Universidade de Aveiro

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Exercício e efeitos da Doxorubicina na função testicular

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica do Professor Doutor António Ascensão, Professor na Faculdade de Desporto da Universidade do Porto e co-orientação da Professora Doutora Margarida Sâncio da Cruz Fardilha, Professora auxiliar convidada da Secção Autónoma de Ciências da Saúde da Universidade de Aveiro.

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agradecimentos

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palavras-chave

Doxorubicina, Stress oxidativo, antioxidantes, exercício físico, apoptose, função testicular

resumo

A Doxorubicina (DOX) é um agente antineoplásico de grande eficácia utilizado no tratamento de vários tipos de tumores. No entanto, a sua utilização clínica é limitada devido à sua toxicidade em vários órgãos, com destaque para o coração. Outros órgãos afectados por este fármaco incluem fígado, cérebro, rins e testículos. Algumas estratégias farmacológicas e não farmacológicas têm sido desenvolvidas de forma a contrariar os seus efeitos secundários tóxicos, incluindo suplementação com antioxidantes e, mais recentemente, exercício físico. Assim, o objectivo do presente estudo é avaliar o efeito da actividade física na funcionalidade testicular, bem como no stress oxidativo e apoptose, sugeridos para a acção tóxica da DOX.

Trinta e seis ratos macho Sprag-Dawley foram divididos em 6 grupos: salino sedentário (SAL+SED), sedentáros tratados com doses sub-crónicas de DOX – injecções de 2mg.Kg-1 durante sete semanas (DOX+SED), salinos treinados na passadeira durante 12 semanas (SAL+TM), treinados tratados com DOX (DOX+TM), salinos realizando exercício voluntário em roda livre (SAL+FW) e tratados realizando exercício voluntário em roda livre (DOX+FW). Vinte e quatro horas depois da última sessão de exercício, os animais foram sacrificados, os espermatozóides foram obtidos e tratados para estudos de contagem e de motilidade. Os testículos foram recolhidos para posterior análise de marcadores de stress oxidativo (actividade da aconitase, concentrações de substâncias reactivas de ácido tiobarbiturico, malondialdeido (MDA) e de grupos sulfidril (-SH) e sinalização apoptotica (actividades das caspases 3,8 e 9). O tratamento com DOX induziu uma diminuição significativa na contagem e motilidade dos espermatozóides, independentemente da actividade física. Apesar de existir uma tendência para um aumento de MDA e diminuição de –SH com o tratamento com DOX, não foi detectado qualquer efeito significativo nos marcadores de stress oxidativo e apoptose. Não foi observado qualquer efeito do exercício nestes parâmetros. Concluindo, o exercício físico não influenciou o impacto que a DOX teve na funcionalidade testicular. Surpreendentemente, nem a DOX nem o exercício modularam o ambiente redox e a sinalização apoptótica nos testículos, considerando os marcadores analisados.

keywords

Doxorubicin, Oxidative stress, apoptosis antioxidants, physical exercise; testicular function.

abstract

The anthracycline Doxorubicin (DOX) is a widely used antineoplastic agent against several tumors with high efficacy. However, the clinical use of this drug is limited by its dose-related toxicity in several organs with particular emphasis on the heart. Other organs affected by DOX include liver, brain, kidney and testes. Several pharmacological and non-pharmacological strategies have been designed to antagonize the toxic side effects of DOX, including antioxidant supplementation and, recently, physical exercise. Therefore, the aim of the present study is to analyze the effect of physical exercise in testes function as well as oxidative damage and apoptosis, suggested mechanisms by which DOX exerts its toxic effects.

Thirty-six Sprag Dawley male rats were randomly divided into 6 groups as follows: Saline Sedentary (SAL+SED), Sedentary sub-chronically treated with DOX – 2mg.Kg-1 injections for 7 weeks (DOX+SED), Saline endurance treadmill trained for 12 weeks (SAL+TM), trained receiving DOX (DOX+TM), saline voluntary exercised in a free-wheel (SAL+FW) and voluntary exercised receiving DOX (DOX+FW). Twenty-four hours after the last exercise bout, animals were sacrificed; sperm was obtained and treated for counting and motility studies. Testes were harvested for tissues analysis of markers of oxidative stress and damage (aconitase activity, thiobarbituric acid reactive substances, as MDA, and sulfhydryl –SH groups content) and apoptotic signaling (caspases 3,8 and 9 activities). DOX treatment induced significant decrease in sperm count and motility, irrespective of exercise training status. Despite a tendency for MDA increase and –SH decrease with DOX treatment, no significant effect was detected in either markers of oxidative damage or apoptosis. No exercise effect was observed as well.

In summary, chronic physical exercise did not influence DOX-induced testes dysfunction. Surprisingly, neither DOX nor exercise modulated testes redox environment and apoptotic signaling, at least seen by the measured markers.

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Abbreviations:

DOX-(Doxorubicin); RONS-(Reactive Oxygen Nitrogen Species); OS-(Oxidative stress); FSH-(Follicle stimulating hormone); LH-(Luteinizing hormone); GnRH-(Gonadotropin-releasing hormone); mDNA-(mitochondrial DNA); SHBG-(Sex Hormone Binding Globulin); NSAID´s-(Non Steroid anti-inflammatory drugs); HPT-(Hypothalamic-pituitary-testicular); GSH-(glutathione); GSSG-(glutathione disulfide); CAT-(catalase); SOD-(superoxide dismutase); GSH-Px-(glutathione peroxidase); GSH-R-(Glutathione reductase); IV-(Intra venous); UV-(ultra-violet); CYC-(Cyclophosphamide); G6PD-(Glucose-6-phosphate dehydrogenase); NADPH-(nicotinamide adenine dinucleotide phosphate-oxidade); O_2 - (Oxygen); H_2O - (Water); MDA-(malondialdheyde); AIF-(Apoptosis inducing factor); MPTP-membrane permeability transition pore); ETC-(electron transporter chain); Mn-SOD-(manganese superoxide dismutase); VDAC-(voltage dependent ion channel); CYPD-(cyclophilin D); MPTP-(membrane permeability transition pore); TBAR´s-(Thiobarbituric acid reactive species); TBA-(Thiobarbituric acid);Sal-(Saline); FW-(Free-wheel); TM-(Treadmill); SED-(Sedentary); DNA-(Deoxyribonucleic acid); EDTA-(Ethylenediamine tetraacetic acid); LP-(Lipid peroxidation); NaCl-(Sodium Chloride); PUFA´s-(Polyunsaturated fatty acids); -SH-(Sulfhydryl groups); WHO-(World Health Organization).

1 Introduction

One of the mostly used and effective chemotherapeutic drugs is the anthracycline Doxorubicin (DOX). However, its clinical use is limited by a dose-related cardiotoxicity and consequent dysfunction [1]. DOX increases the interest of many researchers since they try to understand the exact mechanism by which DOX induces toxicity, and, on the other hand, they claim for new strategies to prevent the harmful effects of this drug. Several studies report DOX toxicity in other organs such as kidney and liver as well as in testes, in which testicular dysfunction and male infertility is reported [2-5].

Due to the importance of DOX in treating various types of cancer, many pharmacological and non-pharmacological strategies have been carried out to counteract its toxicity, namely antioxidant supplements and physical exercise. Growing experimental evidence suggests that DOX treatment induces testes dysfunction mainly through the increased production of RONS and increased susceptibility to apoptosis [2, 6]. Exercise has been proposed as an important strategy to counteract DOX induced toxicity predominantly in heart tissue [1]. Although the exact mechanisms responsible for this protection continue to be debated, it has been argued that they are in part, associated with the decreased free radical production and with increased response of antioxidant defense systems. Therefore, it seems reasonable to hypothesize that physical exercise training provides some protection against DOX-induced dysfunction by possibly attenuating DOX-induced increased oxidative damage and apoptotic signaling in other tissues as well, including testes.

Therefore, the aim of this study was to analyze the effect of endurance training and voluntary physical exercise in testicular function of rats submitted to a sub-chronic treatment of DOX, which resembles cancer treatments in human patients. Sperm motility, the levels of oxidative stress and damage and apoptosis markers in testes were analyzed. Accordingly, the treatment with DOX can be considered an appropriate model to accomplish the above referred features and hence to exercise-induced adaptations, as DOX induces a dose-related and potentially lethal toxicity [2, 6, 7] that may be in part be minimized by physical exercise. We hypothesized that endurance training and voluntary physical exercise may antagonize the expected deleterious effects caused by DOX treatment on testes function, oxidative damage and apoptotic signaling.

2 A State of Art

2.1 Male reproductive system

Nowadays, some alteration in lifestyle and environment lead to a variety of health problems that include chronic diseases such as obesity, diabetes and insulin resistance, cardiovascular diseases among others. Such chronic diseases are usually related with risk factors for other pathological conditions of which infertility is an example.

Infertility affects about 15% of married couples and in about half of cases the predominant causative factor is the male [8]. Some lifestyle conditions such as intense tobacco consumption, alcohol, diet, stress or the intake of some drugs can interfere with normal function of male reproductive system affecting spermatogenesis and normal hormonal regulation [8-10]. At a cellular level, those conditions can interfere with normal development of spermatozoa, particularly morphology with implications in sperm counting, motility and DNA integrity [8-10]. These alterations are responsible for the decline of fertilizing capacity of the individual.

The primary sex organs of the male reproductive system are the two testes in which sperm cells are produced. Other structures of the male reproductive system are divided in internal and external organs. Internal accessory organs include the epididymis, genital ducts, seminal vesicles and prostate. The external reproductive organs are the scrotum that encloses the testes, and the penis [11]. The human testis are two organs of the shape of rotation ellipsoids with diameters of $2.5^{\times 4}$ cm engulfed by a capsule (tunica albuginea) of strong connective tissue [12].

The main process that occurs in testis is called spermatogenesis, which is initiated in the male testis with the beginning of puberty. This comprises the entire development of the spermatogonia (former primordial germ cells) up to sperm cells [13, 14]. In testis, germ cells are at different development stages: spermatogonia, primary and secondary spermatocytes and spermatids. Spermatids are located within invaginations of somatic Sertoli cells, with which they maintain an intimate and cooperative relationship [11, 12]. Spermatogenesis takes place in the seminiferous tubules and this process is a highly dynamic and metabolically active process during which haploid spermatozoa are

produced through a gradual transformation of an interdependent population of germ cells. Spermatogenesis can be divided in three main phases: spermatogoniogenesis, meiosis (maturation of spermatocytes) and spermiogenesis [11, 12, 15]. The final product of spermatogenesis is the mature male gametes called spermatozoa which are constituted by 3 major parts: head, midpiece and tail. The head comprises the DNA, which is in the nucleus rounded by histones. Normal spermatozoa exhibit an ovalshaped head with a regular outline and an acrosomal capcovering more than one-third of the head surface. The midpiece is slender, less than one third of the width of the head, straight and regular in outline. Tail is about 50 µm long and it is responsible for sperm motility and it is constituted by microtubules network connected by dinein [16-18]. The main development phases and the mechanism of division of distinct type of germ cells are presented in figure 1.

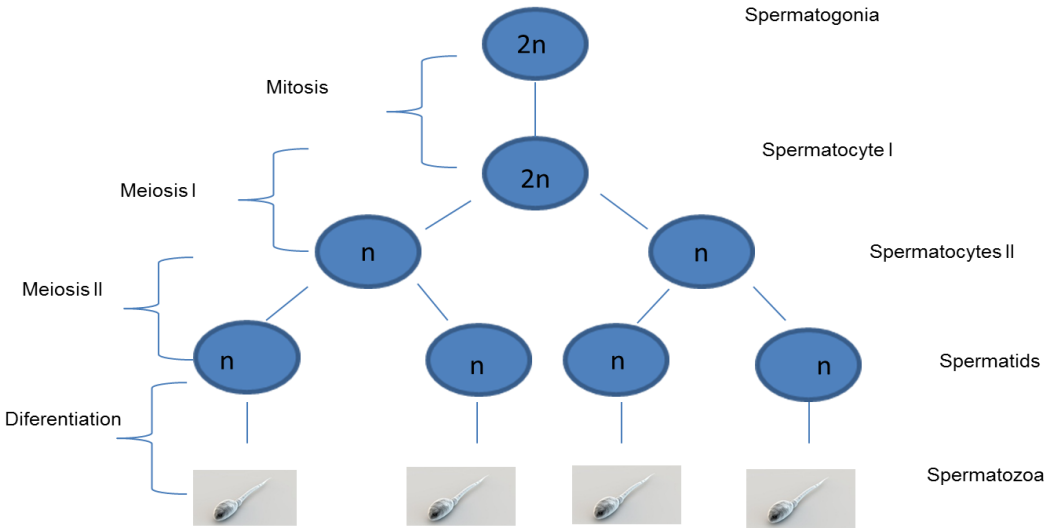


Figure 1 Schematic representation of Spermatogenesis

Some hormones involved in the regulation of spermatogenesis through endocrine, paracrine and autocrine pathways include pituitary gonadothrophins, follicle stimulating hormone (FSH) luteinizing hormone (LH) and androgens [12, 19, 20]. The process of spermatogenesis in seminiferous tubules is maintained by different intrinsic and extrinsic influences. During intrinsic regulation, the Leydig cells in the intertubular space secrete testosterone and additional neuroendocrine substances and growth factors. These hormones, transmitters and growth factors are: i) directed to neighboring Leydig cells, to

blood vessels, to the lamina propria of the seminiferous tubules and to Sertoli cells; ii) involved in maintenance of the trophic of Sertoli cells and the cells of peritubular tissue; iii) important to the contractility of myofibroblasts and in that way regulates the peristaltic movements of seminiferous tubules and the transport of spermatozoa and iv) also contribute to the regulation of blood flow in the intertubular microvasculature. On the other hand, the external regulation of spermatogenesis in the testes requires the well-known extratesticular stimuli provided by the hypothalamus and hypophysis. Pulsatile secretion of gonadotropin releasing hormone (GnRH) of the hypothalamus initiates the release of LH from the hypophysis. As a result of this stimulus, Leydig cells produce testosterone, which directly influences spermatogenesis but is also distributed through the body in order to provide feedback to the hypophysis related to the secretory activity of the Leydig cells. In fact, the combined and inter-related function of these two regulatory paths act synergistically such that extratesticular influences are a necessary basis for the function of intratesticular regulations [12, 19, 20].

