against changes of mitochondrial dynamics and auto(mito)phagy.

4. Boosting effects of exercises in extra-heart mitochondria during DOX therapy

DOX treatment causes a variety of side effects and systemic disturbances that extend beyond the heart. Adverse effects of DOX treatment to other tissues rather than cardiac, including the brain, liver and

skeletal muscle, have also been described. Actually, neurological disturbances, changes in cognitive function, compromised liver function and decrease in muscle-specific force production are common reported side effects caused by DOX-containing chemotherapies [90–94].

Although the exact mechanisms of DOX-induced systemic changes in metabolism and tissue homeostasis are complex and still somewhat unclear, the increased production of ROS leading to oxidative damage and impairments in major mitochondrial functions are common features [for an extensive review on the topic see 95]. Importantly, some differences have been reported concerning the mechanisms behind DOX-induced mitochondrial dysfunction depending on the studied tissue.

Exercise-induced adaptations depend and require the coordination of multiple cellular events and one of the most important modulatory effects at the subcellular level probably occurs in the mitochondrion/mitochondrial network. Indeed, the protective effect of exercise against DOX has been attributed to mechanisms and signalling pathways related to mitochondria.

4.1. Skeletal muscle

The impact of endurance exercise on DOX-induced skeletal muscle toxicity is also a matter of study. DOX exposure impaired muscle function in a dose-dependent manner [96] and exhibited a time-dependent progressive decline in function during the 5 days following DOX treatment [97]. At a mitochondrial level, it has been reported a biphasic response of skeletal muscle mitochondria to a single doxorubicin injection (20 mg/kg) [98]. The authors suggested an inhibition in mitochondrial respiration and a marked increase in H_2O_2 emission 2 h after DOX exposure. Although these changes were rescued 24 h after DOX exposure, the respiratory capacity is further decreased at a later timepoint (72 h) along with increased H_2O_2 generation and an increased sensitivity to mPTP opening.

Long-term (10 weeks) resistance and endurance training preconditioning may be effective in preserving skeletal muscle function and minimizing fatigue as a consequence of acute DOX exposure [99]. Similarly to cardiac tissue, short-term exercise has also been suggested to mitigate acute DOX-induced skeletal muscle toxicity. Studies reported that exercise protected against DOX-induced compromised *soleus* levels of antioxidant enzymes and heat shock protein 72 (HSP72), oxidative damage, activation of calpain and caspase-3 [100]. Exercise also led to increased autophagy signalling [101], and protected against DOX-induced increases of FoxO transcriptional activity (FoxO3) and FoxO-target genes (MuRF-1 and BNIP3) and increased PGC-1α [76]. These findings are consistent with the notion that exercise training also protects against DOX-induced myopathy in skeletal muscle.

Recently, Dickinson et al. [102] reported that skeletal muscle size alterations caused by DOX treatment was limited by exercise training performed before and during the course DOX treatment. These improvements in skeletal muscle mass were associated with exercise-induced favorable changes in biomarkers of oxidative DNA damage and mTOR signalling pathway, whereas no alterations were observed regarding autophagy signalling proteins and in the master regulator of oxidative metabolism PGC1α gene expression.

4.2. Liver

As liver is one of the principal organ responsible for drug metabolic transformation and clearance, it is expected to be one of the most affected by DOX treatment. Increased oxidative stress and compromised antioxidant activity [103,104], decreased mitochondrial membrane potential and ATP levels in liver mitochondria [105], as well as altered mitochondrial dynamics and mitophagy [106] have been reported in rats submitted to DOX treatment.

In an attempt to ascertain whether acute exercise afford liver protection against DOX treatment in rodents, data from our group showed that exercise improved liver mitochondrial complex activities in DOX treated animals with no detected effects on endpoints of mitochondrial functionality, and apoptotic signalling markers [64]. The effects of chronic exercise performed before and during DOX treatment on liver mitochondrial bioenergetics, biogenesis, dynamics, apoptotic and autophagy signalling remain to be analyzed.

4.3. Brain

Given that DOX does not cross the blood-brain barrier [107,108], it has been proposed that DOX-induced brain mitochondrial dysfunction is independent from cellular drug entering. Although presently unclear, it has been proposed that the chemotherapy-related neurotoxicity is mediated by increased inflammatory response, as DOX increases the systemic production of tumor necrosis factor-α (TNFα) [108,109], which can migrate into the brain and stimulate locally neurotoxic-related pathways [110]. 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 [108,111,112]. This systemic and consequently pro-inflammatory environment in the brain caused by DOX induces oxidative damage driven by several cellular sources, including mitochondria and eventually lead to the increased susceptibility to mPTP and apoptosis [113]. These mechanisms seem to be central to explain DOX-induced brain mitochondrial toxicity.

Exercise has been reported to play a significant role in brain and cerebellum mitochondrial machinery improvements [114] and likely serves to attenuate mitochondrial dysfunction that characterizes, for instance, aging and neurodegenerative diseases [115]. Brain cortex and cerebellum mitochondrial modifications associated with cumulative sub-chronic DOX administration, including disturbances in mitochondrial oxidative phosphorylation capacity, modifications in redox state, in mPTP opening susceptibility, apoptotic signalling and mitochondrial quality control were mitigated by long-term physical exercise performed during treatments [116].

4.4. Testis

Besides the above-mentioned deleterious effects in several organs and tissues, DOX treatment also affects negatively testicular function and male fertility, and these effects are closely related to increased oxidative stress and apoptotic signalling [117]. Whether physical exercise could mitigate these consequences is so far not understood. Despite no changes were observed in oxidative damage markers and in apoptotic signalling proteins, proteomic analysis revealed that physical exercise performed before and during DOX treatment positively modulated testis proteome susceptibility to oxidation, particularly proteins involved in metabolic and stress response [118].

5. Exercise: what type, when and how much?

Physical exercise has been associated with the modulation of important physiological mechanisms, up-regulating some important tissue and particularly 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.

