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Short Communication

Exercise training can induce cardiac autophagy at end-stage chronic conditions: Insights from a graft-versus-host-disease mouse model

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ABSTRACT

Introduction: Chronic graft-versus-host disease (cGVHD) is a frequent cause of morbimortality after allogeneic hematopoietic stem cell transplantation (allo-HSCT), and severely compromises patients' physical capacity. Despite the aggressive nature of the disease, aerobic exercise training can positively impact survival as well as clinical and functional parameters. We analyzed potential mechanisms underlying the recently reported cardiac function improvement in an exercise-trained cGVHD murine model receiving lethal total body irradiation and immunosuppressant treatment (Fiuza-Luces et al., 2013. *Med Sci Sports Exerc* 45, 1703–1711). We hypothesized that a cellular quality-control mechanism that is receiving growing attention in biomedicine, autophagy, was involved in such improvement.
Methods: BALB/C female mice (aged 8 wk) with cGVHD were randomly assigned to a control/exercise group (n = 12/11); the exercise group underwent moderate-intensity treadmill training during 11 wk after allo-HSCT. In the hearts of those few mice surviving the entire 11 wk period (n = 2/5), we studied molecular markers of: macroautophagy induction, preservation of contractile/structural proteins, oxidative capacity, oxidative stress, antioxidant defense, and mitochondrial dynamics.
Results: Mainly, exercise training increased the myocardial content of the macroautophagy markers LC3BII, Atg12, SQSTM1/p62 and phospho-ULK1 (S555), as well as of α-tubuline, catalase and glutathione reductase (all $p < 0.05$).
Conclusions: Our results suggest that exercise training elicits a positive autophagic adaptation in the myocardium that may help preserve cardiac function even at the end-stage of a devastating disease like cGVHD. These preliminary findings might provide new insights into the cardiac exercise benefits in chronic/debilitating conditions.

1. Introduction

Chronic graft-versus-host disease (cGVHD) is a frequent cause of morbimortality following allogeneic hematopoietic stem cell transplantation (allo-HSCT). Despite the debilitating nature of the disease, a recent murine aerobic training model positively impacted survival and clinical disease indicators, which was accompanied by improved cardiovascular fitness at the end of the training period (as reflected by decreased basal heart rate and the ability to tolerate higher exercise loads) (Fiuza-Luces et al., 2013b).

In the last years, autophagy, a catabolic route of degradation and recycling of cellular components, has received great attention partly due to its role in longevity promotion and defense against chronic diseases (Moran et al., 2012). There are several routes of autophagy including microautophagy, chaperone-mediated autophagy and macroautophagy. The latter involves the formation autophagosomes that enwrap and sequester the cellular component to be eliminated, and is the only currently known route to degrade organelles or large protein aggregates. Although there is still some debate, the bulk of evidence supports that autophagy is an important cardiac protective mechanism that helps to 'declutter' the cell and restore its functionality, including in pathological conditions (Gottlieb and Mentzer, 2012). For instance, autophagy

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can protect against hypertrophic cardiomyopathy (Frey and Olson, 2003; Chen et al., 2013) or apoptosis activated by chronic myocardial ischemia (Yan et al., 2005). Some genetic heart diseases, *e.g.* Danon disease ('lysosomal glycogen storage disease with normal acid maltase') are associated with chronic perturbation in the autophagy process (Saftig et al., 2001). Autophagy is also gaining attention for its potential involvement in the beneficial adaptations induced by regular exercise (Fiuza-Luces et al., 2013a). In this report, we have mainly analyzed if macroautophagy is associated with cardiac training adaptations shown by mice with cGVHD.

2. Methods

The study protocol received animal experimentation IRB approval. We studied the heart tissue of those mice surviving at the end of an 11 wk-cGVHD model. The experimental animals were BALB/C (recipient) female and B10.D2 (donor) male mice. Briefly, after a myeloablative regimen consisting of 2 sessions of lethal total body irradiation (4.75 Gy/session, with a 24-h interval between sessions to minimize gastrointestinal toxicity), all recipient mice ($n = 23$, aged 8 wk) underwent allo-HSCT to induce cGVHD and were randomly assigned to an exercise/control group ($n = 11/12$). All mice from the two groups received cyclosporine A [15 mg/kg/day, prepared in sterilized conditions and diluted in a final volume of 100 μL PBS 1X (Sigma Chemical Co, EEUU)] every day of the week (Monday–Sunday), from the day before allo-HSCT (*i.e.* Day −1, with allo-HSCT corresponding to 'Day 0') to Day +82. The exercise program consisted of moderate-intensity treadmill running [total duration ~11 wk (from Day +2 to Day +80)], with gradual increases in treadmill speed and inclination, *i.e.* starting at Day +2 with very low workloads [25 min at 35% of the maximal velocity obtained during a baseline incremental test (V_{max}) and 0% gradient] and ending at Day +80 with 60 min at 70%V_{max} and 25% gradient (Fiuza-Luces et al., 2013b).

This program elicited a significantly ($p = 0.011$) higher survival rate after the 11 wk period in the exercise group ($n = 11 \rightarrow n = 5$ final survivors, *i.e.* 45.5% survival) compared with the control group ($n = 12 \rightarrow n = 2$, *i.e.* 16.7%) (Fiuza-Luces et al., 2013b) (**Fig. 1**). Basal heart rate, as measured consistently by the same experienced researcher (CF-L) during the animal's dark cycle using a throat sensor for anesthetized mice (MouseOx® STARR Life Sciences Corp.), remained ~stable in the surviving controls (356 ± 4 *vs* 346 ± 29 beats/min at pre- and post-transplant) but decreased in trained survivors (395 ± 45 and 316 ± 70 beats/min, *i.e.* -20%), indirectly reflecting improved fitness status in the latter (Fiuza-Luces et al., 2013b).