2.2 Pathophysiological conditions leading to testis dysfunction

Current epidemiological evidence suggests that 15% of couples experience infertility and in half of these patients, the problem remains unresolved [21-23]. Background prevalence rates now appear to be reasonably stable, but there is evidence of an increase in the rate of referrals for medical help. Actually, testicular dysfunction is responsible for about 25% of infertility problems [24]. In the following sections, some pathophysiological conditions that may lead to testis dysfunction and infertility are addressed.

2.2.1 Aging

Aging can be defined as a time-dependent general decrease of physiological functions of an organism, associated with an increasing risk of morbidity and mortality [11, 25]. Aging is associated with structural and functional alterations in all organs of the human body, including the reproductive male organs. The aging of gonads represents a special case, as these organs are not functional for the whole lifespan of an individual and their normal

function is not indispensable for the maintenance and execution of other body functions [26, 27].

Aging post-mitotic cells progressively accumulate waste products resulting in degeneration and decreased functionality. Several cellular deleterious alterations are described as a result of aging process. For instance, at the membrane level, aging induce damage to lipids and proteins that can compromise membrane fluidity and consequent disturbs in molecular transport, membrane permeability and other cell functions [25, 27-29].

The nuclei of aged cells are characterized by increased heterochromatin and damage in DNA and nuclear proteins. However, the most notable features observed in aging cells are changes at mitochondria level, including structural deterioration, loss of cristae, destruction and homogenization of the matrix and mitochondrial membranes and mutations in mDNA (mitochondrial DNA) [25, 27-29]. One of the major causes of male infertility associated with advancing age is the decline of testicular function and testosterone concentration. This progressive decline begins about the age of 30 [30] and is accompanied with many symptoms associated with the decrease of testosterone production and release, including decreased bone and muscle mass, body hair and beard growth and increased abdominal fat mass [12, 29, 31, 32]. In parallel with testicular mass and testosterone concentration decrease, carrying sperm tubes become less elastic and sperm production rate is slower. The sperm quality and number as well as the muscles responsible for ejaculation also get weaker over time [33, 34]. This decline in testosterone levels with age may be explained by changes in all the components and regulatory levels of the hypothalamus-pituitary- gonadal axis [31, 32].

2.2.2 Diabetes

Diabetes is a chronic metabolic disease characterized by absolute or relative deficiencies in insulin secretion and/or insulin action, which lead to an increase in blood glucose levels [35]. When glucose is at high concentrations in the body, it can lead to sleep disturbances, fatigue, stroke, renal failure and many other complications [35]. About 90% of diabetic patients have disturbances in several testis functions, decreased in libido, impotence and infertility [36]. In fact, infertility is one of the common complications in diabetic men mainly due to the loss of germ cells by apoptotic cell death [37]. In literature, it has been reported

that diabetic induction of testicular oxidative stress and damage may be the predominant mechanism responsible for the testicular cell death in diabetic patients. Results in literature suggest that diabetes-induced testicular cell death that may cause infertility in men is mediated by oxidative stress and damage [37, 38]. It is known that the increased oxidative stress, which characterizes diabetes mellitus, has deleterious effects on male reproductive function. Sperm DNA damage and lower semen volume, despite normal sperm count, shape and motility are observed in type 1 diabetic patients [36].

2.2.3 Obesity

Obesity, a common disease in sedentary societies resulting from an excess of caloric intake over energy expenditure, increases the risk for other pathologies such as hypertension, heart disease and diabetes.

In addition, obesity in men is associated with lower testosterone, free testosterone and sex hormone binding globulin (SHBG), which is a glycoprotein synthesized in the liver with high affinity binding for 17 beta-hydroxysteroid hormones such as testosterone and estradiol [39]. It is known that obese men have reduced sperm concentration and total sperm count compared to lean counterparts, although sperm motility and morphology appear unaffected [39].

Obesity is linked with increased activity of aromatase, an enzyme involved in the conversion of testosterone into estradiol, which causes a negative feedback at the hypothalamic-pituitary axis to decrease the secretion of both FSH and LH. This leads to a reduced stimulation of Sertoli and Leydig cells in testes and hence, a decreased production of testosterone and sperm. The fat deposited around the testes in an obese individual also raises the temperature of the testes leading to a destruction of the sperm and lowered sperm counts [39, 40].

2.2.4 Rheumatic diseases

Sexuality is an often neglected concern related to the life quality of patients with rheumatic disease. Manifestations and symptoms of disease can impair sexual functioning, but this can be much improved by adequate intervention and counseling [41, 42].

Rheumatic diseases are characterized by functional changes in musculoskeletal system of nontraumatic cause. The most common manifestation of this disease is pain and limited mobility.

Diseases such as rheumatoid arthritis, systemic lupus and spondylitis can affect the fertility of men. Autoantibodies and hormonal disorders found in many of these diseases as well as some drugs used in disease treatment can adversely affect reproductive capacity system that includes male and female host [41, 43] and can interfere with fertilization, implantation, embryonic development and placental function [41].

Gonadal dysfunction and infertility in patients of both sexes with chronic rheumatic illness are multifactorial [41, 42]. Furthermore, hypothalamic–pituitary–adrenal axis dysfunction autoimmunity with production of *anti–corpus luteum*, antiendometrial or antisperm antibodies high disease activity or chronic renal failure and immunosuppressive drugs can induce impairment of fertility in patients with rheumatic diseases [43]. However, the main contributing factor for infertility associated with rheumatic diseases seems to be the drug therapy. The risk of infertility in both male and female can be aggravated by the use of NSAIDs (non-steroidal anti-inflammatory drugs), immunosuppressive drugs, and biologic agents [42].

2.3 Oxidative stress (OS)

Among the causes associated with pathophysiology of male infertility, increased OS has been identified as one factor and thus, has been extensively studied [44]. OS is characterized by an imbalance between production of oxidants and antioxidants, which leads to the loss of capacity of our body to defend our organism from the oxidants [16, 45], in other words OS is a condition in which the delicate balance existing between RONS production and the neutralization of their reactive action via the antioxidant defence system becomes skewed in favour of free radical expression [46]. The body's antioxidant defence system serves to protect the cells from excessive RONS production and is comprised of both endogenous (bilirubin, uric acid, glutathione, superoxide dismutases,

catalase, glutathione reductase, peroxidase and others also inducible) and exogenous (carotenoids, tocopherols, ascorbate, bioflavonoids, as well as many others primarily coming from the diet particularly from ingestion of fruits and vegetables) compounds [47].

It is known that oxygen is essential to sustain life and physiological levels of RONS are necessary to maintain normal cell function. Conversely, RONS can be detrimental to cell function and survival as essential signaling molecules for cellular adaptation. Radicals are the chemical species that have one or more unpaired electrons and intermediates the process of oxidative stress [16]. This chemical condition causes an electronically labile state and results in extreme reactivity of the respective molecules. Biologically important and relevant are free radical derivatives of oxygen (O_2) and nitrogen (N_2) [16, 45]. Some RONS are relevant for spermatozoa like hydroxyl radicals (·OH), superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2). These radicals react at the site of generation. On the other hand, some other oxygen species can exert their actions at other target sites distant to their generation subcellular location, such as peroxyl (ROO-) and alkoxyl (RO-) radicals [16, 45]. In addition, hydrogen peroxide (H_2O_2), which is chemically a non-radical as it does not have unpaired electrons, but is also highly reactive, is persistent and can freely penetrate plasma membranes because it is electronically not charged. It is harmful to spermatozoa from different species and their functions at high concentrations. In contrast, other RONS like superoxide anion or the hydroxyl radical are non-membrane permeable [16]. By the other hand RONS play an essential role in several physiological conditions and cell functions like gene expression or signal transduction. When, by many causes, they are overproduced some normal cell function could be compromised due to changes in DNA, lipid peroxidation or directly membrane damage [8, 16, 45].

In living systems, this delicate balance (free radical production vs. antioxidant defences) serves to determine the intracellular redox state [48], which in turn plays a role in optimizing cellular function. The redox state and/or redox balance is representative of the oxidation/reduction potential present within the cell and is tightly regulated similar to that of pH, and is commonly accessed via the ratio between reduced GSH (glutathione) and oxidized GSSG (glutathione dissulfide) glutathione (the major non-enzymatic antioxidant) or other thiol/disulfide compounds [49].

In physiological conditions, RONS maintain equilibrium with the antioxidants, based on the oxidation-reduction balance. This balance minimizes the risks caused by the excess of RONS. In this case the antioxidants play an important role. There are some endogenous antioxidants like calatase (CAT), superoxide dismutase (SOD) or glutathione peroxidase

(GSH-Px) which play an important role. Some exogenous antioxidants like vitamins from the diet are also important in order to minimize some RONS attack.[16, 49, 50]. As shown in table I, at low level RONS facilitate hyperactivation, capacitation, acrosome reaction, fertilization and oocyte adhesion of spermatozoa [8, 51]. On the other hand, high levels of RONS damages a large variety of biomolecules like lipids, amino acids, carbohydrates, proteins and DNA thereby affecting sperm function [8, 51, 52]. Recent studies focused on the impact of RONS in human sperm and they have shown that sperm dysfunction can arise either by damaging sperm plasma membrane or DNA [8, 52, 53]. Elevated amounts of RONS in sperm are related with morphological alterations and deficiency in mitochondrial activity. The oxidative phosphorylation system of mitochondria is suspected to be both the production and target site of RONS, as it is closely associated with the inner mitochondrial membrane [8].

Table I Pathological and physiological role of RONS

Physiological Role	Pathological Role
✓ Hyperactivation	✘ Lipid peroxidation
✓ Capacitation	✘ Protein Oxidation
✓ Acrosome reaction	✘ Apoptosis
✓ Fertilization	✘ DNA damage
✓ Oocyte adhesion	

Overproduction of RONS can result from a variety of stressors, such as exposure to drugs, UV(ultra-violet) radiation, environmental pollutants [54], excessive nutrient intake [55] or acute intense physical exercise [56]. However, any situation in which the consumption of oxygen is increased, as during physical exercise, could result in an acute state of oxidative stress. Depending on the type, intensity and duration of exercise, RONS generation in response to acute exercise can primary occur via several pathways. These include mitochondrial respiration (electron leakage from electron transport chain with univalent reduction of molecular oxygen and subsequent production of the superoxide radical), prostanoid metabolism, the autooxidation of catecholoamines, and through

enzymatic activity of NAD(P)H (nicotinamide adenine dinucleotide phosphatse-oxidase) oxidase and xanthine oxidase [57].

2.3.1 Oxidative damage and testes dysfunction

Sperm cell have ability to generate RONS as well as its great susceptibility to such molecules [58]. Not only do sperm cells contain high levels of polyunsaturated fatty acids and endogenous RONS, but they have a limited store of cytoplasmic defences that leaves them nearly incapable of membrane repair. Thus, sperm cells are particularly susceptible to oxidative damage [58, 59]. In literature it has been reported that OS can inflict direct damage to genomic DNA or up-regulate apoptotic proteins. Both pathways lead to impaired spermatogenesis and germ cell death [58, 60, 61].

In sperm cells, RONS can be originated by endogenous or exogenous sources. For instance, tobacco, alcohol and environmental pollutants are three exogenous sources reported in literature that originate additional RONS by decreasing antioxidant levels, affecting the sperm motility, morphology and concentration and DNA damage [62]. On the other hand there are two main sources of RONS in semen: leucocytes and immature spermatozoa. As already stated, RONS can alter cell function and affect various organelles. As in any other living cell, in spermatozoa, energy is largely aerobically produced by means of enzymatically controlled mitochondrial oxidative phosphorylation. Subsequently, the chemical energy is conserved as adenosine triphosphate. In the course of this stepwise process of the electron transfer chain, elementary oxygen (O_2) is taking up four electrons and is thus reduced to highly reactive free radicals as intermediate products. At the end of this process of oxygen reduction, water (H_2O) is formed. Yet, mammalian spermatozoa may also obtain the metabolic energy in form of adenosine triphosphate by glycolysis. Disruption and subsequent leakage of electrons from the mitochondrial electron transfer chain resulted in the generation of RONS from Complex I or III. Even in the course of normal physiological aerobic metabolism, about 1–5% of the consumed oxygen is converted into free radicals and RONS produced via this mechanism are normally regarded as cytotoxic byproducts that are involved in the etiology of disease and aging. So, spermatozoa are very professional and effective producers of RONS like superoxide and H_2O_2 [16, 49, 50]. Leucocytes are present in both male and female genital tract, even in healthy, fertile individuals not having an infection. In cases of male

genital tract infection and inflammations, fertility is seriously affected as clinical findings show azoospermia, oligozoospermia and asthenozoospermia. As a result of male genital tract infections/inflammations, leucocytes infiltrate the infected organs releasing high amounts of RONS which are associated to infertility by stimulation of lipid peroxidation through oxidative stress. By this mechanism, infections/inflammations do not only damage sperm DNA and reduce sperm count, but also impair sperm functions like motility and acrosome reaction [16, 63, 64]. Some studies support that leucocytes are endogenous sources of RONS because they increase pro-inflammatory cytokines, decrease antioxidant levels and cause lipid peroxidation in sperm cells membrane. Immature spermatozoa are a source of RONS since they have an excess of cytoplasm which activates NADPH system which provides electrons for free radical production [44].