Several evidences from the literature have shown preconditioning-like effect in rodents of a single exercise bout against cardiac dysfunction and mitochondrionopathy characterizing DOX toxicity [64,119]. Moreover, some authors frequently described very short-term exercise running programs comprising only five exercise days followed by the induction of skeletal muscle toxicity caused by DOX [76,100,101], cardiac pathological states including myocardial ischemia-reperfusion

injury or DOX-related cardiac dysfunction [44,74,76,120,121]. Recently, two studies conducted in human cancer patients reported protective effects of single vigorous-intensity exercise bout(s) performed 24 h only before the first DOX treatment session [122] and before each DOX session [123] observed in systemic hemodynamic parameters, mood and skeletal muscle symptoms. Acute or short-term exercise preconditioning interventions may be particularly appealing as it does not conflict with work or family obligations. Although useful to identify potential mechanisms related to protection against acute cardiotoxicity, the short duration preconditioning models are unlikely to result in prolonged functional and general health benefits to the 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 in rodents. Additionally, there has been a growing interest on the effects, applicability and safety of chronic exercise interventions performed with cancer patients in the last decades.

Patients undergoing DOX-based chemotherapy exhibit debilitating fatigue and considerable exercise intolerance, interfering with their daily activity and quality of life. DOX treatment leads to disturbances to the global cardiovascular system that extend beyond the cardiac muscle and include endothelial dysfunction [124,125], decreased hemoglobin concentration [126], changes in plasma lipid and apolipoprotein levels [127] and upregulation of inflammatory response [128]. These, along with the deterioration of cardiac function can compromise the ability to deliver oxygen to exercising skeletal muscle. Indeed, decreased cardiorespiratory fitness has been reported after DOX chemotherapy treatment with an associated reduction of exercise tolerance [129,130]. Moreover, it has been suggested that cardiorespiratory fitness may be an independent predictor of survival in metastatic disease [131]. Patients undergoing DOX-based chemotherapy can experience skeletal muscle wasting with a reduction in type I and II muscle fibers sizes [132,133], which trigger significant functional changes leading to skeletal muscle fatigue and/or muscle weakness [93,99,132,134]. Although the physiological mechanisms underlying cancer-related fatigue remain to be elucidated, evidence suggests that this is a complex multifactorial phenomenon, including both tumor and therapy–related outcomes [for refs see 92].

Cancer-related fatigue may possibly limit the usefulness of exercise as a co-adjuvant therapy in patients undergoing DOX treatment [135]. However, several international institutions/agencies have published cancer-specific guidelines and recommendations for exercise enrolment following diagnosis [136]. Physical activity confers cardiovascular-specific benefits in women with breast cancer with an elevated cardiovascular risk phenotype resulting from the therapy side-effects [137]. Yu and Jones [138] summarized the clinical studies highlighting the beneficial effects of exercise interventions during or after completion of breast cancer treatment in the cardiovascular system. It has been suggested that moderate-intensity exercise not only maintains but significantly improves aerobic capacity and muscle strength in women undergoing chemotherapy (including with DOX) [139]. Importantly, a supervised aerobic training program comprising high-intensity aerobic interval training was considered safe (and relatively well tolerated) during adjunct therapy in women undergoing anthracycline-containing chemotherapy [140]. This interval-training model may present some resemblance 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 [141,142], these contradictory results/opinions justify the need to further investigate different exercise models to optimize cardiovascular and skeletal muscle 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 of exercise preconditioning *i.e.*, performed prior to DOX bolus, prevented DOX-induced impairments in left ventricle (LV) systolic and diastolic function [75,143], the increased plasma cardiac troponin I (cTnI) levels [48,63], and attenuated vascular smooth muscle dysfunction [144] and the severe morphological and histological signs of cardiac muscle injury [48]. Exercise preconditioning was shown to be protective, attenuating deficits in coronary flow [75], transmitral and transaortic flow [145,146], end-systolic pressure, left ventricular developed pressure, and the maximal rate of left ventricular pressure [119] and preserving myosin heavy chain (MHC) isoform distribution [145], even 5 to 10 days after DOX treatment. Importantly, most studies addressing exercise preconditioning used a single DOX bolus instead of a cumulative DOX treatment, which would closely mimic atypical treatment involving small doses administered over time to reach a cumulative dose. In an attempt to respond to this concern, Hydock and coworkers [147] reported that 10 weeks of exercise preconditioning followed by 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.

As the interval between cancer diagnosis and treatment enrolment can be shorter than the length of most studied preconditioning training programs, the feasibility of the application of this preventive strategy in humans has been questioned. Therefore, 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 [77] and protected/attenuated cardiac hemodynamic changes [60,148,149].

Another additional concern is the effect of exercise in the efficacy of DOX-induced decreased tumor growth. Data suggested that exercise is considered a safe cardioprotective intervention without reducing the antitumor efficacy of DOX [150,151]. However, there are also data showing that exercise increases DOX efficacy in inhibiting tumor growth without mitigating subclinical DOX-induced cardiotoxicity in a murine model of melanoma [152]. Importantly, a pilot study conducted in 20 women with early breast cancer receiving doxorubicin–cyclophosphamide therapy suggested that supervised aerobic exercise training interventions can improve several host (including cardiorespiratory fitness) and tumor-related pathways (beyond standard chemotherapy) that may have implications for cancer-related events and therapeutic response [153]. Collectively, the evidence supports that chronic exercise can be a promising strategy to prevent or treat DOX cardiotoxic effects.

6. Concluding remarks

The mitigation of DOX-induced tissue and mitochondrial toxicity remains an actual challenge. Data here discussed suggest that chronic physical exercise is a promising strategy to prevent DOX toxicity (preconditioning), or to act as an adjuvant therapy (during the course of DOX treatment) in several tissues, with particular effectiveness against DOX-mediated 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 the ability to reestablish cardiac mitochondrial network. The plasticity and ability of heart mitochondria and from other tissues such as liver, brain and skeletal muscle 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 molecular mechanisms underlying the mitochondrial-driven 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.

Transparency document

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5.

