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

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ABSTRACT

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

Male-young Sprague–Dawley rats were divided into six groups (n = 6 per group): SAL + SED (saline sedentary), SAL + TM (12-weeks TM), SAL + FW (12-weeks FW), DOX + SED (7-weeks of chronic DOX treatment 2 mg/kg per week), DOX + TM and DOX + FW. DOX administration started 5 weeks after the beginning of the exercise protocol. Heart mitochondrial ultrastructural alterations, mitochondrial function (oxygen consumption and membrane potential), semi-quantification of oxidative phosphorylation (OXPHOS) proteins and their in-gel activity, as well as proteins involved in mitochondrial oxidative stress (SIRT3, p66shc and UCP2), biogenesis (PGC1α and TFAM), acetylation and markers for oxidative damage (carbonyl groups, MDA, – SH, aconitase, Mn-SOD activity) were evaluated. DOX treatment resulted in ultrastructural and functional alterations and decreased OXPHOS. Moreover, DOX decreased complex I activity and content, mitochondrial biogenesis (TFAM), increased acetylation and oxidative stress. TM and FW prevented DOX-induced alteration in OXPHOS, the increase in oxidative stress, the decrease in complex V activity and in complex I activity and content. DOX-induced decreases in TFAM and SIRT3 content were prevented by TM only.

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

1. Introduction

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

Among the strategies proposed as effective in counteracting the cardiac side effects associated with DOX treatment, physical exercise has been recommended as a non-pharmacological tool against myocardial injury (Ascensao et al., 2006c; Ascensao et al., 2007; Ascensao et al., 2011b; Powers et al., 2008). Previous work suggested that the advantage of both acute (Ascensao et al., 2011a; Wonders et al., 2008) and chronic exercise models (Ascensao et al., 2005a,b; Ascensao et al., 2006a; Chicco et al., 2005, 2006; Dolinsky et al., 2013) on triggering a preconditioning-like effect on DOX-treated rats with acute single doses includes the protection of cardiac tissue and especially mitochondria against negative remodeling. Recent studies investigated the effects of exercise performed during and following late-onset DOX-induced cardiotoxicity which showed improvements in hemodynamic

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parameters (Hayward et al., 2012; Hydock et al., 2012a). However, the cellular and molecular mechanisms underlying this protective phenotype induced by exercise against sub-chronic DOX administration, particularly those targeting mitochondria, are unknown. Specifically, whether perturbations in heart mitochondrial oxidative phosphorylation capacity and oxidative modifications associated with sub-chronic cumulative DOX administration are mitigated by "forced" or "voluntary" long-term exercise models performed prior and during the course of treatments have not been determined and represents the novelty of the present study. As patients undergoing chemotherapy experience severe fatigue and display severe exercise intolerance, the intensity and duration of tolerable exercise are likely to be severely limited (Emter and Bowles, 2008). Facing this, we aimed at analyzing the effects of two types of long-term exercise with distinct characteristics regarding volume and intensity on cardiac mitochondrial bioenergetics and oxidative stress markers in rats sub-chronically treated with DOX.

2. Methods

2.1. Animals

All experimental procedures were conducted in accordance with 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). Thirty-six male 6-weeks old Sprague–Dawley rats were housed in individual cages in 12 h light/dark cycles with free access to food and water.

Animals were randomly divided into six groups (n = 6 per group): saline sedentary (SAL + SED), saline treadmill endurance training (SAL + TM), saline free wheel voluntary physical activity (SAL + FW), doxorubicin sedentary (DOX + SED), doxorubicin treadmill endurance training (DOX + TM) and doxorubicin free wheel voluntary running (DOX + FW).

2.2. Exercise protocols and doxorubicin treatment

Animals from TM groups were exercised 5 days/week (Monday–Friday) in the morning (between 10:00 and 12:00 AM) for 12 weeks on a LE8700 motor driven treadmill (Panlab, Harvard, USA). The protocol included 5 days of habituation (week 0) followed by continuous running (60 min/day) with a gradual increase in velocity from 18 m/min to 27 m/min (week 7). SAL + TM continued to increase velocity to 30 m/min while in DOX + TM, animals' velocity was gradually adjusted until 20 m/min. The animals from FW groups were housed in polyethylene cages equipped with a running wheel [perimeter = 1.05 m, Type 304 Stainless steel (2154F0106-1284L0106) (Tecniplast, Casale Litta, Italy)]. The rats were allowed to exercise with an unlimited access to the running wheel 24 h/day. Running distance was recorded using ECO 701 from Hengstler (Lancashire, UK).