The production of RONS by sperm at low levels plays a positive role in fertilization; however when produced at high levels it can lead to potential toxic effects on sperm quality and function [65]. An excessively RONS-producing sperm seems to damage themselves, especially their own DNA, basically through modification of all bases, production of base free sites, deletions, frame shifts, DNA cross-links and chromosomal rearrangements [34, 66, 67]. OS is also associated with high frequencies of single and double strand DNA breaks. RONS can also cause various types of gene mutations such as point mutations and polymorphisms, resulting in decreased semen quality [48]. RONS damage in sperm mitochondrial membrane can also decrease sperm motility and its ability to fuse with the oocyte [68] and alter the sperm DNA resulting in the passage of defective paternal DNA on the conceptus [69].

The male germ cells have one important mechanism to be protected against these oxidant [16]. In testes, Sertoli cells are responsible for this protection which provides a high level of glutathione-dependent defense. In epididymis potential diminishes of RONS are, for instance, members of glutathione peroxidase family and catalase [16, 51]. In contrast, the seminal plasma is the biological fluid with more antioxidants which guarantees sperm cells protection after ejaculation [16]. Additionally, environmental and lifestyle factors as well as pathologies of the reproductive system and chronic diseases are associated with increase sperm oxidative damage. Two mechanisms have been suggested by which RONS can cause infertility, by reducing sperm motility and its ability to fuse with the oocyte, compromising paternal genome contribution to the embryo [65, 69]. Until recently, RONS was exclusively considered toxic to human spermatozoa. However, as in the majority of physiological functions, low levels of RONS have been shown to be essential for

fertilization, acrossome reaction, hyperactivation, motility and capacitacion [44, 57, 69]. Increased RONS levels also have been correlated with decrease sperm motility [45]. However, the exact mechanism for this association remains unclear. One hypothesis is that H_2O_2 can diffuse across membrane into the cells and inhibit the activity of some vital enzymes such as glucose-6-phosphate dehydrogenase [44, 53], which is a cytosolic enzyme of the pentose phosphate cycle pathway that supplies energy and reducing equivalents to various types of cells. Another hypothesis involves a series of redox-dependent interrelated events resulting in a decrease in axonemal protein phosphorylation and sperm immobilization [44].

A scheme summarizing the factors that contributes to oxidative-stress induced infertility is depicted in figure 2.

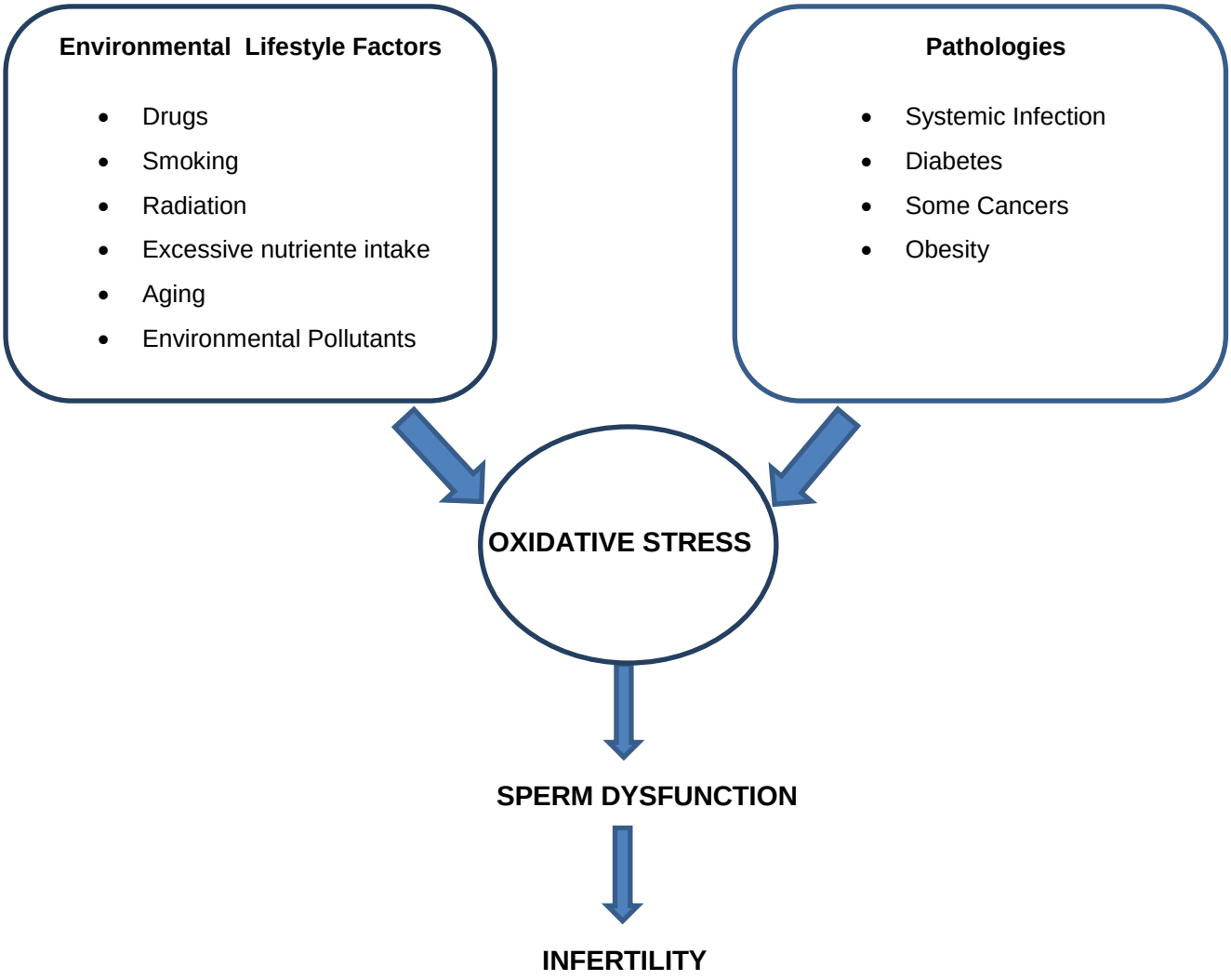


Figure 2 Factors contributing to oxidative stress-induced infertility.

2.3.2 Lipid Peroxidation

Lipids are considered to be the most susceptible macromolecules to oxidative damage and are present in sperm plasma membrane. RONS can react with polyunsaturated fatty acids in the cell membranes leading to a cascade of reactions called lipid peroxidation [70]. One of the byproducts of lipid peroxidation is malondialdehyde (MDA) [71]. Other important markers of lipid peroxidation are conjugated dienes, and lipid hydroperoxides. Lipid peroxidation can also be estimated by breath analysis of certain hydrocarbons, such as pentane and ethane [72, 73]. These products have been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa [74].

In sperm cells, lipid peroxidation decreases membrane fluidity of both plasma and organelle membranes and, as a result, damages membrane function, ion gradients, receptors-mediated signal transductions, as many others. In fact, with the loss of membrane function, spermatozoa lose the ability to function correctly and, thus, fertility is impaired [16].

Lipid peroxidation is a three-phase process [16]. In the initiation phase RONS react with carbon atoms leading to hydrogen removal from neighboring methylene groups, resulting in the creation of a lipid radical and water. The free electron is transferred to the lipid, which forms a lipid radical reacting with molecular oxygen to form lipid peroxide. Second phase is called propagation and in this phase the lipid peroxide radical molecule reacts with a neighboring fatty acid producing another fatty acid radical which reacts with molecular oxygen in order to form another lipid peroxide. This step is called “radical chain reaction” and results in a propagation of damage to numerous molecules. By this process, almost 60% of fatty acids presents in membrane can be oxidized. Propagation phases ends when two radicals react in order to produce a stable product, and the two free electrons from the two radicals form a covalent bond [16]. The last phase is termination and is initiated when lipid radicals are available to enter in chemical reactions in order to form a non-radical toxic stable product, for example, lipid molecules are broken down and molecules containing carbonyl byproducts are formed like MDA [75-77].

2.3.3 Activation of cell death through apoptotic signaling

Apoptosis is a process that reinforces tissue homeostasis but can also participate in organ dysfunction and disease, in particular, the induction of apoptosis is essential to promote male infertility. [58]

Apoptosis is a non-inflammatory and energy independent response to tissue damage characterized by a series of morphological and biochemical changes resulting in cell death [44, 78] and proceeds in response to different stimuli, for instance, environmental stimuli such as radiation, chemotherapeutic drugs and RONS and deprivation of survival factors, such as testosterone. By activating caspases, RONS may initiate a series of events that in apoptosis, which may be induced by cell injury or stress [58]. Specifically, it is a process characterized by membrane blebbing, cell volume shrinkage, chromatin condensation, cytoplasmic vacuolization and disassembly of the cell into membrane-bound remnants termed apoptotic bodies [78, 79]. Apoptosis is broadly divided into an initiation phase, a signaling phase and an execution phase in which cells rapidly execute a death program [80]. There are two major apoptotic pathways: the extrinsic or death receptor pathway and intrinsic involving the mitochondria and the endoplasmic reticulum [81]. Programed cell death is essential in the development, morphogenesis, tissue remodeling, and immune regulation and it is also connected to some pathologies [82]. The reproductive system involves various cycles of tissue growth and degeneration and spermatogenesis is no exception, so, any factor that affect germ cells and Sertoli cells may directly affect spermatogenesis. In this way, testicular apoptosis is essential to deplete excess germ cells and remove abnormal spermatozoa during normal process of spermatogenesis, so the principal function of testicular apoptosis is to help maintain tissue homeostasis during spermatogenesis [58]. On the other hand, inappropriately occurring apoptosis has been linked to suboptimal male reproductive function. Excessive apoptosis results from impaired regulation or improper activation affecting spermatogenesis and it can even lead to male infertility [58, 83]. In testes, executive apoptosis requires the action of some proteins namely caspases, Apaf-1, anti-apoptosis proteins such as Bcl-2 which exert their action together with p-53 and death receptors in human apoptosis [58, 84].

2.4 Drug- induced dysfunction

There are several pharmacological agents used in many therapeutic schemes that may affect testis and cause testicular dysfunction. DOX, for instance, is an anthracycline antibiotic used for more than 30 years in the treatment of a wide variety of malignancies. However, the clinical use of DOX is limited due to its serious side effects, inducing testicular toxicity [85]. It alters sperm development, production, structural integrity and motility, being associated with increased cellular death through apoptosis [86].

Another example widely described are steroids, as they can also cause testicular dysfunction and lead to infertility [87, 88]. Anabolic steroids are derived from the male hormone testosterone and as a result, the use of steroids can dramatically affect male reproductive system. In men, the use of steroids can lead to decreased sperm production, increased number of abnormal sperm, erectile dysfunction and atrophy of testis [87, 88].

Among the drugs that may cause reversible infertility in men is sulfasalazine [89, 90]. Sulfasalazine can induce spermatozoa abnormalities, oligozoospermia asthenozoospermia, teratozoospermia and infertility [90]. These sperm abnormalities are caused by sulfapyridine, a metabolite of this drug. As folate and other antioxidants do not prevent these abnormalities, it seems that oxidative damage may not have a causative relationship with the dysfunction in reproductive system [41, 89, 90]. Sperm abnormalities may also occur in some cases due to the use of methotrexate [91]. Most of times, monotherapy with methotrexate does not induce infertility in men. In fact, sperm abnormalities may occur in rare cases, as described in a young man with severe psoriasis treated with methotrexate [91]. Oligospermia was reported with this treatment, but a normal sperm concentration was observed after the discontinuation of the drug. In a recent study, the administration of folic and folinic acids together with methotrexate therapy attenuated germ cell toxicity in mice [15, 92, 93].

Reports of irreversible infertility in both sexes were observed after treatment with alkylating agents, of which cyclophosphamide (CYC) and chlorambucil are examples [43, 92].

2.4.1 DOX-induced dysfunction

The DOX (trade name: adriamycin) is an antineoplastic drug of the anthracycline family, which is widely used because of their spectrum of activity and has been commonly used in clinical practice since 1969. It is a drug indicated for the treatment of different types of tumors, including breast carcinoma, microcytic carcinoma of the lung, gastric carcinoma or gynecologic tumors [94, 95].