Summary of Results and General Discussion

Non-communicable diseases typical from modern industrialized countries represent nowadays serious health concerns, not only for the economy but also negatively influencing the well-being of people of distinct age ranges. These health problems comprise cardiovascular and metabolic as well as neurodegenerative diseases, which usually result in aggravated signs with the increasing age (Fontana, 2009). Among these pathological conditions are liver diseases, which result from a myriad of factors including excessive caloric intake in diet, inadequate levels of physical activity and physical fitness leading to the so-called non-alcoholic fatty liver diseases (NAFLD), and also from the exaggerated consumption of pharmacological agents, generally leading to toxicity at several levels of tissue and cellular organization (Pessayre et al., 2010). Actually, being the liver a key organ in the metabolization of drugs and clearance of body toxicity, it is expected that it can suffer from serious alterations regarding its structure and function resulting from the toxic interaction of the ingested toxic drug compounds and hepatic cell molecules (Chiang, 2014). With the increasing development and efficacy of pharmacological therapeutics, drug consumption has also concomitantly increased, with some of them (or even the majority) having side-effects and consequences in the target but mainly in non-target tissues. In this regard, DILI contributes to this scenario of increased hepatic diseases that can progress in extreme cases to cirrhosis and cancer. There are several drugs in the market known to induce harmful effects to the liver, including anti-inflammatory, anti-

diabetic, anticancer and antiretroviral agents (Pessayre et al., 2008, 2010). The biological mechanisms of toxicity of these drugs have been studied and, among others, mitochondrial dysfunction has been described as a common feature characterizing the hepatic phenotype of subjects submitted to treatments with these agents (Labbe et al., 2008; Vuda and Kamath, 2016). Being an organ highly dependent on energy metabolism for performing its detoxifying functions, the liver mostly relies on mitochondrial energy production through the oxidation of carbohydrates and fats. In addition, as mitochondrial network are key cellular substructures with important roles in many other mechanisms rather than energy production, such as redox regulation, establishment of ion homeostasis, control of cellular death and cellular signaling (Jonckheere et al., 2012; Kong et al., 2010; Moreira et al., 2011), the interaction of harmful drugs with hepatic mitochondria translates into serious deleterious consequences for the liver. Therefore, studies reporting perturbations in liver mitochondrial capacity to oxidate substrates (Gómez-Lechón et al., 2003; Pessayre et al., 2010) increased levels of oxidative stress and damage in mitochondrial macromolecules (Bort et al., 1999; Ponsoda et al., 1995), augmented levels of apoptotic cell death signaling driven by mitochondria as well as marked signs of cellular and subcellular remodeling suggested by alterations observed in signaling markers of mitochondrial dynamics, namely biogenesis, fusion and fission and also of auto(mito)phagy as a consequence, for instance, of anti-

inflammatory and anticancer treatments (Dirks-Naylor et al., 2014; Gómez-Lechón et al., 2003).

As a consequence of these deleterious effects in the liver after treatments, several pharmacological and non-pharmacological strategies to mitigate these effects have been proposed including antioxidants, AMPK activators, diet and exercise (Alshabanah et al., 2010; Gonçalves et al., 2013; Rector et al., 2011; Singh et al., 2018; Yang et al., 2018). In fact, physical exercise, particularly chronic programs of endurance-based stimuli, afford protection by increasing tissue resistance against deleterious consequences of many physiopathological conditions including cardiovascular, metabolic and neurodegenerative diseases (Ascensão et al., 2007; Bernardo et al., 2016; Lira et al., 2012). In contractile tissues such as skeletal and cardiac muscle, mechanical and metabolic demands imposed by exercise are the main responsible triggering stimuli for most of the described adaptations (Ploug et al., 1990; Schnyder and Handschin, 2015), and a lot of beneficial outcomes have been described as well as several related mechanisms (Hoffmann and Weigert, 2017). Regarding the exercise-induced adaptations in non-contractile tissues such as liver, adipose tissue or brain, it is well known that the contractile activity developed by skeletal muscle results in the release of hundreds of molecules (proteins, growth factors and myokines), which exert paracrine and endocrine effects (Banzet et al., 2009; Beleza et al., 2018; Rocha-Rodrigues et al., 2018). In the particular context of the present thesis, the liver seems to be clearly benefited from chronic exercise

programs. Several studies have consistently reported that aerobic fitness phenotypes demonstrated increased liver resistance, both under basal situations and against many additional deleterious conditions, associated with diseases such as NASH (Gonçalves et al., 2014a, 2015), NAFLD (Rector et al., 2008, 2011), obesity (Linden et al., 2013; Rector et al., 2008; Thyfault et al., 2009). Increased mitochondrial performance to oxidize substrates including fats and mitochondrial plasticity-related mechanisms are important common features associated with these cross-tolerance effects (Beleza et al., 2018; Rocha-Rodrigues et al., 2018). The general purposes of the experimental work done in the context of the present thesis are focused to fill the missing data on the effects of chronic physical exercise on: (i) liver mitochondrial remodeling through following signaling markers associated with oxidative stress, biogenesis, dynamics and auto(mito)phagy, known mechanisms intimately related to mitochondrial plasticity (**paper I**); (ii) on the impact of exercise against *in vitro* (Diclofenac – **paper II**) and (iii) *in vivo* (DOX – **paper III**) deleterious effects on liver mitochondrial function, which were. Additionally, a review focused on the current understanding of the mitochondrial-mediated mechanisms underlying the protective effects of physical exercise against DOX-induced toxicity in different tissues, including liver is also presented (**paper IV**).