Surviving mice were sacrificed 3 days after the last training session, *i.e.* at Day +83 post-transplant (with i.p. Avertin injection). Heart muscle tissue was dissected, immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Activities of respiratory chain complexes II, III, IV, I + III and II + III were determined in cardiac-muscle homogenates prepared in 225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA and 10 mM Tris–HCl pH 7.4, according to recent standardized protocols for spectrophotometric assays (Medja et al., 2009). Citrate synthase activity was also spectrophotometrically determined (at 30 °C, with 0.1% Triton X-100 following the formation of 5-thio-2-nitrobenzoic acid at 412 nm) (Trounce et al., 1996).

Total ventricular extracts (right and left ventricles) were obtained by homogenization in 6.5 volume of ice-cold extraction buffer (10 mM TRIS–HCl, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and protease and phosphatase inhibitor cocktails) in a Potter–Elvehjem homogenizer. Samples were centrifuged at 800×*g* and 4 °C for 10 min, and the resulting supernatants were collected for protein concentration determination (Bradford

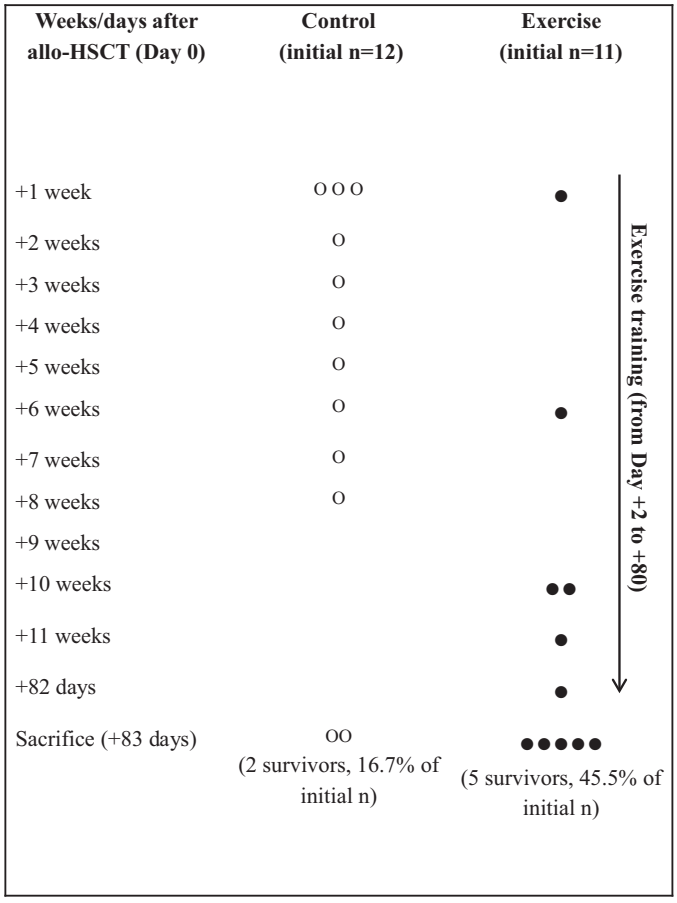

Fig. 1. Individual data of death days in the two groups (O control, ● exercise). Abbreviation: allo-HSCT, allogeneic hematopoietic stem cell transplantation.

method). Samples of cardiac-muscle extracts (10 and 20 mg) were used to perform semi-quantitative analysis of protein levels by immunoblotting. SDS–PAGE was performed on a 15% separation gel for microtubule-associated protein 1 light chain 3 alpha (LC3B), 10% for proteins involved in mitochondrial dynamics, and 7.5% for the remaining proteins. Resolved proteins were transferred to a PVDF or nitrocellulose membrane for detection of 4-hydroxynonenal (HNE) and malondialdehyde (MDA)-modified proteins. Blots were blocked and incubated with the following primary antibodies: anti-glyceraldehyde phosphate dehydrogenase (GAPDH), anti-α-tubulin, anti-LC3B and anti-catalase (CAT) (Sigma–Aldrich, Madrid, Spain); anti-heavy chain cardiac myosin, anti-sequestosome 1 (SQSTM/p62), anti-glutathione reductase (GR), anti-MDA and anti-4-HNE (Abcam, Cambridge, UK); anti-autophagy related gene 12 (Atg12) (MBL International, Woburn, MA, USA); anti-unc-51-like kinase 1 phosphorylated at serine 555 (Phospho-ULK1 S555) and anti-dynamin related protein (Drp1) (Cell Signaling Technology, Inc., Danvers, USA); anti-mitofusin 2 (Mfn2) (Abnova, Taipei, Taiwan); anti-optic atrophy protein 1 (OPA1) (Becton Dickinson, San Agustín, Spain); and anti-mitochondrial superoxide dismutase (mtSOD) (*Millipore Ibérica*, Madrid, Spain).

Band densities were evaluated by densitometric scanning (Image J software), and were expressed as optical density (O.D) arbitrary units. To verify that the total protein amount loaded in each lane was the same, GAPDH was used as loading control and was immune detected with anti-GAPDH (Sigma–Aldrich, Madrid, Spain) coupled to peroxidase-conjugated goat anti-rabbit antibody (GE Healthcare, Buckinghamshire, UK).

Data are shown as means ± SD. The Mann–Whitney *U* test was used for between-group comparisons ($\alpha = 0.05$). All statistical