This drug has piqued the interest of various researchers as it is a potent antineoplasic agent, while gathers characteristics that induce oxidative damage and toxicity in various tissues [96]. There are some hard work attempting to understand the exact mechanisms of action of this drug and many researchers trying to find some preventive strategies in order to moderate the harmful effects of this drug. The history of DOX began when a bacterium called *Streptomyces peucetius* was isolated and the antibiotic was produced from this [2, 7]. The first drug name was Daunorubicin. However, after many trials and some treatments it was found that this type of drug caused a fatal cardiac toxicity. Thus, the bacteria *Streptomyces peucetius* was mutated and a new drug was proposed named Adriamycin, later called Doxorubicin [2, 7]. The new drug showed better activity and efficacy against several types of solid tumors as well as an increased spectrum of therapeutic action. However, the severe tissue toxicity, particularly in heart is still reporting exerting its cytotoxic effect by oxidative stress, which leads, among other to cell death.

Nowadays the investigation is based in the search for responses to its high therapeutic efficacy as well as for the discovery of certain preventive strategies that help to decrease / moderate the toxic effects of the drug. In our study we evaluated the physical exercise in order to moderate the harmful effects of DOX. It should be noted the importance of acute exercise since it is important in the regulation of antioxidant defenses [97]. The understanding of the mechanisms of action of this drug in order to try to minimize its harmful effects and the proper strategies to counteract its side effects are of extreme importance. Despite its extensive clinical use, the exact mechanism of action of anthracyclines in cancer cells remains a matter of controversy, however, in literature some mechanisms are described i) intercalating DNA leading to inhibition of the synthesis of macromolecules; ii) formation of free radicals which induce DNA damage and leads to lipid peroxidation; iii) DNA binding and alkylating ; iv) DNA “cross-linking”; v) interference with DNA strand separation and helicase activity and vi) direct effects in membrane. Despite its high use in clinical practice, the mechanisms by which this drug acts still

remain a matter of controversy, and in many literature, the processes are not yet fully understood.

As stated, DOX is a widely used chemotherapeutic agent causing serious dose-dependent toxicity to non-target tissues. DOX characteristics induce toxicity and oxidative damage in various organs and tissues causing some harmful effects in healthy cells, impairing cardiac function, liver function and possibly testicular function. In literature it has been reported that cardiac dysfunction is a major limitation of anthracycline treatment and the use of this therapy is limited due to the adverse effects of these compounds on cardiovascular and hepatic systems [98, 99]. Its testicular toxicity is mainly due to the induction of oxidative stress [100]. Although, the exact mechanism by which DOX or its toxic metabolites exert organ dysfunction is not yet fully understood, however, in the literature some hypotheses are described that include modifications in calcium homeostasis, formation of iron complexes, RONS synthesis, mitochondrial dysfunction or damage in cell membranes [101].

The main mechanism of DOX toxicity is the formation of potent free radical by reducing the electron transport chain complex I of mitochondria with consequently formation of a semiquinone. The oxidative stress generated by the formation of free radicals is also involved in the toxic action of this drug [102-106]. Literature data associates high doses of DOX with high RONS production and, in turn, when there is an imbalance between the production of RONS and antioxidant defense of the organism itself generates certain types of tissue damage [44, 69, 106]. It has been proposed that DOX causes a further increase in RONS therefore, the main preventive strategy will be the enhance of cellular protection during increased production of free radicals. In this regard the up regulation of antioxidant defenses is a central mechanism in order to attenuate the effects induced by RONS.

Numerous endogenous antioxidant enzymes exist, SOD, CAT, GSH-Px or GSH-R (Glutathione reductase), which exert a neutralizing effect against RONS in order to protect the most diverse tissues [97, 107-109]. Some studies show a decrease of antioxidant enzyme activity after DOX administration due to the ability of the drug to produce free radicals with adverse effects including protein inactivation by oxidative damage while other studies show that there is an increased activity of the endogenous antioxidants as part of the response to alterations in signaling mechanisms produced by the additional production of RONS [5, 110, 111].

Besides the importance of the antioxidant defense system to antagonize DOX-induced toxicity, other strategies have been suggested including optimization of the dose to be administered, the use of analogs of DOX, the use of antioxidants or physical exercise [112].

It is necessary to emphasize the role of the OS in adaptations induced by exercise improvements in the production of an optimal level of RONS-induced signaling may be necessary for optimum adaptation and normal physiological function [113-115]. Despite there are several studies revealing that physical exercise induces a protective phenotype in DOX-treated tissues, particularly in heart at different levels of cellular organization [1, 116, 117] , no references in literature exist examining this cross-tolerance effect on testes.

2.5 Physical exercise and testis (dys)function

It is of consensus among physicians and scientists that the practice of physical activity is essential to weight maintenance and achieve healthy life conditions. In fact, regular exercise provides a variety of beneficial health effects such as reduced risk of cardiovascular disease, osteoporosis, obesity and can result in many positive physiological adaptations that are highly beneficial for general populations. Additionally, physical exercise is a non-pharmacological strategy to counteract the toxicity of drugs of which Doxorubicin induced-tissue and mitochondrial dysfunctions are an example [118, 119].

The aerobic training is intrinsically linked to increased oxygen consumption. Several studies have shown a link between increased consumption of oxygen and the formation of RONS, increasing the cellular conditions of OS [56, 120-122]. During exercise, additional required ATP induces the increases in the flow of electrons through the mitochondrial electron transport chain (ETC), increasing the probabilities to leakage of electrons to molecular oxygen enhancing the rate of generated RONS [120, 123]. However, it has been recognized that low concentration of RONS can positively affect the antioxidant system and the metabolic processes associated with the transport of glucose, ATPase activity or mitochondrial biogenesis [124, 125].

An increasing number of studies point to participation in endurance exercise training as having a significant detrimental effects upon reproductive hormonal profiles in men, as well as the increase of some markers of OS [126].

Some types of acute exercise have been related with a significant reduction in testicular, epididymal, prostatic and seminal vesicle somatic indexes, however some mechanisms remain unclear [127]. At the same time, exercise is associated with release of a number of pituitary and hypothalamic hormones. In fact, concentration of LH decreases but, FSH, generally, is not influenced by exercise [128, 129]. The decrease in LH secretion is believed to be due to changes in GnRH pulse frequency and amplitude, however, males are less susceptible to these changes of LH levels [128]. It has been reported in literature that men chronically exposed to endurance training exhibit persistently reduced basal free and total testosterone concentrations, however, the exact mechanism inducing this reduction remains unclear but is postulated to be a dysfunction or readjustment in the hypothalamic-pituitary-testicular regulatory axis [126]. There are some results that OS develops with the increase of exercise intensity which may interfere in male reproductive capacity, namely, steroidogenesis and spermatogenesis [130].

Many questions regarding the male reproductive endocrine adaptation process to exercise training and the increase of OS still remain unclear. The exercise and all the adaptations that arise from its practice are essential to improve life conditions. Mitochondria seem to, at least in part, mediate the benefic adaptations induced by physical exercise. However, different types of exercise, including chronic, acute and overtraining, induced different responses.

2.5.1 Acute and chronic exercise

It is well accepted that acute exercise, whether it be mostly isometric or rhythmic, increases OS. However, acute exercise is mentioned as a potential regulator of endogenous antioxidant defenses in response to the additionally RONS production derived from pathological conditions [115] . Some authors report that acute exercise seems to promote a sufficient stimulus to activate the GSH-GSSG redox cycle [131].

Indeed, any situation in which the consumption of oxygen is increased, as during physical exercise, could result in an acute state of OS. RONS production in response to physical

exercise can occur via several pathways such as mitochondrial respiration, prostanoid metabolism, autoxidation of catecholamines and oxidase enzyme activity [115]. On the other hand, post exercise, there are muscle fiber injuries and damage which leads to RONS generation since proteolysis, inflammation and calcium homeostasis are affected [57]. However, specifically RONS generation depends on the duration, mode and intensity of exercise as different types of exercise differ in their energy requirements and oxygen consumption. Hence, methods to reduce radical production and oxidative damage during and following physical exercise have been a central issue to many researches. Presently, there exist no “cause and effect” data that the increase in RONS production with acute physical exercise causes ill-health and disease [115, 132].

Different exercise protocols may induce different levels of RONS production. During low-intensity and duration protocols antioxidant defenses seems to be sufficient to stop RONS production but as the intensity/duration increases these defenses are no longer adequate, resulting in oxidative damage to surrounding tissues. In literature, some markers of exercise-induced oxidative damage have been studied. MDA is the most common method to indicate exercise-induced oxidative damage and numerous studies reported an increase in MDA following exercise ,in humans, with values typically returning to baseline one hour after exercise; redox changes in glutathione have also been routinely measured and studies present a decrease in reduced glutathione and an increase in oxidized glutathione (values return to baseline 15-30m after the end of exercise); DNA and protein oxidation are also measured, as well as the antioxidant capacity, which appears to be temporarily reduced during and immediately post-exercise [115, 133, 134].

Chronic exercise is a regular exercise that occurs by a long period of time and results in many adaptations of the circulatory, respiratory and muscular systems [115]. Regular physical exercise of either isometric or rhythmic type results in adaptive responses that protect muscle from OS reactions [135, 136]. The blood levels of LH, FSH, prolactin, testosterone, free testosterone decrease and SHBG increase in high and moderate intensity exercised men. Additionally, the same study reported that the subjects exercising with high intensity demonstrated significantly declined semen parameters compared with those exercising with moderate intensity [137], suggesting that intensity of exercise seems to have an important role in adaptations induced by exercise in men reproductive system.

Endurance training induces conformational changes related to the ability to uptake and transport of oxygen associated with increased oxidative potential. Among others, we can highlight the mass increase, activity of mitochondrial enzymes and oxygen utilization rate

[138]. In this way, there are some important mitochondrial adaptations, associated with endurance training at a morphological and metabolic level related to mitochondrial biogenesis, the balance between oxidants and antioxidants and apoptosis with mitochondrial origin, functional response and MPTP (membrane permeability transition pore), mostly in heart and skeletal muscle [139].

Some authors identified a number of specific adaptations resulting from endurance training in skeletal muscle mitochondria and the respiratory capacity, which play a crucial role in improving the performance [140-142]. Indeed, in response to the endurance training there are an increasing of oxidation capacity of pyruvate and the activity of mitochondrial enzymes of ETC suggesting a general increase of the oxidative capacity [143]. In agreement, several studies have reported significant increases in the levels of mRNA, protein content and activity of certain key enzymes of oxidative metabolism in skeletal muscle [144-146]. Thus, we can mention for example, the Krebs cycle enzymes such as citrate synthase, aconitase and some desidrogenases as malate dehydrogenase or succinate dehydrogenase [147]. Such adjustment related to physical activity is associated with increased protein content and activity of the components of the ECT, during and after endurance training, including the increase of cytochrome C concentration, are indicative of the increase in the oxidative capacity of transformation of energy substrates [147-149].

Using a mix of histological techniques, in trained individuals compared with the active untrained individuals of the same age, it has been experimentally shown that the volume and mitochondrial density is approximately 50% higher [150]. In fact, as a result of the endurance training, it has been suggested an expansion of mitochondrial network in various tissues such as skeletal muscle, which involves innumerous steps which began with the first exercise sessions and continue with a training program [151]. In fact, some proteins concentrations increase in the initial response to exercise, like PGC1α and TFAM (mitochondrial transcription factor) which are key activators of mitochondrial biogenesis [152-155].

Exercise has huge anti-apoptotic effects and some authors reported that exercise training enhances cardiac IGFI-R/PI3K/Akt and Bcl-2 family associated pro-survival pathways, which provides one of the new beneficial effects for exercise [156] and at the same time chronic physical activity attenuated markedly the MPTP-induced akinesia/hypokinesia [157].

There are some evidences that low but significant biological levels of RONS are important antioxidant signaling molecules that help the regulation of a variety of antioxidative molecular mechanisms [158]. Indeed, some literature support that endurance training is a stimuli to increase RONS production and, at the same time, stimulate the expression of antioxidant enzymes and help to maintain redox equilibrium [159, 160]. In fact, some studies demonstrate an increase in antioxidant activity after a program of endurance training [161, 162] while others reveal no alterations in molecules with antioxidant characteristics [163]. Despite this controversial in literature it is important to highlight that exercise seems to be an important strategy to counteract increased OS induced by aging, some drugs and pathologies, through the beneficial antioxidant adaptations [164-168].

Exercise increases Mn-SOD activity, which is ultimately responsible for some protective actions of exercise [169, 170]. At the same time, the levels of catalase also increase with physical exercise [50, 171, 172]. Terblanche showed that the activity levels of catalase in the liver, heart, kidney and lung were significantly elevated in both male and female rats after one hour of intensive exercise. The increase in the activity levels of catalase in the various tissues investigated for both male and female rats was 417%. In fact, the higher activity levels of catalase as a result of exercise might be indicative of a compensatory measure to counteract the possible detrimental effects associated with additional RONS production [173].

2.5.2 Exercise-induced overtraining

Overtraining represents a physical stress that challenges homeostasis. The excess of physical exercise or uncontrolled exercise can lead to serious infertility problems both in men and women [137]. The uncontrolled intensive exercise in male can lead to a decreased sperm production because the endorphin release inhibits the pituitary gland which controls the secretion of endocrine glands in the body affecting spermatogenesis [174, 175]. Overtraining can result in dysfunction in male reproductive system (low testosterone levels) and many experiments involving animals have demonstrated that intensive exercise causes an additional increase in oxygen consumption which is accompanied by an over production of RONS as well as induced OS [174].