5.1 Summary of results

During the setups performed trough the experimental work of this thesis, some additional contributions to better understand the role of chronic physical exercise and liver capacity for mitigating harmful conditions were provided. Both used exercise models, namely forced endurance treadmill training and voluntary physical activity in the form of free-wheel running, augmented the expression of proteins related to mitochondrial biogenesis and altered protein expression involved in mitochondrial dynamics and autophagy signaling, suggesting that exercise can induce liver mitochondrial adaptive remodeling and hepatocyte renewal (**paper I**). Moreover, exercise augmented hepatic mitochondrial resistance to *in vitro* diclofenac-induced mitochondrial alterations, including increased mitochondrial permeability transition pore opening susceptibility, possibly by modulating oxidative stress and MPTP regulators (**paper II**). Exercise also positively reversed the deleterious effects caused by sub-chronic *in vivo* DOX treatment, such as oxidative damage, mitochondrial dysfunction, and altered mitochondrial dynamics toward fission, thus contributing to increase liver resistance against DOX administration (**paper III**). Furthermore, basal liver ultrastructural alterations induced by the exercise programs applied suggest mitochondrial remodeling aimed at facing increased metabolic demands and counteracting stimuli-induced injury (**papers I, II and III**).

5.2 Effect of physical exercise on hepatic mitochondrial function

As previously mentioned, physical exercise has been reported to regulate and improve metabolic and mitochondrial functions in skeletal muscle and in other tissues, including the liver. The upregulation of key proteins and genes during and after physical exercise, of which PGC-1α is a hallmark, seems to be critical for the underling of hepatic adaptations. Morris et al., (2016) showed that the overexpression of PGC-1α in primary hepatocytes produced an increase in markers of mitochondrial content and function (citrate synthase and electron transport system complex proteins) and increased fatty acid oxidation capacity, reducing triacylglycerol storage. Thus, PGC-1α has emerged as a major metabolic co-regulator and it is considered a master piece of mitochondrial function and biogenesis (Liang and Ward, 2006; Liang et al., 2009). Under normal conditions, liver PGC-1α expression is relatively low when compared with other tissues such brain or heart (Liang et al., 2009). However, during chronic sub-lethal stressful conditions, such as physical exercise or prolonged fasting, the overexpression of PGC-1α is observed (Haase et al., 2011). Indeed, our results showed that both VPA and ET induced an increase in PGC-1α (**paper I**: Fig. 2). The increase in PGC-1α was accompanied by the increase of one of its target gene products, the mitochondrial transcription factor A (TFAM), which is essential for mtDNA transcription and replication (Ekstrand et al., 2004). Concomitantly, an increase of other canonic mitochondrial content hallmarks

such as citrate synthase activity (**paper I**: Fig. 2) and TOM20 content (**annex**: Fig. 1A) was observed, clearly suggesting that physical exercise promoted hepatic mitochondrial biogenesis.

5.2.1 Mitochondrial dynamics

The formation of new mitochondria is necessary to keep an adequate and healthy mitochondrial network through a constant and dynamic turnover. This mitochondrial turnover is accomplished by a dynamic process of fusion and fission of the reticulum network. Mitochondrial fusion allows the integration of new mitochondria (elongation of mitochondrial network) as well as the efficient mixing of their content (Westermann, 2012). Both effects (fusion and content mixture) are advantageous under conditions of high-energy demand, such as physical exercise. Decreased fusion levels can result in loss of mitochondrial respiratory capacity and dysfunction. This process is dependent of three GTPases with distinct mitochondrial sub-localization: mitofusins (MFN1 and MFN2) in the outer membrane and OPA1 in the inner membrane (Shirihai et al., 2015; Westermann, 2012). On the other hand, mitochondria can also undergo a process of fission. This process is necessary to facilitate the removal of damaged organelles from the mitochondrial reticulum, contributing to the preservation of the mitochondrial bioenergetics (Twig and Shirihai, 2011; Westermann, 2012) and require a large GTPase dynamin-related protein 1 (DRP1). This protein is present in cytoplasm and binds to the mitochondria

through mitochondrial fission 1 protein (Fis1) where after activation start the scission of organelles (Gomes and Scorrano, 2008; Shirihai et al., 2015). The interplay and balance between fusion and fission is essential to maintain the homeostasis of the mitochondrial network. For instance, excessive mitochondrial fragmentation has been associated with several diseases such as cancer, metabolic and cardiovascular disorders and neurodegenerative diseases (Alzheirmer´s or amyotrophic lateral sclerosis) (Barsoum et al., 2006; Liu et al., 2014; Reddy et al., 2011). Some studies showed that the excessive mitochondrial fission is intimately related to the increase of ROS (Barsoum et al., 2006; Manczak et al., 2010). Indeed, in a study conducted by Solesio et al., (2013) in cell culture showed that the use of mitoQ reduce dramatically the mitochondrial fission induced by a neurotoxin and prevent the translocation of DRP1 to mitochondria, suggesting that mitochondrial fission is mediated by ROS. Concomitantly, excessive mitochondrial fission can in turn led to elevated ROS production thus begetting a vicious cycle (Ježek et al., 2018).

Although mitochondrial adaptations caused by physical exercise are well-known in contractile tissues, hepatic mitochondrial dynamics are so far poorly understood, and the literature is scarce on this particular issue (Gonçalves et al., 2016). In the first study of this thesis (**paper I**) it was reported that physical exercise promotes an alteration in the hepatic mitochondrial dynamic signaling markers, as pointed by increased OPA1 and DRP1 proteins in the ET group (**paper I**: Fig. 3B, C), although no alterations were observed in MFN1 content.

Despite that both pro-fusion and -fission proteins were increased, the ultrastructural analysis of the mitochondrial morphology (**paper I**: Fig 1) suggested an imbalance towards fusion. Electron microphotographs analysis showed an increase in the mean mitochondrial area (probably because of increased fusion of new mitochondria) without alterations in mitochondrial density, which is usually increased in cells with excessive mitochondrial fission (Manczak et al., 2010; Reddy et al., 2011). Although the molecular signaling pathways underlying the effects between physical exercise and mitochondrial dynamics in the liver are still poorly understood, studies in other non-contractile tissues suggest that PGC-1α plays an important role. Dabrowska et al., (2015) showed that the overexpression of PGC-1α induced an increase of mitochondrial MFN and DRP1 expression in *substantia nigra* of rats. Moreover, this study confirms the close interplay between fusion, fission and biogenesis-related proteins in the dynamics of mitochondrial reticulum as immunoprecipitation data reveals that binds PGC-1α to DRP1 in the process (Dabrowska et al., 2015). Thus, the increase of PGC-1α found in our studies (**paper I and III**) could explain, at least partially, the modulation of mitochondrial dynamics above described.

