Histol Histopathol (2015) 30: 801-811
DOI: 10.14670/HH-11-581

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

Aerobic training attenuates nicotinic acetylcholine receptor changes in the diaphragm muscle during heart failure

Paula Aiello Tomé de Souza[1], Rodrigo Wagner Alves de Souza[1], Luana Campos Soares[1], Warlen Pereira Piedade[1], Dijon Henrique S. Campos[2], Robson Francisco Carvalho[1], Carlos Roberto Padovani[3], Katashi Okoshi[2], Antônio Carlos Cicogna[2], Selma Maria Michelin Matheus[4] and Maeli Dal-Pai-Silva[1]

[1]Department of Morphology, Bioscience Institute, [2]Department of Internal Medicine, Faculty of Medicine, [3]Department of Statistic, Bioscience Institute and [4]Department of Anatomy, Bioscience Institute, Sao Paulo State University, UNESP, Botucatu, Sao Paulo, Brazil

Summary. Introduction: Heart failure (HF) is a progressive myopathy, with clinical signs of fatigue and limb weakness that can damage the nerve-muscle interaction, altering synaptic transmission and nicotinic acetylcholine receptors (nAChR) in neuromuscular junctions (NMJs). The diaphragm is composed of a mixed proportion of muscle fibres, and during HF, this muscle becomes slower and can alter its function. As exercise training is an accepted practice to minimise abnormalities of skeletal muscle during HF, in this study, we evaluated the hypothesis that aerobic training attenuates alterations in the expression of nAChR subunits in NMJs diaphragm during heart failure. Objective: The aim of this study was to evaluate the distribution and expression of nAChR subunits in the diaphragm muscle fibres of rats subjected to an aerobic training programme during HF. Methods: Control (Sham), control training (ShamTR), aortic stenosis (AS) and aortic stenosis training (ASTR) groups were evaluated. The expression of nAChR subunits (γ, $\alpha1$, ε, $\beta1$ and δ) was determined by qRT-PCR, and NMJs were analysed using confocal microscopy. Results: We observed increased expression of the γ, $\alpha1$ and $\beta1$ subunits in the AS group compared with the ASTR group. The distribution of NMJs was modulated in these groups. Discussion: HF alters the mRNA expression of nAChR subunits and the structural characteristics of diaphragm NMJs. In addition, aerobic training did not alter NMJs morphology but attenuated the alterations in heart structure and function and in nAChR subunit mRNA expression. Our findings demonstrate the beneficial effects of aerobic exercise training in maintaining the integrity of the neuromuscular system in the diaphragm muscle during HF and may be critical for non-pharmacological therapy to improve the quality of life for patients with this syndrome.

Key words: Skeletal muscle, Heart failure, Aerobic training, Neuromuscular junction, Nicotinic acetylcholine receptors

Offprint requests to: Maeli Dal-Pai-Silva, Department of Morphology, Bioscience Institute, Sao Paulo State University, UNESP, Botucatu, Sao Paulo, CEP: 18618-970, Brazil. e-mail: maeli@ibb.unesp.br

Introduction

Heart failure (HF) is defined as the inability of the heart to meet the oxygen and nutrient demands of peripheral tissues and results in fatigue or tachypnea upon exertion, which progresses to laboured respiration at rest (Piña et al., 2003). These symptoms may partially result from specific skeletal muscle myopathy, which is characterised by a reduced oxidative capacity and shift from myosin heavy chain (MyHC) I 'slow' to MyHC II 'fast', which may culminate in limb-muscle fatigue (Simonini et al., 1996; Vescovo et al., 1998; Carvalho et al., 2003), and a shift from MyHC II to MyHC I in the diaphragm muscle that may contribute to laboured respiration in this syndrome (Tikunov et al., 1996; De Sousa et al., 2001). Additionally, HF alters nAChR gene expression in NMJs (Souza et al., 2011).