Many studies were carried out in order to evaluate the consequences of overtraining in men fertility. These studies show that basal levels of testosterone decreased immediately

after overtraining and basal cortisol levels increase. However, both testosterone and cortisol levels returned to pre-training values 3 months after resumption of previous training volume [174]. Sperm count decreased significantly immediately after overtraining. These results indicate that overtraining reduces testosterone levels, which is highly correlated with an increase in levels of cortisol and possibly a subsequent decrease in sperm [174, 175].

Table II Principal conclusions in some studies regarding relationship between moderate/high intensity exercise and fertility.

Moderate/high intensity exercise	**Consequences to (In)fertility**	**Reference(s)**
• Increase serum SHBG	Low serum testosterone	[137]
• Decrease serum LH, FSH and testosterone	Impaired spermatogenesis	[137, 174]
• Suppresses GnRH endogenous stimulation	Low LH and FSH secretion	[137]
• May increase RONS production	Interact with lipids, DNA and proteins	[176, 177]
• Enhanced secretion of cortisol	Inhibits GnRH secretion	[174, 178]
• Releasing of CRH (Corticotropin-releasing hormone)	Innibitory actions on testosterone synthesis	[179]
• External heating of testes	Decrease sperm count	[137]

3 Methods

3.1 Reagents

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

3.2 Animals

All experiments involving animals were conducted in accordance with the European Convention for the Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes (CETS no. 123 of 18 march 1986 and 2005 revision) and the Commission Recommendation of 18 June 2007 on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (C (2007) 2525). Supervisors are accredited by the Federation of Laboratory Animal Science Associations (FELASA) for animal experimentation. Thirty-six Sprague-Dawley male rats obtained from Charles River, France (aged 3-4 weeks and 200 g at the beginning of the experiments) were used. During the experimental protocol, animals were housed in collective cages (two rats per cage) and were maintained in a room at normal atmosphere (21–22 ºC; 50–60% humidity) receiving food (Scientific Animal Food and Engineering, A04) and water ad libitum in 12-hour light/dark cycles. The animals were randomly divided into six groups (n = 6 per group): saline sedentary (SAL+SED), sedentary receiving DOX (DOX+SED), saline endurance trained in a treadmill (SAL+TM); endurance trained treated with DOX (DOX+TM); saline exercised voluntarily in free wheel (SAL+FW) and exercised voluntarily treated with DOX (DOX+FW).

3.3 Endurance Training Protocol

The animals from TM groups were exercised 5 days/week (Monday–Friday) in the morning (between 10:00 and 12:00 A.M.), for 12 weeks on a LE8700 motor driven treadmill (*Panlab,Harvard, U.S.A*). The treadmill speed was gradually increased over the course of the 12-week training period (Table II). The protocol included 5 days of habituation to the treadmill with 10 min of running at 15 m/min, with daily increases of 5–10 min until 30 min was achieved. Habituation was followed by one consecutive week of continuous running (30 min/day) at 15 m/min and was gradually increased until 60 min/day on the second week. The animals from SED groups were not exercised but were placed on a non-moving treadmill five times per week (10–30 min/session) with the purpose of habituate animals to the possible environment stress induced by treadmill without promoting any physical training adaptations.

Table II Endurance treadmill training protocol

							Treatment							
	Weeks	0	1	2	3	4	5	6	7	8	9	10	11	12
SAL+TM	velocity (m/min)	15	18	20	22	24	25	25	27	27	28	28	30	30
DOX+TM	velocity (m/min)	15	18	20	22	24	25	25	27	27	25	25	22	20
	duration (min/day)	30	60	60	60	60	60	60	60	60	60	60	60	60

Legend: Training velocities through the entire protocol for saline and DOX-treated treadmill trained groups are shown.

3.4 Voluntary exercise

The animals from FW groups were housed in a polyethylene cage equipped with a running wheel (perimeter=1,05m, Type 304 Stainless steel (2154F0106-1284L0106) *Technicplast, Casale Litta, Italy*). Rats were allowed to exercise *ad libitum* with an unlimited access to the running wheel 24h/day. Running distance was recorded using, ECO 701 *Hengstler, Lancashire, U.K.).*

3.5 DOX administration

The animals from DOX groups received seven weekly intraperitoneal injections of DOX (2 $mg.kg^{-1}$) whereas saline groups received an equivalent volume of vehicle solution (NaCl 0.9%, 1 $ml.kg^{-1}$), during seven weeks. All animals were injected on Saturdays to avoid conflicts with treadmill training and during the light phase of the cycle. They were also observed daily and weighed at the beginning and at the end of the experimental treatment period, being also weekly weighed at the time of injection.

3.6 Animal Sacrifice

Animal sacrifices were carried 24 h after the last exercise bout for exercised groups and one week after the last DOX injection for DOX groups. Non-fasted rats were euthanized by cervical dislocation between 9:00 and 10:00 AM to eliminate possible effects due to diurnal variation, followed by decapitation to confirm death. After organ collection, fresh testes were immediately stored at -80ºC until further use in the biochemical assays described below. Three portions of testes were frozen separately for further homogenization and distinct assays as described.

A brief scheme resuming the experimental is shown in figure 3:

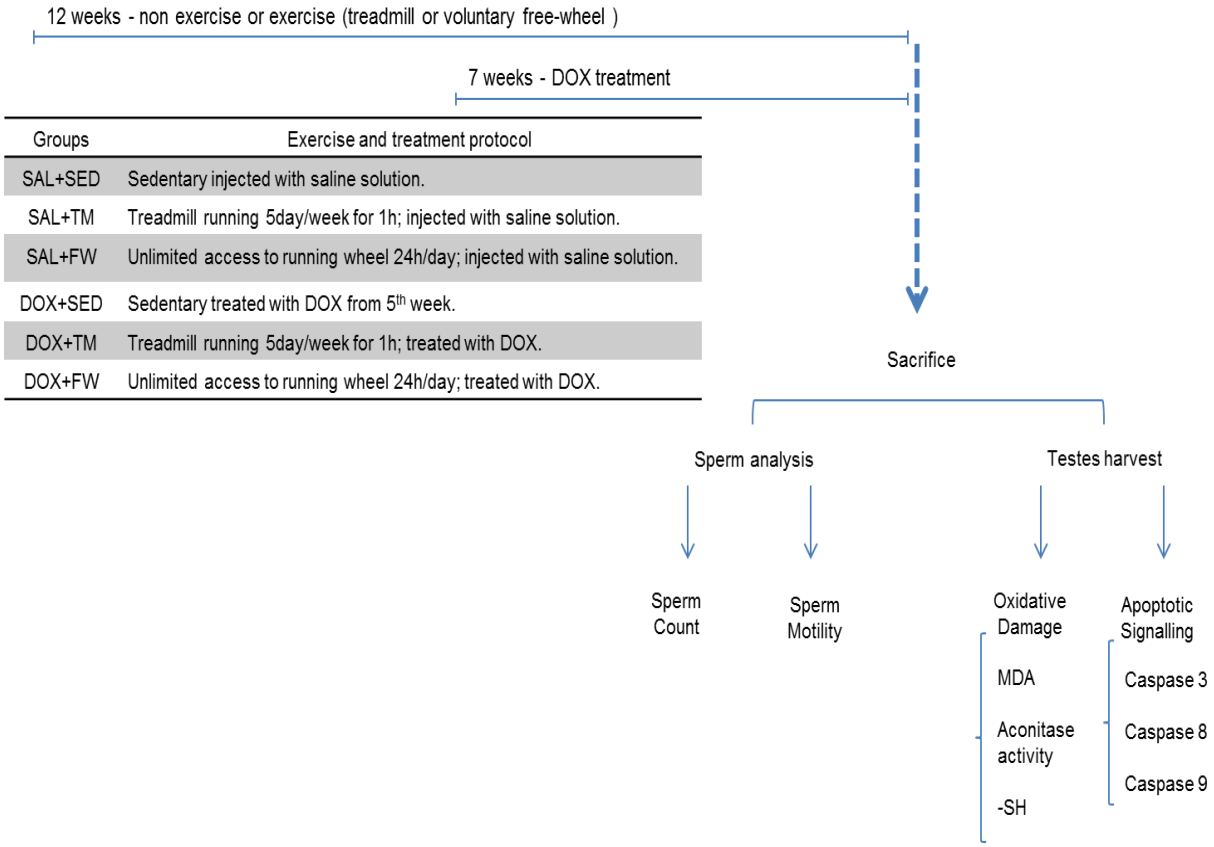


Figure 3 Experimental Setup – sedentary, exercised and DOX treated animals (left table); sperm analysis and biochemical assays are depicted in the right scheme.

3.7 Motility analysis

Sperm motility within semen was assessed as soon as possible after liquefaction of the sample, preferably at 30 minutes and no more than 1 hour following the sample collection, to limit the deleterious effects of dehydration, pH or temperature change [67, 180].

To evaluate the motility, the semen sample was well mixed. A wet preparation was performed to allow the direct observation of spermatozoa. For proper evaluation of sperm motility, it was essential that the depth of the preparation is about 20 µm (Using a 22 × 22 mm coverslip requires a volume of 10 µl) [180, 181]. After mixing the sample, a 10 µl volume was placed on a microscope slide and the coverslip immediately applied. Within 60 seconds, the sample should stop drifting, allowing slide examination with phase-contrast optics at ×400 magnification. Approximately 200 spermatozoa were assessed for

the percentage of different motile categories to be registered (motile progressive, motile non progressive and immotile) [180-182].

3.8 Sperm Count (Concentration)

A well-mixed undiluted preparation of liquefied semen on a glass slide under a coverslip was examined to determine the appropriate dilution. The semen sample was mixed and the appropriate volume of semen was added to the fixative (5 g of $NaHCO_3$ and 1 ml of 35% (v/v) formalin in 100 ml purified water) (table III). The dilution was vortexed for 10 sec and the improved Neubauer chambers were immediately filled with 10 µl of the fixed solution. The chamber was stored for approximately 15 min at room temperature in a humid chamber. Then, the improved Neubauer chamber was examined at 400x magnification and at least 200 spermatozoa were counted per replicate. First, the central grid of one side of the improved Neubauer chamber was assessed. The same number of rows was assessed in the other chamber. Then, the concentration in spermatozoa per milliliter was calculated according to the formula: C= (N/n) x (1/20) x dilution factor where N is the number of spermatozoa, divided by the volume in which they were found (volume of the total number (n) of rows examined) and multiplied by the dilution factor. Finally, the total number of spermatozoa was calculated multiplying the sperm concentration by the semen volume.

Table III Semen dilutions required

Spermatozoa per x400 field	Dilution required	Semen (µl)	Fixative (µl)
>200	1:50	50	2450
40-200	1:20	50	950
15-40	1:10	50	450
2-15	1:5	100	100
<2	1:2	100	100

3.9 Oxidative damage and antioxidants

Before thiobarbituric acid reactive substances (TBARs) analysis and content of oxidative modified -SH groups determination, testis were homogenized in a homogenization buffer (1M HEPES, 1Mm, EDTA, 100mM NaCl, 0,05% Triton X100), centrifuged (7000xg for 10 minutes) and the supernatant was used. Protein concentration was spectrophotometrically determinated by using the biuret method using bovine serum albumin as standard [183].

The assay used to detect the levels of lipid peroxidation is based on the principle that TBARs, such as MDA, react with thiobarbituric acid (TBA) producing a pink chromogen color detectable spectrophotometrically. The extent of lipid peroxidation in testis tissue was determined by measuring MDA contents by colorimetric assay, according to a modified procedure described previously [184]. The tissue from the six experimental groups were mixed with 2 volumes of trichloroacetic acid (10%) and 2 volumes of thiobarbituric acid (1%). The mixtures were heated at 80–90 °C for 10 minutes and re-cooled in ice for 10 minutes before centrifugation (4000xg for 10 minutes). The supernatants were collected and the absorbance measured at 535 nm. The amount of MDA content formed was calculated using an MDA standard curve as nanomoles of MDA per milligram of protein.

The basal testes content of oxidative modified -SH groups, including GSH and other -SH-containing proteins, was quantified by spectrophotometric measurement according to *Hu* [185]. Briefly, testis homogenized containing 5 mg/mL protein was mixed with 0.25 M Tris buffer pH 8.2 and 10 mM DTNB and the volume was adjusted to 1mL with absolute methanol. Subsequently, the samples were incubated for 30 minutes in the dark at room temperature and centrifuged at 3000xg for 10 minutes. The colorimetric assay of supernatant was performed at 414 nm against a blank test. Total -SH content was expressed in nanomoles per milligrams of protein ($\varepsilon 414=13.6\ mM^{-1}.cm^{-1}$).

Before aconitase activity analysis testis were homogenized in a homogenization buffer (50mM Tris HCl and 600µM of $MnCl_2$, pH 7.4), centrifuged (800xg for 10 minutes) and the supernatant was used. Protein concentration was spectrophotometrically determinated by using the biuret method using bovine serum albumin as standard [183].

Aconitase activity was measured spectrophotometrically by monitoring the formation of *cis*-aconitate from isocitrate at 240 nm and 25°C as previously described [186]. Testes homogeneized (50 µL) of each sample was collected and isocitrate (200 µM) was added.