5.2.2 Autophagic signaling

The maintenance of a healthy and functional mitochondrial network requires an equilibrium between mitochondrial fusion, fission as well as biogenesis and

degradation, the latter being accomplished by auto(mito)phagy. Several studies showed that the dysregulation of the autophagic signaling can lead to the accumulation of damaged organelles and therefore to cell death (Thiessen et al., 2017). Thus, dysfunctional fragmented mitochondria are removed from the cell by mitophagy (Twig et al., 2008; Westermann, 2012). This is a highly complex and dynamic process involving several steps such as the formation of a double-membrane structure (the autophagosome) where the autophagic target is included; and the fusion of the autophagosome with a lysosome to form autolysosomes where the autophagic target is degraded (Bartlett et al., 2017). There has been little research investigating the role of physical exercise in hepatic autophagic signaling. Indeed, to our knowledge, the **paper I** of the present thesis is the first published study showing that physical exercise *per se* modulates some autophagic signaling markers in liver. Both chronic exercise types promoted an increase of Beclin-1 and LC3II proteins (**paper I**: Fig. 4A and E). Beclin-1 is a central positive regulator of autophagy (Kang et al., 2011) and LC3II is an important marker of autophagosome formation (Gomes et al., 2011). Additionally, our results showed an increase of p62 in ET rats (**paper I**: Fig 4G), which has a significant role in the formation of ubiquinone aggregates that identity the autophagy targets (Liu et al., 2016). Furthermore, the evaluation of mitophagic markers showed that ET promoted a decrease of PINK and an increase of Parkin (**paper I**: Fig 4H and I). Briefly, Parkin is translocated from the cytoplasm to the dysfunctional mitochondria by PINK-dependent

mechanisms. Once in the mitochondria, Parkin promotes fragment degradation via ubiquitin-proteasome system and mediates the recruitment of the autophagic adaptor p62 (Chan et al., 2011; Jin et al., 2010). Vainshtein et al., (2015) demonstrated that acute exercise modulates the expression of several of these autophagy-related genes in a PGC-1α-dependent manner in skeletal muscle. Thus, the observed increase of PGC-1α in our results could, at least partially, explain the alterations in autophagic signaling in the liver of exercised rats. Therefore, our results seem to indicate that physical exercise promoted a coordinated upregulation between the different hepatic mitochondrial quality control mechanisms (namely mitochondrial biogenesis, dynamics and autophagy) which could potentially lead to a healthier and more plastic mitochondrial network. Autophagy is a dynamic process resulting from a plea of intricate and coordinated mechanisms of difficult analysis and understanding. In the studies comprised in the present thesis, only molecular signaling markers of these different processes were evaluated complemented with descriptive morphological analysis, which is limited in the context of such complex mechanism. Further studies are therefore needed to deeply analyze the interplay between the processes behind mitochondrial plasticity induced by physical exercise in the liver.

5.2.3 Oxidative and antioxidant biomarkers

As previously mentioned, physical exercise is associated with an increase of ROS production (Radak et al., 2017), which in high levels can be dangerous to the cell. However, chronic and moderate release of ROS is linked with beneficial metabolic adaptations such as mitochondrial biogenesis and expression of antioxidant enzymes in contractile tissues (Powers and Jackson, 2008). Thus, like in contractile tissues, although through upstream extracellular "motive forces", including inflammatory mediators, one of the possible mechanisms underlying the beneficial effects of physical exercise in non-contractile tissues is the oxidative stress regulation (Ascensão et al., 2013). Our results showed a decrease in the levels of the oxidative stress marker MDA (**paper II**: Fig. 3C) in both exercise protocols, which was associated with an increase of aconitase activity (**paper II**: Fig. 3A), a frequently used biochemical marker of oxidative stress in the cell due to its vulnerability to oxidative damage (Vasquez-Vivar et al., 2000). Despite these biomarkers suggest that exercised rats had lower oxidative stress than sedentary animals, our results did not show an increase of the antioxidant enzyme MnSOD (**paper II**: Fig. 3B), which seems contrary to what was founded in Lima et al., (2013). However, and similarly to what has been found in Ascensão et al., (2012), our results showed that both models of physical exercise increased SIRT3 content (**paper II**: Fig. 3E). Similarly to aconitase, SIRT3 is a very ROS-sensitive enzyme, which is found decreased under elevated oxidative stress (Wu. et al 2014). SIRT3 is a downstream target

gene of PGC-1α (Kong et al., 2010) that has an important role in metabolic reprogramming (such as mitochondrial biogenesis) and ROS scavenging through the deacetylayion and acetylation of mitochondrial and antioxidant enzymes such as glutathione peroxidase-1 (GPx1) or catalase (Ahn et al., 2008; Kong et al., 2010). Moreover, the evaluation of OXPHOS-related proteins showed that both exercise models induced an increase in complex IV content (**paper II**: Fig. 2D), which is associated with an indirect reduction of ROS production by decreasing the leak of electrons (Parise et al., 2005).

5.2.4 Apoptotic signaling

One of the most deleterious effects associated to exacerbated oxidative stress is the activation of cell death signaling (Redza-Dutordoir and Averill-Bates, 2016). Thus, in agreement with the abovementioned findings, our results showed that exercised rats had increased levels of the anti-apoptotic protein Bcl-2 (**paper II**: Fig. 5 B and C) and lower susceptibility against calcium-induced mitochondrial MPTP induction with consequent swelling (**paper II**: Fig. 4 A-D). Mitochondria cooperate intimately with endoplasmic reticulum in the maintenance of cytosolic calcium homeostasis, participating in the regulation of calcium-dependent pathways. However, excessive calcium uptake can act as a trigger for the apoptosis signaling through the MPTP opening, which results in mitochondrial cytochrome c release, Bax and pro-caspases activation (Ascensão et al., 2013). The activation and upregulation of anti-apoptotic

proteins, such as Bcl-2, can prevent the MPTP formation (Shamas-Din et al., 2013). Consequently, our data suggested that the hepatic tissue of trained animals exhibited a more protective phenotype against oxidative stress and apoptotic signaling.