The nAChR is a pentameric membrane protein

complex composed of homologous subunits (γ [foetal], α1, ε [adult]), β1 and δ) (Wu et al., 2010; Wood and Slater, 1997). In adult innervated skeletal muscles, nAChR are highly concentrated in the post-synaptic membrane but are virtually absent from the rest of the muscle plasma membrane. In mammalian muscle, nAChR are expressed as a foetal and an adult isoform (Brenner and Sakmann, 1978) that differ in their functional properties and possess a different subunit composition (Mishina et al., 1986). During development, the foetal isoform, the adult form, nAChRε, in which the ε subunit is substituted by the γ subunit, substitutes nAChRγ, which is composed of α1, β1, γ and δ subunits; thus, the adult-type receptors are composed of α1, β1, ε and δ subunits. As a result of this γ/ε substitution, endplate nAChR exhibit reduced channel open times, increased ion conductance and higher Ca^{2+} permeability (Jin et al., 2008). The interaction between nAChR and sarcolemma proteins is important for the maintenance of nAChR clustering (Martyn et al., 2009, Souza et al., 2011). To achieve efficient neuromuscular transmission, nAChR must be densely clustered on the muscle membrane of the NMJs; thus, a lack of nAChR clustering is associated with disorders of neuromuscular transmission (Witzemann et al., 1991).

Dispersion or disruption of nAChR clusters at NMJs resulting from acute peripheral nerve injury (Charbonnier et al., 2003) and alterations in nAChR expression have been demonstrated in both young and old rats (Willmann and Fuhrer, 2002) as well as in monocrotaline-induced HF (Souza et al., 2011). Because peripheral synapses are large and readily experimentally accessible, these synapses have greatly contributed to the general understanding of the development of potential therapeutic strategies for muscular disorders (Higuchi and Yamanashi, 2011).

The diaphragm muscle fibre is an important inspiratory muscle that remains active throughout life and sustains a high-duty cycle relative to other skeletal muscles (~40% compared with 2-15% for limb muscles) (Kang et al., 2011).

During HF, the effort exerted by the skeletal muscle tends to increase both at rest and during exercise (Apel et al., 2009), and a shift from MyHC II to MyHC I may contribute to laboured respiration in this syndrome (Hernsbergen and Kernell, 1993; Sieck et al., 1989). Lopes et al. (2008) demonstrated that MyoD is selectively downregulated during HF; this alteration in MyoD expression may be associated with alterations in the MyHC content.

There is a growing consensus that exercise training is beneficial for patients with cardiovascular diseases, including those with severe alterations in cardiac function (Sieck et al., 1995; Prakash and Sieck, 1998; Giannuzzi et al., 2003). In patients with aortic stenosis, recommendations for physical activity are based on clinical examinations with special emphasis on haemodynamic data (Smart, 2011; Erbs et al., 2010; Linke et al., 2005). Aerobic training improves the exercise capacity of HF patients and can partially reverse abnormalities of the skeletal muscle (Hambrecht et al., 2000; Magnusson et al., 1996) by improving its oxidative capacity (Williams et al., 2007; Pu et al., 2012, Selig et al., 2004) and facilitating the redistribution of blood and oxygen to the skeletal muscle (Ding et al., 1999).

In this study, we evaluated the hypothesis that aerobic training attenuates alterations in the expression of nAChR subunits in NMJs diaphragm during HF. The aim of this study was to evaluate the distribution and expression of nAChR subunits in diaphragm muscle fibres of rats subjected to an aerobic training program during HF.

Materials and methods

Experimental animals and study protocol

All experiments and procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996; http://www.nap.edu/openbook.%20php?record_id=5140) and were approved by the Animal Ethics Committee (Sao Paulo State University, UNESP, CEEA - Protocol 210).