One unit was defined as the amount of enzyme necessary to produce 1 μmol of cis-aconitate per minute (ε240=3.6 mM^{-1} cm^{-1}) and he content of aconitase was expressed in nanomoles per minute per miligrames of protein.

3.10 Caspase activity assay

Before caspase activity assay testis were homogenized in a homogenization buffer (Tris HCl 200mM, NaCl 137mM, EDTA 0,2mM, EGTA 0,5mM, Triton 1%, glycerol 10%, pH 7,4), centrifuged (7000xg for 3 minutes) and the supernatant was used. Protein concentration was spectrophotometrically determinated by using the Bradford method using bovine serum albumin as standard [187].

To measure caspase 3, 8 and 9 activities, aliquots of testis homogenate were incubated in a reaction buffer [25 mM Hepes, pH 7.4, 10% (w/v) sucrose; 10 mM DTT (dithiothreitol), 0.1%CHAPS and 100 μM of the caspase substrate Ac (N-acetyl)-LEHD-pNA (p-nitroaniline) [Caspase 3 Substrate I colorimetric (235400), caspase 8 Granzyme B Substrate I colorimetric (368057), Caspase 9 Substrate II colorimetric (218805) (*Calbiochem, Darmstadt ,Germany*)] for 2 h at 37 ◦C. Caspase activity was determined by following the detection of the chromophore pNA after cleavage from the caspase substrate. Calibration was performed with known concentrations of pNA (*Calbiochem, Darmstadt, Germany*).

4 Results

After the beginning of treatment, there was a significant ($p<0,05$) decrease in food intake in animals treated with DOX (figure 4). A stabilizing tendency was observed over the 12 weeks of protocol in saline rats. In both groups treated and saline rats with access to FW, food intake was higher along the protocol, meaning that SAL+FW and DOX+FW are those eating higher quantities within saline and treated groups, respectively.

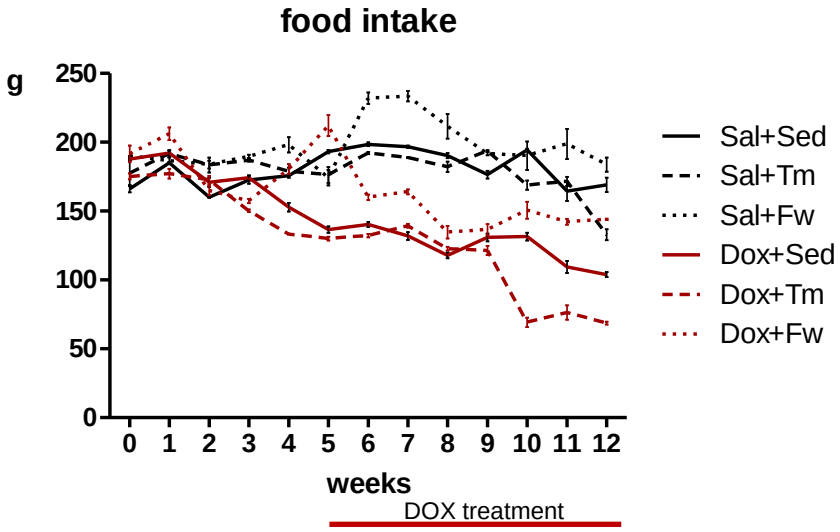


Figure 4 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on weekly rat food intake during the overall protocol. Data are presented as mean ± SEM. The significant differences between groups over the weekly time points are as follows: Sal+Sed vs. DOX+SED – weeks 5 to 10 and week 12 ; DOX+SED vs. DOX+TM – weeks 3,10 and 12 ; DOX +TM vs. SAL+TM – weeks 3,4 and 6 to 12 ; DOX+FW vs. SAL+FW – weeks 3, 6 to 9 and week 12 ; DOX+TM vs DOX+FW – weeks 5, 6 and 10 to 12 and SAL+TM vs SAL+ FW – weeks 6,7 and 12.

Regarding water consumption along the protocol, SAL+FW and DOX+FW are those that consume more water within saline and treated groups, respectively (figure 5).

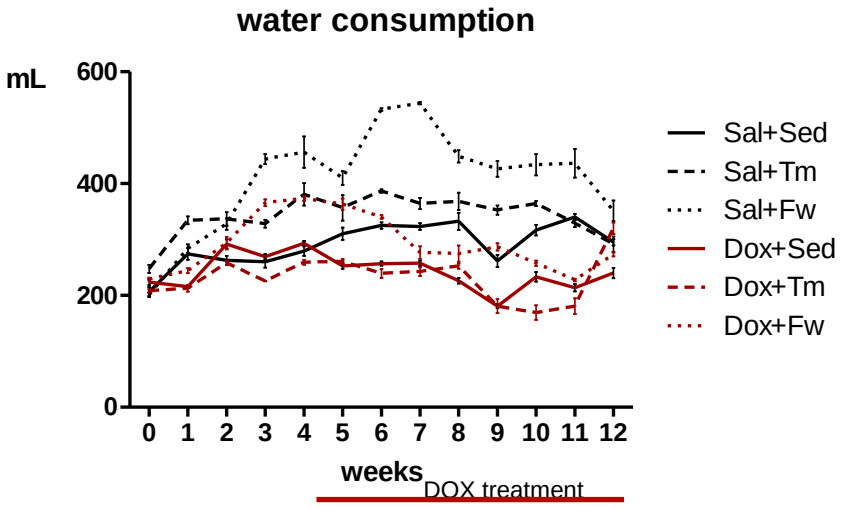


Figure 5 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on weekly rat water consumption during the overall protocol. Data are presented as mean ± SEM. The significant differences between groups over the weekly time points are as follows: SAL+SED vs. DOX+SED – weeks 6 and 11; DOX+SED vs. DOX+TM – no differences; DOX +TM vs. SAL+TM – weeks 1, 6, 7, 9, 10 and 11; DOX+FW vs. SAL+FW - weeks 6 to 11; DOX+TM vs DOX+ FW – weeks 3, 6, 9 and SAL+TM vs SAL+ FW – weeks 3, 6 and 7.

Figure 6 shows the forced and voluntary distance, respectively, covered during endurance training protocol and voluntary physical exercise by treated and saline animals. In the first four weeks of protocol, there was a gradual increase in distance covered in all groups. In the last weeks of protocol a tendency to decreased distance covered in SAL+FW animals was observed. In DOX+FW animals there was a decrease in distance covered from 5th week (beginning of treatment) until the end of protocol in DOX+FW compared with their saline counterparts. SAL+TM animals progressively increased distance covered through the 12 weeks. DOX+TM animals also increase distance covered until the beginning of DOX treatment at 8th week. From weeks 8 to 12, DOX+TM decrease the training velocity and consequently distance covered compared with their saline counterparts.

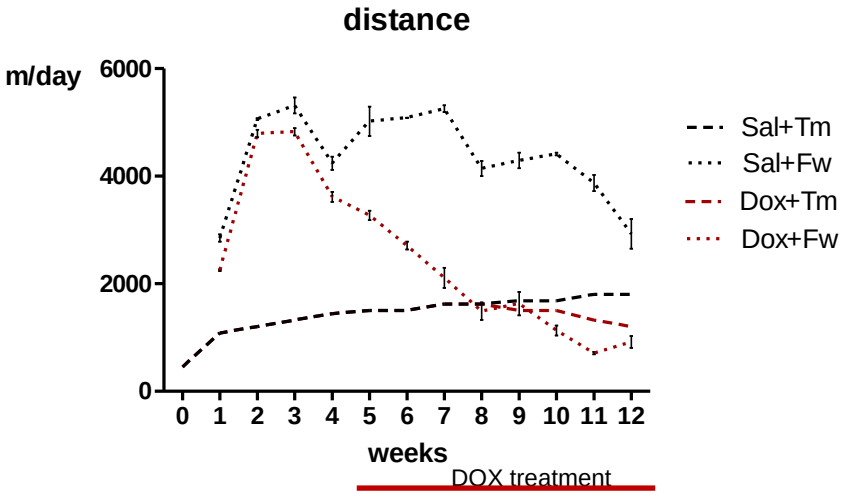


Figure 6 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on weekly distance covered during the overall protocol. Data are presented as mean ± SEM. The significant differences between groups over the weekly time points are as follows: SAL+TM vs DOX+TM – week 11; SAL+TM vs SAL+ FW – weeks 1 to 12; SAL+TM vs DOX+ FW – weeks 1 to 6 and weeks 10 and 11, DOX+TM vs DOX FW – weeks 1 to 6 and week 11; SAL+FW vs DOX+FW – week 1 and weeks 5 to 12.

Figure 7 presents body weight alterations induced by DOX treatment, exercise training and voluntary exercise. As depicted in the figure, after the beginning of DOX treatment a progressively increase in body weight of all the animals was observed through the 12 weeks. After the beginning of DOX treatments, a blunted effect on animal weights was observed in DOX treated animals compared with their respective saline counterparts. Before DOX treatment no differences in body weights between groups were observed.

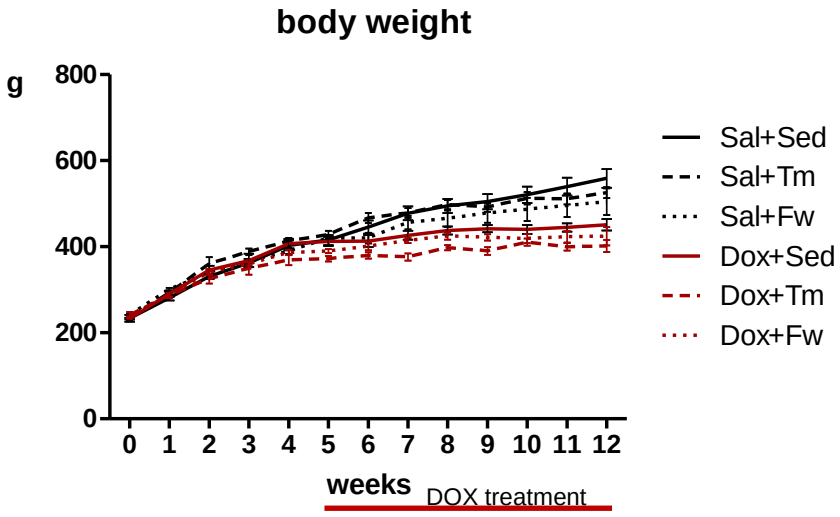


Figure 7 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on body weight during the overall protocol. Data are presented as mean ± SEM. The significant differences between groups over the weekly time points are as follows: Sal+Sed vs. DOX+SED - week 7 to 12; DOX+SED vs. DOX+TM - weeks 5, 7 and 9; DOX +TM vs. SAL+TM - week 5 to 12; DOX+FW vs. SAL+FW - weeks 7, 8 and 11.

The final body weights ,testes and hearth weight, as well as the ratio between testes, hearth and body weight is presented in table IV. Treated animals present a lower final weight when compared with saline. There were significant decreases between saline and DOX groups in testes weight. Accordingly, testes/body weight ratio is lower in treated animals. Hearth weight increased in saline exercised groups and ratio between hearth and body weights also increase in exercised saline groups. No differences between exercised and sedentary animals submitted to DOX treatment were observed in heart/body weight ratio.

Table IV Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on body weight (initial and at the end of the protocol) testis weight, testis/body weight ratio, heart weight and heart/body weight ratio.

	Body weight initial (g)	**Body weight final (g)**	**Testes weight (g)**	**Testes/body weight (mg/g)**	**Heart weight (g)**	**Heart/body weight (mg/g)**
SAL+SED	205.50±3.98	583.00±24.02^{a}	3.58±0.13^{a}	5.95±0.31^{a}	1.39±0.04^{a,c}	2.32±0.08^{a}
SAL+TM	218,50±3,77	539,33±13.21^{a}	3.45±0.12^{a}	6.48±0.14^{a}	1.92±0.08^{b}	3.63±0.12^{b}
SAL+FW	211.50±1.56	527.30±40.22^{a}	3.50±0.22^{a}	6.73±0.98^{a}	1.85±0.14^{b}	3.68±0.18^{b}
DOX+SED	208.90±4.68	444.4±22.99^{b}	1.21±0.10^{b}	2.74±0.25^{b}	1.13±0.05^{a}	2.62±0.13^{a,c}
DOX+TM	207.00±4.63	409.2±13.83^{b}	1.90±0.09^{b}	4.69±0.49^{b}	1.33±0.13^{a,c}	3.16±0.12^{b,c}
DOX+FW	208.80±2.60	464.5±28.02^{a,b}	1.61±0.26^{b}	3.23±0.45^{b}	1.54±0.14^{b,c}	3.12±0.28^{b,c}

Values (mean ± SEM) with different letters are significantly different ($p<0.05$).

Figure 8 show us the sperm count of saline and treated animals of sedentary, treadmill and free-wheel groups. As observed, sperm count in DOX-treated groups showed a significant decrease in sedentary and free-wheel groups compared with their saline counterparts. No exercise effect was observed in sperm count on DOX treated animals from sedentary and free-wheel groups.