5.2.5 Mitochondrial bioenergetics

Considering the beneficial mitochondrial adaptations found at a molecular level in exercised rats, it would be expected that the *ex vivo* mitochondrial function was significantly improved. Surprisingly, physical exercise did not improve any mitochondrial function parameters, such as RCR, ADP/O and lag phase (**paper II**: Fig. 1A-F), which reflects the ADP phosphorylation efficiency and electric potential fluctuations.

Nevertheless, both exercise models seem to positively modulate molecular and cellular hepatic features related to a more resistant and plastic phenotype, which could emerge under stress conditions such as drug administration. Consequently, the next section will further discuss the beneficial effects of physical exercise against drug-induced hepatic mitochondrial toxicity, specifically in an *in vitro* Diclofenac toxicity model and in an *in vivo* model of subchronic administration of DOX.

5.3 Effects of physical exercise against drugs-induced mitochondrial dysfunction

Previous studies showed that physical exercise is an effective non-pharmacological strategy to increase liver resistance against drug toxicity. Indeed, a previous study from our group showed that 5 weeks of ET revert the mitochondrial uncoupling induced by salicylate *in vitro* toxicity (Ascensão et al., 2012), suggesting that physical exercise can be a viable non-pharmacological strategy in patients with clinical risk of drug hepatotoxicity development.

5.3.1 Cross-talk between physical exercise and Diclofenac-induced mitochondrial dysfunction

In **paper II,** the effect of two models of exercise against mitochondrial toxicity induced by *in vitro* diclofenac (15 μM and 25 μM) was evaluated. The hepatic mitochondrial fraction of trained and sedentary rats was incubated with a diclofenac-containing solution during the bioenergetic and swelling assays.

Exercised groups exhibited higher resistance against mitochondrial dysfunction induced by diclofenac incubation. Our results showed that diclofenac compromised the mitochondrial function, as observed by increased state 4, a measure of oxygen consumption not associated to ADP phosphorylation, which reflects mitochondrial uncoupling (**paper II**: Fig. 1B). Concomitantly, the RCR, a hallmark of respiratory efficiency, was found decreased (**paper II**: Fig. 1C).

Furthermore, increased susceptibility to MPTP opening (**paper II**: Fig. 4C and D) and decreased of mitochondrial potential (**paper II**: Fig. 1D) were found. These potential negative alterations were counteracted by both ET and VPA chronic exercise models (**paper II**: Fig. 1A-F and Fig. 4 C and D), further reinforcing the hypothesis that physical exercise promoted hepatic mitochondrial adaptations that could increase liver tissue resistance against drug toxicity.

5.3.2 Cross-talk between physical exercise and DOX-induced mitochondrial dysfunction

In **paper III** the hepatic and mitochondrial resistance of exercised animals to a subchronic intraperitoneal administration of DOX was evaluated. As summarized in **paper IV**, previous studies from our group using the same model showed that physical exercise attenuates mitochondrial dysfunction induced by DOX in heart and brain tissues (Marques-Aleixo et al., 2015b, 2015a, 2016, 2017). Similarly, **paper III** showed that DOX compromised mitochondrial ultrastructure and function and induced hepatic damage as observed by the presence of elevated hepatic transaminases levels in serum (**paper III**: Table 1). One of the main mechanisms associated to DOX toxicity is the exacerbated production of ROS, which has been extensively described in tissues like heart (Lahoti et al., 2012; Zhou et al., 2001). Here, we demonstrated that DOX treatment also induces an increase of oxidative damage markers (MDA, protein carbonylation, aconitase) in liver with a concomitant reduction of the

antioxidant enzyme MnSOD (**paper III**: Table 2), contrasting with the results obtained by Dirks-Naylor (2013). Dirks-Naylor study showed an increase in glutathione and a decrease in carbonylated protein content in liver 24 h after acute DOX administration. A possible explanation for the different results found in both studies could be associated to the distinct DOX treatment protocols (acute vs. sub-chronic) and the possible adaptive capacity of liver against acute insults. As observed in healthy trained animals (**paper II**), both exercise models promoted a decrease in MDA and carbonylated proteins, although MnSOD activity remained similar to SED+DOX animals, contrary to what has been observed in brain (Marques-Aleixo et al., 2016), suggesting that other mechanisms could be behind the hepatic antioxidant modulation exerted by physical exercise in rats treated with DOX. Furthermore, other antioxidant systems, such as glutathione, should be analyzed to further understand the protective role of exercise against oxidative stress in liver.

Exacerbated oxidative stress can lead (and/or be a consequence of) to impaired mitochondrial biogenesis and dynamics (Wu et al., 2011). Indeed, DOX induced a decrease of PGC-1α protein expression and its downstream target TFAM (**paper III**: Fig. 3A-B), leading to decreased mitochondrial density (**paper III** Fig. 5I). In contrast to what has been described in healthy animals in **paper I**, only ET increased the protein content of PGC-1α and TFAM, concomitantly reverting the decrease of the mitochondrial biogenesis markers observed in SED+DOX (**paper III**: Fig. 3A and B) and mitochondrial density (**paper III**: Fig. 5I). In

agreement with previous works in non-contractile tissues performed by our group (Marques-Aleixo et al., 2016), DOX treatment promoted a shift of mitochondrial dynamics towards fission, as reflected by the decrease in fusion-related proteins (OPA1 and MFN1) and an increase in fission protein DRP1 (**paper III**: Fig. 2A-C). The above-mentioned study conducted by Dirks-Naylor et al (2014) using an acute model of DOX administration also reported an imbalance towards mitochondrial fission in hepatic tissue after DOX treatment. Excessive mitochondrial fragmentation can result in a bioenergetic impairment and lead to cellular damage or even cell death.