After the 5th week of endurance training or free wheel exercise, the animals were treated with sub-chronic protocol of seven weekly injections with doxorubicin (Ferrer Farma, Barcelona, Spain) or sterile saline solution of NaCl 0.9% (intraperitoneal injection 2 mg/kg of body weight). The animals assigned to the TM groups received DOX or SAL injections during the weekend in the day-off training (Sunday morning: 48 h after the last training session and 24 h before the next one).

2.3. Animal euthanasia, blood, soleus extraction and isolation of heart mitochondria

Forty-eight hours after the last TM exercise session, non-fasted rats were injected with ketamine/xylazine (90 and 10 mg/kg, i.p., respectively). Upon absence of eye-blink, toe-pinch and righting reflexes, blood was collected and determination of cardiac troponin I (cTnI) assayed by chemiluminescent microparticle immunoassay. The hearts were then harvested and excised, and mitochondria were isolated using conventional methods of differential centrifugation (Bhattacharya et al., 1991). A portion of the left ventricle papillary muscle was prepared for later semi-quantification of protein expression by Western blotting. Right *soleus* muscles were homogenized for spectrophotometrical determination of citrate synthase, as previously described (Coore et al., 1971).

2.4. Mitochondrial respiratory activity and transmembrane electric potential

Mitochondrial respiratory function was measured polarographically at 25 °C using a Biological Oxygen Monitor System (Hansatech Instruments, Norfolk, UK) and a Clark type oxygen electrode (Hansatech DW1, Norfolk, UK). Mitochondrial respiration was initiated by adding glutamate/malate (G/M, 5 and 2.5 mM, respectively). State 3 respiration was determined after adding ADP (150 nmol); state 4 was measured as the rate of oxygen consumption after ADP phosphorylation. The RCR and ADP/O ratios, were calculated according to Estabrook (1967).

Mitochondrial transmembrane electrical potential ($\Delta\psi$) was monitored using lipophilic cation tetraphenylphosphonium (TPP^+) as previously described by Ascensao et al. (2011a) and according to Kamo et al (1979). $\Delta\psi$ was measured at 25 °C, energization was carried out with G/M (5 and 2.5 mM, respectively) and ADP (150 nmol) was used to produce a phosphorylation cycle. The phosphorylative lag phase was also measured.

2.5. Blue-native PAGE separation of mitochondrial membrane complexes and in-gel activity of complexes IV and V

The blue-native PAGE separation of mitochondrial membrane complexes was performed using the method described by Schagger and von Jagow (1991). The in-gel activity and histochemical staining assays of complexes IV and V were determined using the methods described by Zerbetto et al. (1997).

2.6. Respiratory chain complex I and V activity

Mitochondrial fractions were disrupted by a combination of freeze–thawing. Complex I activity was determined by rotenone sensitive reduction of NADH absorbance as previously described by Janssen et al (2007) and ATP synthase activity was estimated by the blue color intensity formed by the reaction of the phosphate produced by hydrolysis of ATP and ammonium molybdate, according to Simon et al. (2003).

2.7. Mitochondrial oxidative damage and antioxidant quantification

Before analysis, mitochondrial membranes of isolated mitochondrial fractions were disrupted by a combination of freeze–thawing cycles. The extent of lipid peroxidation was determined by measuring MDA contents through a colorimetric assay, according to Marques-Aleixo et al. (2012) and based on procedures described previously (Buege and Aust, 1978). The content of oxidative modified –SH groups was quantified by a spectrophotometric measurement according to the method proposed by Hu (1990). Aconitase activity was measured spectrophotometrically by monitoring the formation of cis-aconitate from isocitrate as previously described (Ascensao et al., 2005b). Manganese-dependent superoxide dismutase (Mn-SOD) activity was measured using a commercial kit (RANSOD, Randox Labs, Crumlin, UK), according to the manufacturer's instructions.

2.8. Immunoblotting for detection of mitochondrial and heart tissue proteins

Equivalent amounts of heart mitochondria or tissue were separated by SDS/PAGE (12% gels) followed by blotting on PVDF membranes (Millipore, Massachusetts, USA). Membranes containing