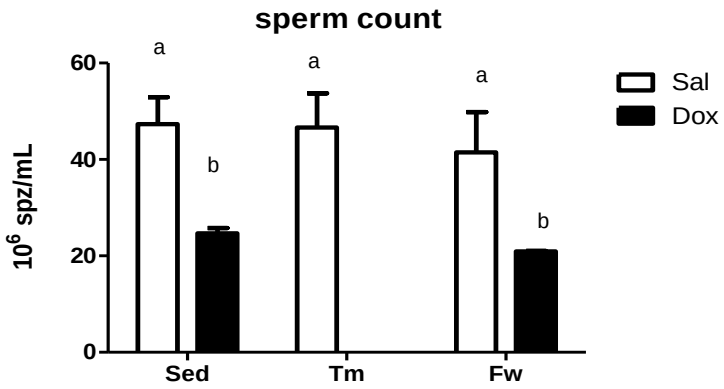


Figure 8 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on sperm count. Values (mean ± SEM) with different letters are significantly different ($p\leq0.05$). Due to technical limitations (eg. DOX+TM animal premature death) sperm count were not possible to acquire.

The sperm motility analysis of treated and non-treated animals of sedentary, treadmill and free-wheel groups is presented in the figure 9. A significant decrease in sperm motility as seen in progressive, non-progressive and immotile spermatozoa was observed in treated compared to saline animals. In immotile sperm when compared with Sal sedentary animals. Similarly, the DOX effect was seen in treadmill groups since treated animals present a significant decrease in immotile sperm when compared with their saline counterparts.

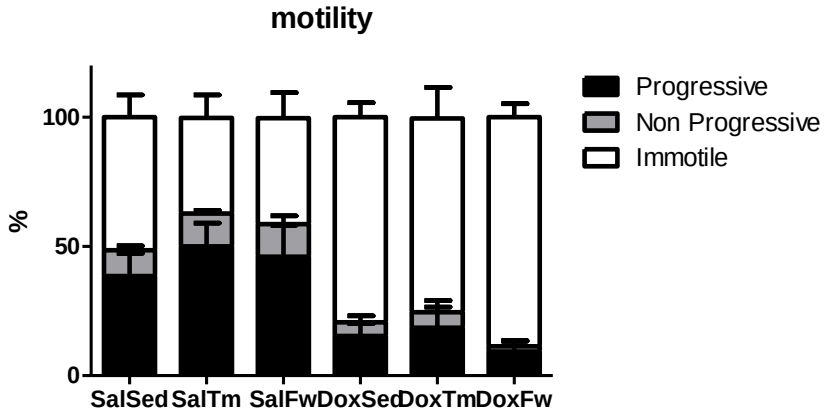


Figure 9 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on sperm motility (progressive, non-progressive and immotile) during the overall protocol. The significant differences between groups are as follows: SAL+FW vs DOX+FW in progressive motile, non-progressive motile and immotile; SAL+SED vs DOX+SED; SAL+SED vs SAL+TM in immotile.

We also measured the extent of oxidative stress and damage in rats of all groups. MDA levels were used as an index of lipid peroxidation in testes (figure 10). There were no significant differences in MDA between groups. A tendency to an increase of lipid peroxidation in treated groups, compared with their respective saline counterparts seems to occur as well as for MDA decrease in exercise groups, particularly in those trained on the treadmill.

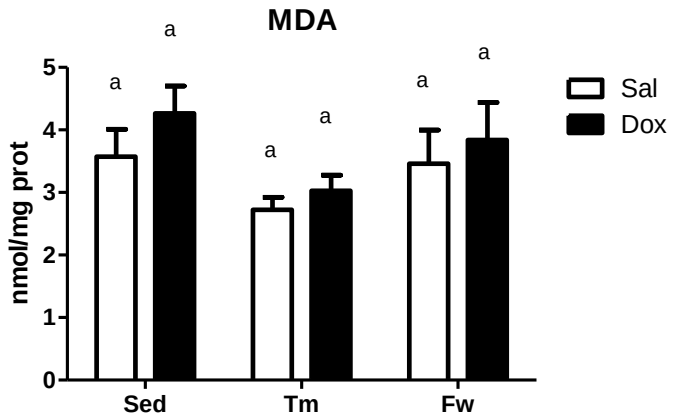


Figure 10 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on levels of MDA. Values (mean ± SEM) with different letters are significantly different ($p<0.05$).

The levels of protein oxidation in testes were determined by –SH groups content (figure 11).

There were no statistical differences in the content of –SH groups between treated and saline groups, in sedentary and treadmill subgroups. A decrease in –SH content in testes of DOX treated animals from the FW group when compared with the respective saline group (DOX+FW vs SAL+FW) was observed.

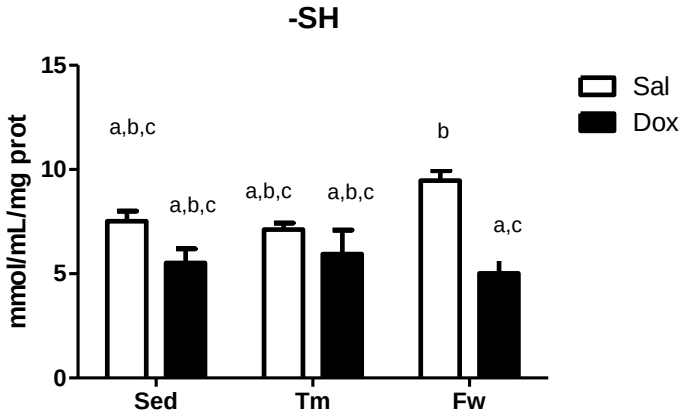


Figure 11 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on levels of –SH groups. Values (mean ± SEM) with different letters are significantly different ($p<0.05$).

Oxidative damage is paralleled by a loss of catalytic activity of aconitase, an enzyme that is critical in energy metabolism. Aconitase catalyzes the interconversion of citrate and isocitrate in citric acid cycle, a reaction essential to normal metabolic function. It is inactivated by RONS and thereby is used as marker of OS.

No differences in testes aconitase activity were observed between treated and saline groups, sedentary and treadmill. However, there was an increase in aconitase activity in testes of DOX treated animals from the FW group when compared with the respective saline group.

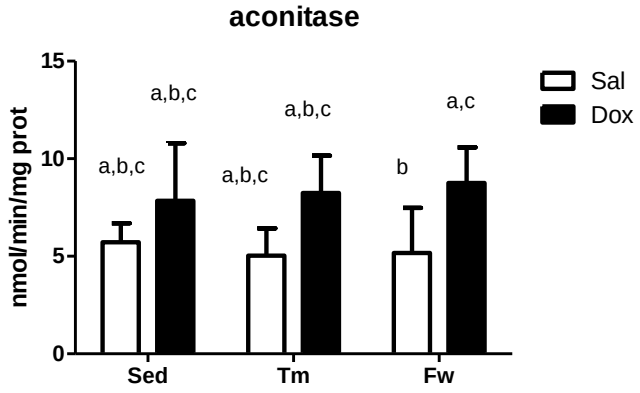
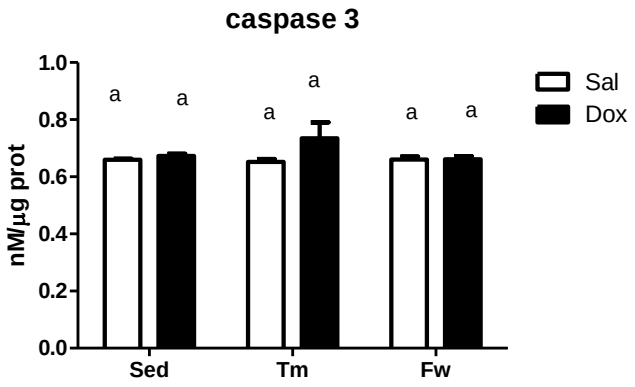


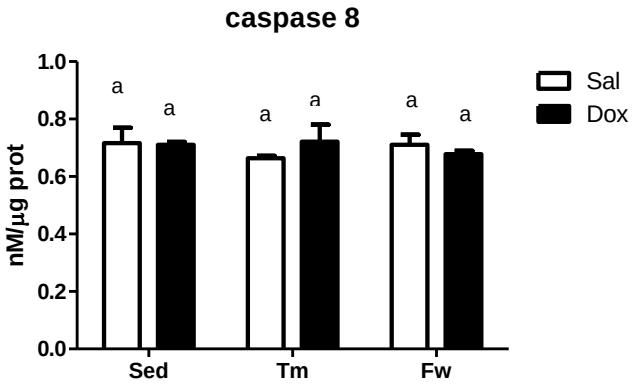
Figure 12 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on aconitase activity. Values (mean ± SEM) with different letters are significantly different ($p<0.05$).

Apoptotic signaling was followed by the measurement of testes caspases 3, 8 and 9 activities (figures 13–A,B,C). As seen, no DOX treatment effect was detectable on the activity of such caspases irrespective to training status.

A



B



C

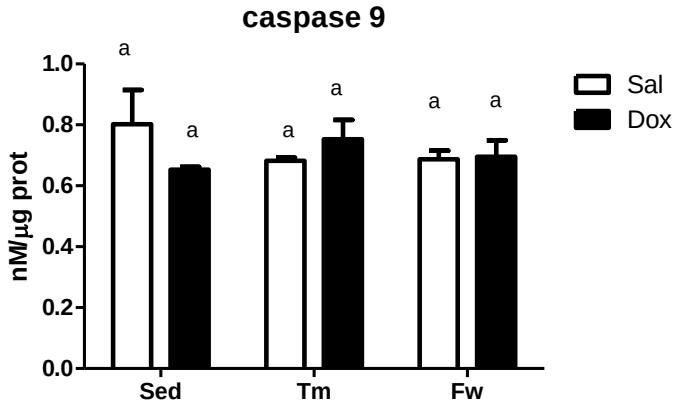


Figure 13 Effects of Doxorubicin treatment, endurance treadmill training and free wheel voluntary activity on caspase 3 (A), 8(B) and 9(C) activities. Values (mean ± SEM) with different letters are significantly different ($p<0.05$)

A brief resume of the results are shown in the next figure.

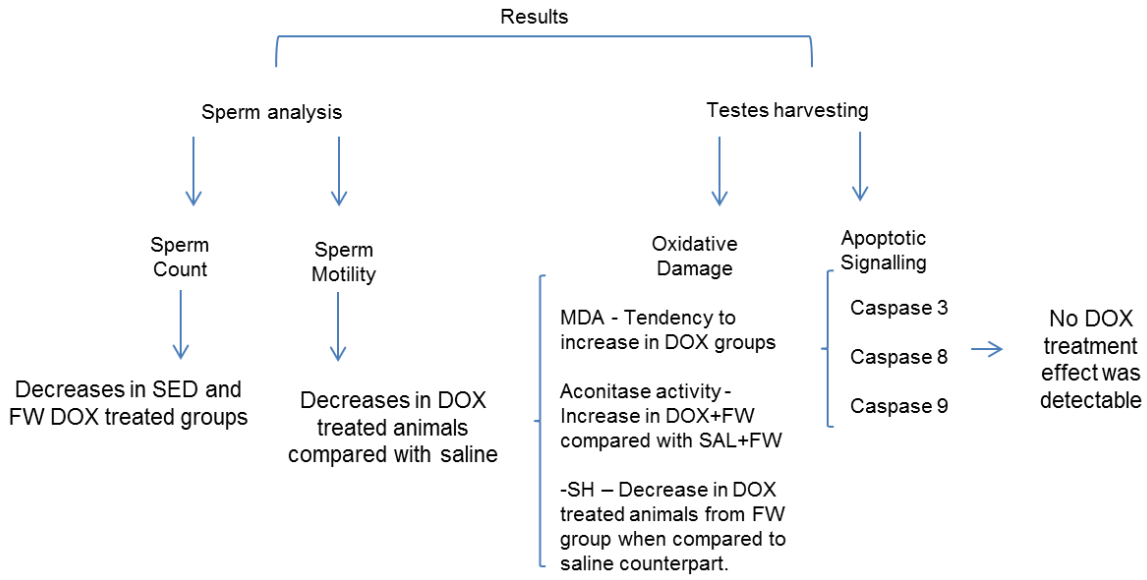


Figure 14 Summary of the results

5 Discussion

In 2009 American Cancer Society (ACS) estimated that there were nearly 1,5 million new cases of cancer diagnosed in United States. Improved prognosis on the basis of earlier detection and newer treatments has created a welcomed new challenge of addressing the unique needs of cancer survivors. In the last years, it has become clear that exercise plays a vital role in cancer prevention and control [188]. There are growing evidences suggesting that exercise decreases the risk of many cancers and data supporting that exercise may extend survival in some types of cancer is emerging [189]. Numerous studies have hypothesized that some of the psychological and physiological concerns faced by cancer survivors can be prevented, attenuated and treated through exercise [188, 190].

Physical activity has numerous proven benefits and its long-contested ability to keep cancer at bug is now being put into test. Some evidences suggest that exercise can prevent breast cancer in about 20/30 per cent if more women followed the physical activity guidelines [190]. In this sense, exercise affect two groups of hormones that are thought to influence breast cancer risk – estrogens and insulin. Hormonal changes, decreases in insulin levels and decreases in adipokines produced by fat cells seem to have relationship with reduced breast cancer risk [190].