After submitting DOX-treated rats to physical exercise, we found that ET reverted the content decrease of fusion-related proteins induced by DOX but did not attenuated the increase of DRP1 (**paper III**: Fig 2A-C). The fact that both pro-fusion and pro-fission proteins were found increased suggests that ET promoted a mitochondrial reticulum remodeling that could enhance the integration of new mitochondria, while still removing the damaged ones. Indeed, the results obtained from the analysis of autophagic-related markers in ET+DOX rats showed an increase of PINK and Parkin proteins, which are directly associated to mitophagy (**paper III**: Fig. 4H and I).

The DOX-induced disturbances observed at a molecular level translated into impaired mitochondrial function. Indeed, and similarly to what was found in **paper II** in mitochondria incubated with diclofenac, **paper III** showed that DOX led to an increase in state 4 and lag phase (**paper III**: Fig. B and H) and a

decrease in RCR and ADP/O (**paper III**: Fig. C and D), suggesting mitochondrial uncoupling and lower respiratory efficiency compared to the SED+SAL group. Oxygen consumption after incubation with the ATP synthase inhibitor oligomycin was higher in SED+DOX rats than in SED+SAL animals, indicating an uncoupling between mitochondrial respiration and oxidative phosphorylation (**paper III**: Fig. 1E).

As discussed above (**paper I**), positive alterations at a molecular level do not forcibly imply improved mitochondrial function. Thus, and contrary to what was found in **paper II**, ET (but not VPA) partially reverted the DOX-induced mitochondrial dysfunction, as reflected by the decrease in lag phase time and oligomycin rate found in ET+DOX rats. Lag phase represents the time necessary to phosphorylate ADP. The decrease of this parameter in ET+DOX, together with a non-significant state 4 decrease and RCR increase, suggested greater mitochondrial membrane integrity stability and a more efficient oxidative phosphorylation, which could lead to a more preserved mitochondrial function in ET rats during a DOX treatment.

5.3.3 Differences between ET and VPA

Results in **paper I** and **II** suggested that VPA could be an interesting tool to promote mitochondrial molecular and functional adaptations in DOX models of toxicity. Indeed, our results showed that VPA+DOX animals had a lower oxidative stress observed by the decrease MDA and carbonylated proteins

(**paper III**: Table 2), which suggest a greater resistance against oxidative damage induced by DOX. However, and contrary to observed in DOX+ET rats, VPA rats exposed to DOX treatment showed less consistent results. For instance, the VPA+DOX group did not show significant alterations in the mitochondrial biogenesis-related markers, although increased mitochondrial density was observed (**paper III**: Fig. 5I). This apparently divergent results could be partially explained by the downregulation of remodeling processes such as mitochondrial dynamics and auto(mito)phagy (**paper III**: Fig. 2 and 4), which could lead to the accumulation of damaged mitochondria.

One of the possible explanations for the divergent results between VPA healthy animals (**paper I** and **II**) and VPA+DOX animals is the decrease of physical activity observed after the first injection of DOX (**Annex:** Fig. 2A). Specifically, the present VPA model consisted in a 24-h access to a freewheel (FW). Thus, the drop of daily running distance induced by the subchronic administration of a drug as *strong* as DOX reflects the decrease of the frequency, duration and intensity of the exercise bouts[1]. As reviewed in **paper IV** these three parameters are paramount in the development of cellular metabolic adaptation to exercise. Therefore, the drastic DOX-induced decrease in FW running time could prevent the activation of several signaling pathways that underlie the

[1] The exercise performed by rats in a FW is characterized by short bouts (< 1 min) at high speed followed by short-to-long lasting pauses, which in a certain way mimics the exercise bouts performed during high intensity interval training. Thus, the very nature of FW-VPA is completely different from ET.

beneficial adaptations to exercise. On the other hand, ET, as a forced treadmill exercise, was kept at a constant intensity, duration and frequency during the entire exercise protocol (except in the last two weeks), leading to the mitochondrial adaptions described in the previous section. Indeed, one of the most relevant differences between ET+DOX and VPA+DOX groups was the hepatic PGC-1α content, which was found downregulated in the later (**paper III**: Fig. 3A). Considering the pivotal role of PGC-1α in a wide range of metabolic and mitochondrial adaptations (to the point of being considered a master regulator of the mitochondrial function) (Haase et al., 2011; Vainshtein et al., 2015), the inability of VPA to revert DOX-induced PGC-1α downregulation could explain the lack of further adaptations and a protective phenotype in VPA+DOX animals compared to ET+DOX rats. In the face of these results, one can perhaps say that some adaptations of hepatic tissue to mitigate DOX-induced toxicity may be intensity-dependent.

5.4 Summary

This thesis presents several evidences demonstrating that physical exercise promotes mitochondrial molecular adaptations on a non-contractile tissue such as liver. These adaptations lead to increase hepatic resistance against drug-induced mitochondrial dysfunction both in in vitro diclofenac incubation and in in vivo subchronic DOX administration. Thus, an adequate protocol of physical exercise could be a potential non-pharmacological tool against drug-

induced hepatic toxicity. Future studies, such as those involving cell cultures or knockout animals, will collaborate to a better understanding of the underlying mechanisms of exercise-induced hepatic adaptations. Figure 3 shows a graphic summary of the main findings of this work.

