Toxicology Letters 280 (2017) 57–69

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Can exercise training counteract doxorubicin-induced oxidative damage of testis proteome?

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ARTICLE INFO

Keywords:
Testes
Treadmill exercise
Adriamycin
Protein oxidation
Proteomics

ABSTRACT

The use of the chemotherapeutic drug doxorubicin (DOX) is limited by its toxicity in several organs such as testes. So, we analyzed the effect of endurance treadmill exercise training (EX) performed before sub-chronic DOX treatment on sperm count and motility, testes markers of oxidative damage and apoptosis. Tissue profiling of proteins more susceptible to oxidation was made to identify the molecular pathways regulated by oxidative modifications, as nitration and carbonylation.

Twenty-four adult male rats were divided into four groups (n = 6/group): sedentary saline (SED + SAL), sedentary sub-chronically injected with DOX (2 mg-kg-1 per week, during 7 weeks; SED + DOX), 12 weeks trained saline (EX + SAL) and trained treated with DOX (EX + DOX). DOX treatment started 5 weeks after the beginning of the exercise program. Testes caspase-3, −8 and −9, as well as aconitase activities, the content of malondialdehyde (MDA), sulfhydryl groups (-SH), carbonyl and nitrotyrosine derivatives were determined. Modified proteins were identified by 2D-Western blot followed by MALDI-TOF/TOF mass spectrometry, and bioinformatic analysis was performed to assess the biological processes regulated by these chemical modifications.

The decreased sperm motility induced by DOX was not modified by exercise. Significant increases in MDA content in SED + DOX and in caspase-3 and −9 activities in EX + DOX were found. Despite no significant differences in the levels of carbonylated and nitrated proteins, exercise modulated testis proteome susceptibility to oxidation in DOX-treated group, with less modified proteins identified. Zinc finger Ran-binding domain-containing protein 2 (ZRAB2) and AN1-type zinc finger protein 3 (ZFAN3) were among the proteins found oxidatively modified.

Although no marked alterations in testes oxidative damage were noticed, proteomic analysis of oxidatively modified proteins highlighted the protective role of exercise against oxidative damage of some proteins involved in metabolism and stress response against DOX.

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 (Ascensao et al., 2012; Gharanei et al., 2013; Volkova and Russell, 2011). In addition to the heart, several studies reported DOX toxicity in other organs such as kidney and liver as well as in testis, in which testicular dysfunction and male infertility is reported (Ayla et al., 2011; Hou et al., 2005; Yagmurca et al., 2007). Among the mechanisms underlying DOX toxicity in testis are the increased oxidative stress with consequent lipid peroxidation and cellular apoptosis, impaired sperm motility, decrease in testis weight and sperm concentrations, increased percentage of abnormal spermatozoon, germ cell apoptosis, decreased

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http://dx.doi.org/10.1016/j.toxlet.2017.08.010
Received 25 February 2017; Received in revised form 2 July 2017; Accepted 10 August 2017
Available online 14 August 2017
0378-4274/

testosterone levels and, eventually, testicular failure (Uygur et al., 2014). Indeed, particularly due to its chemotherapeutic efficacy against various types of cancer, DOX has attracted the interest of many researchers aiming at searching for new strategies to prevent its harmful effects.

Many pharmacological and non-pharmacological strategies have been carried out to counteract its toxicity, namely antioxidant supplements and exercise training (Ascensão et al., 2005; Yagmurca et al., 2007). Growing experimental evidence suggests that DOX treatment induces testis dysfunction mainly through the increased production of reactive oxygen and nitrogen species (RONS) and increased susceptibility to apoptosis (Hou et al., 2005; Shinoda et al., 1999). Exercise has been proposed as an important strategy to counteract DOX induced toxicity, predominantly in heart tissue (Ascensao et al., 2012), but also in brain (Marques-Aleixo et al., 2016) and skeletal muscle (Bredahl et al., 2016). Although the exact mechanisms responsible for this protection continue to be debated, it has been argued that they are in part associated with systemic and tissue specific decrease of free radical production and with increased response of antioxidant defense systems (Ascensao et al., 2012; Marques-Aleixo et al., 2015). Therefore, it seems reasonable to hypothesize that exercise training provides some degree of protection against DOX-induced testis dysfunction by possibly attenuating DOX-induced increased oxidative damage and apoptotic signaling, a matter not yet studied.

In order to add new insights on the effect of endurance training in testicular function of rats submitted to a sub-chronic treatment of DOX, which resembles cancer treatments in human patients (Pereira et al., 2012), the testis proteins more susceptible to oxidative modifications were searched using a proteomic approach and related to sperm motility and apoptosis.

2. Material and methods

2.1. Animals

All experiments involving animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (ILAR, NIH Pub. No. 85-23, Revised 2011), the Directive 2010/63/EU of the European Parliament and were approved by the Ethics Committee of the Research Centre in Physical Activity, Heath and Leisure (Faculty of Sport, University of Porto). Twenty-four 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 °C; 50–60% humidity) receiving food (Scientific Animal Food and Engineering, A04) and water ad libitum in 12-h light/dark cycles. The animals were randomly divided into four groups (n = 6 per group): sedentary saline (SED + SAL), sedentary receiving DOX (SED + DOX), saline endurance trained in a treadmill (EX + SAL); endurance trained treated with DOX (EX + DOX).

The animals from EX 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, USA). The treadmill speed was gradually increased over the course of the 12-week training period. The protocol included 5 days of habituation to the treadmill with 10 min of running at 15 m/min, with daily increases of 5–10 min until 30 min was achieved. 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 (Table 1). 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.

Five weeks after the beginning of the protocol, animals from DOX groups received intraperitoneal injections of DOX (2 mg/kg) whereas saline groups received an equivalent volume of vehicle solution (NaCl 0.9%, 1 mL/kg), during seven weeks (Table 1). 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.

Animals were sacrificed 24 h after the last exercise bout for EX 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. Testis were immediately stored at −80 °C until further use in the biochemical assays described below. Three portions of testis were frozen separately for further homogenization and distinct assays as described.

2.2. Semen analysis

Sperm motility (from spermatozoa recovered from the epididymis) was assessed immediately after liquefaction of the sample, preferably at 30 min and no more than 1 h following the sample collection, to limit the deleterious effects of dehydration, pH or temperature change (Barratt et al., 2010; Bjorndahl and Kvist, 2010). A wet preparation was performed to allow the direct observation of spermatozoa. After mixing the sample, a 10 μL volume was placed on a microscope slide (20 μm depth) and the coverslip immediately applied. Within 60 s, 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) (Barratt et al., 2010; Castilla et al., 2010).

To perform sperm count, the semen sample was mixed and an appropriate volume was added to the fixative (5 g of $NaHCO_3$ and 1 mL of 35% (v/v) formalin in 100 mL purified water). The dilution was vortexed for 10 s 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 400× 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) × (1/20)× 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.

2.3. Markers of apoptosis

Testis were homogenized in a homogenization buffer (Tris HCl 200 mM, NaCl 137 mM, EDTA 0.2 mM, EGTA 0.5 mM, Triton 1%, glycerol 10%, pH 7.4), centrifuged (7000 × *g* for 3 min) and the supernatant was used. Protein concentration was spectrophotometrically determined by using the Bradford method using bovine serum albumin as standard (Bradford, 1976).

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, 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, Germany)] for 2 h at 37 °C. Caspase activity was determined by following the detection of the chromophore pNA after