Some chemotherapy is centered on DOX action, which has a heart-damage effect. Exercise also helps to reduce this heart damage since strengths the heart´s pumping power, helps fighting fatigue and it is a powerful weapon against increased cardiac oxidative damage associated with DOX [190]. Indeed, there are some preliminary evidences that exercise training is a supportive intervention that may attenuate a broad range of deleterious symptoms, like functional decline, fatigue and nausea associated with cytotoxic therapy leading to improvements in quality of life [188, 190]. Most commonly used curative therapies could affect negatively multiple body systems like cardiovascular, endocrine, nervous, musculoskeletal and immune, which may cause fatigue, pain, skin damages, fat mass increase among others. Exercise therapies against these symptoms should be individualized according to cancer survivor's pretreatment, aerobic fitness and response to treatment. A large number of exercise trials in oncology have provided indirect evidence of the potential biological mechanisms that may underlie the interaction

between exercise, tumor and anthracycline therapy, which include exercise-modulated change in hormonal and metabolic profile, angiogenesis and endogenous antioxidant expression [191, 192]

The cancer incidence and prevalence is also associated with some genetic disorders. Thus, some strategies have raised the possibility that exercise might exert an anticancer effect by turning on some tumor-repressor genes like p53 and L3MBTL1 [189, 190]. Once activated, p53 mounts a reactive response to the imposed insult by inducing cell cycle arrest, facilitating DNA repair and promoting apoptosis [189]. Endurance exercise potentiates the systemic activation of the tumor suppressor protein p53 in multiple tissues and how this action retards cancer incidence/growth is not yet known [189]. However, the interaction of p53 protein within the regulatory network that contributes to endurance exercise-mediated metabolic and therapeutic adaptations is a new line of study. Regarding breast cancer, some evidences suggest that, in exercising patients, L3MBTL1 gene was demethylated, which indicates an increase in gene expression, which, in turn, is associated with a low risk of recurrence and improved survival. Indeed, DNA methylation status of L3MBTL1 could be a useful marker for breast cancer survivors.

Overall, regularly performed endurance exercise exerts a protective effect leading to a multiple health benefits such as reduced risk of cardiovascular disease, obesity and type 2 diabetes. Exercise is effective in reducing the burden of several specific cancers including demonstrated benefits related to aerobic fitness, muscle strength and cancer-related fatigue. A sizeable percentage of cancer survivors stand to benefit from well-designed exercise program led by increasingly well-educated and informed fitness professionals.

For the above-mentioned reasons, the study of chronic exercise effects in subjects with cancer related multiple disorders, in which tissue-related side effects from receiving chemotherapy is included, seems to be pertinent. Therefore, the analysis of exercise effects in other tissues rather than cardiac muscle in DOX-treated animals is still a matter for uncovering.

DOX is a highly effective anticancer drug used in the treatment of several types of cancer. However its use is limited due to cardiotoxicity. Other tissues are affected by this toxicity including brain, liver, kidney and testes [193, 194]. Thus, some strategies have been advanced in order to minimize and counteract the toxic effects of this drug, including physical exercise. Indeed, it has been mentioned that exercise is a potential regulator of antioxidant defenses and also plays other important metabolic functions [195, 196]. DOX

toxicity can be attenuated by exercise preconditioning and it has been reported that chronic physical activity may provide some resistance against cardiac dysfunction and oxidative damage associated with DOX exposure [63, 197]. Many studies suggest that training status may be a determining factor in the defense of late-onset cardiotoxicity, probably by improving mitochondrial and cell defense systems, diminishing cell oxidative stress and increasing enzymes that combat free radical damage [186, 198]. Taken together, some studies report that exercise is able to prevent genotoxicity induced by DOX in heart cells whether in other organs, such as liver and kidney, a lack of literature supporting this protective effect against DOX toxicity exists [116]. This study appears in order to analyze the effects of physical activity and also endurance treadmill training against the deleterious DOX consequences in testicular function.

In literature, there are no large studies or great information about this topic. We analyzed for the first time the impact of physical exercise as voluntary wheel activity and forced treadmill running during treatment with DOX in testicular function. Sperm motility testes, oxidative damage and apoptotic signaling were measured. Male Sprague-Dawley rats were used as these animals are considered an appropriate model to study functional, biochemical and structural alterations observed during and following Doxorubicin treatment [199]. According to our goal, recorded data included food and water intake, distance covered during training, body weight variations and sperm motility. An important limitation of this preliminary study was related to the reduced number of animals per group, a fact that did not allowed the reaching of significant alterations between groups in the majority of specific parameters. Furthermore, during the training protocol, we had some premature deaths of animals involved in study.

One of the alterations referred in the literature is a decrease in water and food consumption in rats treated with DOX when compared to their untreated counterparts (figures 4 and 5). This was also observed in our study, particularly in the beginning of the treatment [200]. Regarding water consumption, differences are not consistent, as SAL and DOX+ FW groups consumed higher amounts of water compared to their respective counterparts, a fact that could hypothetically be related to the possibility of voluntarily exercise through the 24h.

In the analysis of voluntary distance covered (Fig 6), we noticed a decrease in FW animals after the beginning of treatment (5^{th} week).

A progressive increase in treadmill training distance was noted in SAL+TM animals, although in DOX+TM animals, the pace of training was required to decrease from the 8th week, since it was impossible to maintain the pace of the group SAL+TM.

One of the issues in this study was the choice and design of the exercise training protocols. Patients undergoing chemotherapy or diagnosed with cardiovascular diseases such as heart failure experience severe fatigue or display severe exercise intolerance. Consequently the intensity and duration of exercise they are able to tolerate or successfully accomplish is likely to be severely limited. The treadmill training regimen used in this study is considered to be of higher intensity and frequency in rat models and raises concerns to the clinical implications of the findings [201]. Some authors propose that the workout pace similar to the used in our study is equivalent to 75% of VO_2 max and puts into question if patients undergoing chemotherapy withstand that intensity of training [202]. For that reasons, and as certain whether different exercise intensities have distinct outcomes, two chronic exercise protocols were implemented in our study with distinct intensities and durations.

The results of animal´s body weight were as expected, i.e. with the normal development of the animal and the advance of the age, all the weights increased progressively over time (Fig 7). However, animals treated with DOX showed a less weight variation in comparison with untreated animals, as previously described [200, 203]. Although the animals initially began with similar weights, after the beginning of DOX treatment, body weight stabilization in treated groups was noted. This, paralleled with a progressive reduction in food intake and water consumption immediately after beginning of DOX treatment. The results were similar to those described elsewhere in rats treated with the same cumulative bolus of DOX. Body weight differences between sedentary and trained groups follow the trend described by others, i.e., a decreased body weight, an adaptation resulting from endurance training [204]. Given the differences found in the two experimental groups (trained and non-trained) relative to body weight and ratio of heart weight / body weight, we can mention that the length and intensity of endurance training protocol applied was sufficient to induce a suitable organic/tissue adaptation, in this particular case, physiological cardiac hypertrophy [141, 142, 205, 206]. Endurance training is associated with a number of physiological adaptations at both muscle and heart [207] and these adjustments result in supply, uptake and more efficient use of energy substrates needed to produce and sustain physical work.

Results related to testes/body weight ratio are consistent with literature. The testes weights of treated animals are lower than untreated, which means that the drug effect is clearly visible in this organ. In this sense, the literature report that this fact can be attributed to the apoptotic effect caused by DOX toxicity which can consequently be translated into compromised spermatogonia and primary spermatocytes [3]. Physical exercise, in turn, had no effect on testes weight reduction caused by DOX.

Sperm abnormalities, which include low sperm count and poor sperm motility, are a central factor in male infertility, being caused by a range of factors including congenital birth defects, disease, drug exposure and lifestyle habits [208]. Sperm motility is the sperm´s ability to move. If movement is slow, not in a straight line, or both, the sperm have difficult in invading the cervical mucus or penetrating the egg. Poor sperm motility may be associated with OS and DNA fragmentation [209, 210]. A reduction of the percentage of motile and progressive sperm was noted in treated rats as described in literature [203, 211]. The development and differentiation of spermatozoa are maintained through the function of Sertoli cells under the action of some key hormones like FSH and testosterone [212]. It has been reported that DOX induces damage in endocrine system showing higher concentrations of serum LH and FSH [4]. Nambu and Kunamoto (1995) suggested that spermatogenic disorders were generated by interaction with impaired DNA synthesis in stem cells and Sertoli cell dysfunction, which are both directly related with DOX treatment schedule. These data suggest that suppression of sperm motility is caused by Sertoli cell dysfunction induced by DOX [203]; morphological degeneration of Sertoli cells was also noted in treated animals [213]. Some studies also report that male infertility is caused by a decreased number of sperms reaching the oviducts after mating due to decreases in the percentage of motile sperms and sperm count [214, 215]. These findings suggest that the interaction between decreased number of sperms and decrease in sperm motility causes impaired infertility on males treated with DOX [203].

Contrarily to what is relatively established for the toxic effect of DOX in spermatogenesis, there is no consensus in literature regarding the effects of physical exercise in spermatogenesis and reproductive hormonal profiles in men. Some studies reported that endurance exercise training induces significant detrimental effects on reproductive hormonal profile and suggest that impairments in spermatogenesis may exist in some cases [126]. Other studies pointed out that reductions in testosterone concentration, impaired spermatogenesis and their potential modification by exercise are still unknown [126, 216].

It is well accepted that DOX induces testicular toxicity through the increase in OS and apoptosis [217, 218]. Various studies report that the decrease in body and testicular weights, decrease sperm counts, increase testicular toxicity paralleled with reduced GSH, increased GSSG and MDA levels and decreased antioxidant enzyme activities as consequence of DOX treatment [218, 219]. On the other hand, others revealed that exercise training has a beneficial effect on drug and/or aging-induced OS through the activation of antioxidant enzymes in specific tissues as heart, lungs, liver, muscle and testes [219]. The oxidative effect of acute exhaustive exercise on rat testes is known to decrease with endurance training i.e., it prevents/attenuates oxidative damage by neutralizing oxygen radicals and inhibiting lipid peroxidation via beneficial modulation of several cellular systems [70].

According to above referred modulator effect of both DOX treatment and the protective effect of chronic exercise against acute deleterious stimuli for a variety of cells and tissues including testes, a protective-like phenotype could be expected for exercise against DOX. However, no significant differences in MDA levels and protein –SH groups were observed in the present study. Although a tendency to increase MDA levels in treated groups and a decrease in exercised treadmill group were noted, no significant differences were reached. We expected significant differences in both cases since literature report increases in MDA levels and decreases in protein –SH groups in animals treated with DOX in various tissues as heart and *gastrocnemius* muscle [205, 220, 221] and decreases in MDA levels and increases in protein –SH groups associated with aerobic exercise [222-224]. Some less probable hypothesis that could explain our results may include a possible testes adaptation to the increase of DOX-induced RONS and a possible adaptation of the antioxidant system of treated animals. However, we believed further measurements of the activity of some antioxidant enzymes (SOD, CAT, among others) could possibly support our rational.

Aconitase is a mitochondrial enzyme, which is inactivated by RONS and, consequently, is often used as an indirect marker of superoxide production. The literature report a significant decrease in aconitase activity in some tissues of DOX-treated animals [225, 226]. On the other hand, some controversial information about aconitase activity has been reported. Some studies suggest that exercise decreases aconitase activity in liver, spleen and other organs [227], while others reported no changes in aconitase activity [228]. Even more, Zhang et al reported an increase in aconitase activity in rats EDL muscle after induced contractions, while no changes in aconitase activity were observed in humans

with same protocol [63, 229]. Our results show an increase in aconitase activity in testes of DOX treated animals from FW group compared with their saline counterparts. This was in contrast to the expected result as it is well accepted that, under conditions of OS, aconitase is inactivated [230]. Some hypotheses that may explain the results are that the amount of RONS generated was not sufficiently high to induce decreases in aconitase activity and/or another mechanism for control of aconitase activity may exist, as recent studies indicate that aconitase can also be controlled by phosphorylation/desphosphorylation [231, 232].

According to our results, there was no influence of physical exercise and drug administration on apoptosis activation, which does not follow literature. Indeed, we expected some significant differences in the measured caspase. It is well accepted that DOX administration induces apoptosis in many tissues such as heart, brain and testes [2, 233, 234]. Some studies suggest that the initiation phase of spermatogenesis is highly sensitive to DOX-induced apoptosis, which may result in impaired spermatogenesis and cause infertility [2]. DOX-induced apoptosis is linked to activation of p-53 protein, increased RONS generation and oxidative DNA damage [235-237].

Physical exercise is a strong physiological stimulus that can influence a number of extracellular and intracellular signaling pathways, which may influence apoptotic processes in skeletal muscle and other organs. Several studies report that exercise training enhances protection against various stresses and attenuate OS-induced apoptotic signaling and prevents age and drug-induced apoptosis in many tissues, of which heart is an example [238, 239]. It is well documented that endurance exercise decreases caspases 3 and 9 levels in hearth, inactivate caspase 3 activity in liver and increases levels of antiapoptotic proteins in cardiac muscle [238-240];i.e., endurance exercise promotes biochemical alterations is some tissues, resulting in a phenotype that resist to apoptotic stimuli [241]. In the present study, neither DOX treatment nor physical activity affected significantly the apoptotic signaling of studied tests. In fact, we expected some significant differences induced by DOX administration and exercise in apoptotic markers, since it is well documented in literature that both DOX and physical exercise alter apoptotic signaling [237, 242-245]. Several mechanisms for apoptosis exercise-protective response have been proposed as decreased expression of proapoptotic factors, improved mitochondrial function, reduced RONS generation and increase of antioxidant defense status [240, 246]. Further studies are needed in order to better understand the precise

mechanisms by which physical exercise modulate testes response to toxic stimuli including DOX.

6 References

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