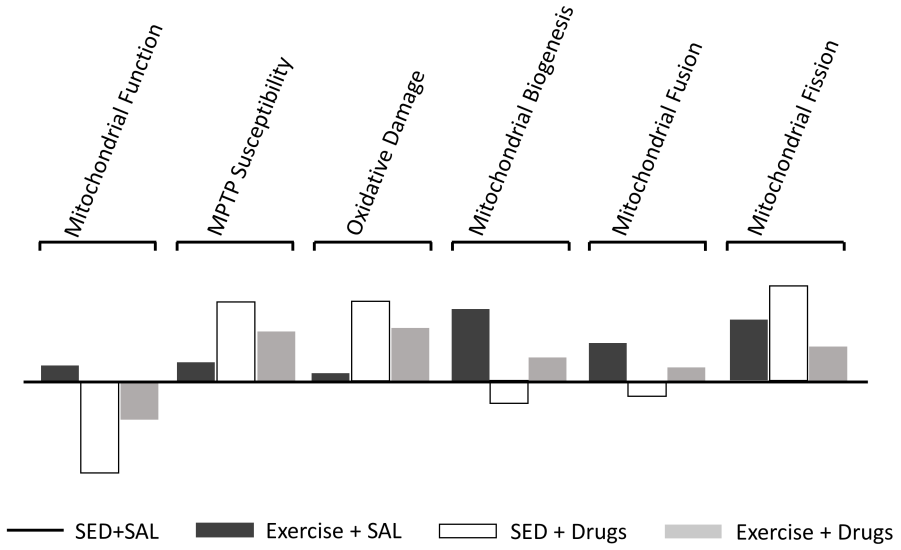

Figure. 3: Visual representation of the relative effects of physical exercise, drug administration and drug administration plus exercise in the main analyzed parameters. This illustration has not statistic value.

6.

Conclusions

1. Physical exercise decreases the hepatic redox environment, which was observed by a decrease of oxidative stress biomarkers and an increase in aconitase activity, a ROS sensitive enzyme.
2. No alterations in the antioxidant enzyme (MnSOD) are observed in exercised groups, although the overexpression of SIRT3 content suggests that an upregulation in other antioxidant systems may have taken place.
3. Physical exercise induces mitochondrial biogenesis, upregulates the expression of fusion and fission-related proteins and modulates the expression of proteins related with autophagic signaling which indicates a mitochondrial network more dynamic and plastic.
4. In vitro incubation with diclofenac induces mitochondrial dysfunction and MPTP opening. This is mitigated by both types of exercise which promote a higher resistance against diclofenac-induced MPTP opening in part due to the upregulation of antiapoptotic biomarkers.
5. Intraperitoneal subchronic administration of DOX leads to hepatic oxidative and mitochondrial damage. Both exercise models mitigate the hepatic damage and decrease the oxidative stress observed by the reduction of transaminases, MDA and carbonylated protein levels.
6. Endurance training partially revert the DOX effects on mitochondrial function whereas no alterations are observed in the voluntary physical exercise group.

7. The expression of several proteins related with mitochondrial biogenesis, dynamic and autophagic signaling are downregulated by DOX treatment. The DOX effect is only reverted in the endurance training group indicating a more resistant and plastic mitochondrial reticulum.

8. These results indicate that physical exercise can be a potential non-pharmacological tool against the hepatic toxicity induced by drugs such as diclofenac and DOX.

7.

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Annex

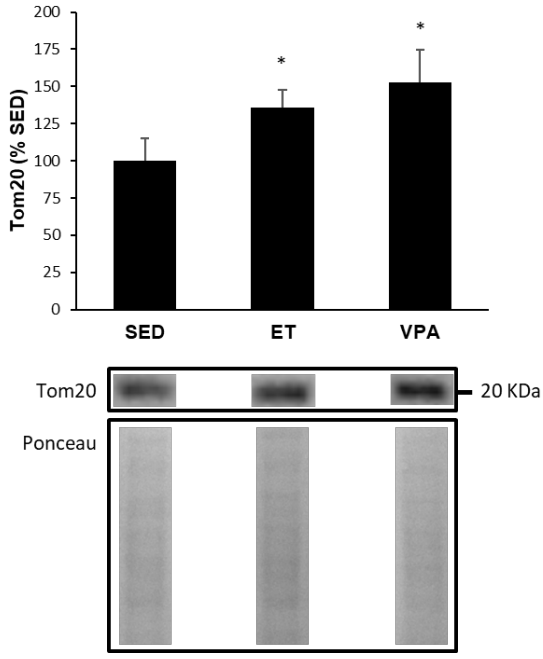

Figure 1A: Effects of ET and VPA on liver mitochondrial import receptor subunit Tom20. Data are expressed as means ± SD (n=4) and in percentage related to SED group. Bands are normalized to Ponceau staining. Significance ($p<0.05$): (*) vs SED group. SED, sedentary animals; ET, endurance training animals; VPA, Voluntary physical activity animals.

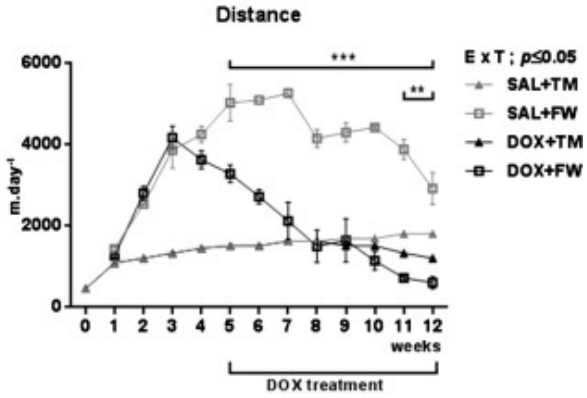

Figure 2A: Effect of exercise and sub-chronic DOX treatment on distance covered per day by animals of the TM and FW groups during the 12 weeks of protocol. SAL+SED — saline sedentary, SAL+TM — saline treadmill, SAL+FW — saline free wheel, DOX+SED — doxorubicin sedentary, DOX+TM — doxorubicin treadmill, and DOX+FW — doxorubicin free wheel, n=6 per group. Significant ($p < 0.05$) interaction effect of exercise and treatment (E × T) is indicated. (*) DOX + SED vs. SAL + SED; (**) DOX + TM vs. SAL + TM; (***) DOX + FW vs. SAL + FW; Figure published in *Physical exercise prior and during treatment reduces sub-chronic doxorubicin-induced mitochondrial toxicity and oxidative stress.* (Marques-Aleixo et al. 2015; Mitochondrion; *20*, 22-23).