Three-to four-week-old male Wistar weaning rats weighing 90-100 g were anaesthetised with a mixture of ketamine (50 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Aortic stenosis was induced by placing a 0.6-mm i.d. stainless steel clip on the ascending aorta via a thoracic incision, as previously described (Ding et al., 1999). Control animals underwent a left thoracotomy without clip placement. Rats were housed under pathogen-free conditions at 23±1°C. Rats were exposed to reverse light conditions (12 h of light and 12 h of darkness) each day with food and water provided ad libitum. Initially, the animals were distributed into two groups: Sham (n=22) and aortic stenosis (n=22). Eighteen weeks after surgery, the AS animals were evaluated by echocardiography and exhibited left ventricular dysfunction (De Paiva et al., 2003). At this point, the animals were redistributed into four groups: control (Sham, n=11), control training (ShamTR, n=11), aortic stenosis (AS, n=11) and aortic stenosis training (ASTR, n=11). The ShamTR and ASTR groups were subjected to exercise for 10 weeks. After this period, echocardiography was performed on the animals in all four groups. Rats were anaesthetised with sodium pentobarbital (50 mg/kg, i.p.), weighed, and decapitated. Five-millimetre-wide samples of the mid-costal region of the left diaphragm muscle fibres were sectioned parallel to the muscle fibre for confocal microscopy analysis. Additional diaphragm muscle fibre samples were immediately frozen in liquid nitrogen and stored at -80°C for gene and protein expression analysis.

Physical training protocol

A previously described training protocol (De Sousa et al., 2002; Siu et al., 2004) was used with some

modifications. The animals in the ASTR and ShamTR groups were subjected to a treadmill-training program five times a week for ten weeks at the same time of day, between 2 and 4 p.m. The duration and intensity (velocity) of the exercise training protocol are summarised in Table 1. The running speed corresponded to the lactate threshold, which was determined by incremental exercise tests performed at the end of weeks 1, 4, and 7 to adjust the training velocities. The lactate threshold was defined as the running velocity that could be maintained without a lactate increase of 1.0 mmol/L above the blood lactate concentration obtained at the previous speed (Ferreira et al., 2007). The incremental exercise test began with warming up the rats for 5 min at a speed of 5 m/min. After 5 min of passive recovery, the animals were subjected to progressive increases in effort with an initial velocity of 6 m/min and increments of 3 m/min every 3 min. The protocol was terminated when the animals reached exhaustion, which was determined by the animal refusing to run even with sound stimulation.

Echocardiography evaluation

Echocardiography was performed 18 and 28 weeks following induction of aortic stenosis. At 28 weeks, echocardiography was performed one day after the end of training. Rats were anaesthetised with a mixture of ketamine (50 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.), their chests were shaved, and they were positioned on their left side. Using an echocardiograph (General Electric Medical Systems, Vivid S6, Tirat Carmel, Israel) equipped with a 5-11.5 MHz multifrequency transducer, two-dimension guided M-mode images were obtained. M-mode tracings were obtained from long-axis views of the left ventricle (LV) at or immediately below the tip of the mitral valve leaflets and at the level of the aortic valve and left atrium (Cicogna et al., 2002; Litwin et al., 1995). M-mode images of the LV were recorded on black-and-white thermal printer paper (UP-890MD; Sony, Tokyo, Japan) at a sweep speed of 100 mm/s. All LV tracings were manually measured by the same observer, who was blinded to the treatment group, according to the leading-edge method of the American Society of Echocardiography (Sahn et al., 1978). The measurements represent the means of at least five cardiac cycles on the M-mode tracings. The following variables were analysed to evaluate cardiac remodelling with systolic and diastolic ventricular dysfunction: LV diastolic dimension (LVDD), LV relative thickness during diastole (LVRWT), the left atrium/aorta (LA/Ao) ratio, LV endocardial fractional shortening (LVEFS), LV posterior wall shortening velocity (LVPWSV) and the E wave transmitral flow (E/A) ratio.

Table 1. Training protocol with the blood lactate threshold during incremental exercise tests.

Weeks	Velocity at lactate threshold S/AS group	Duration (min)
1	5/5	5
2	9/6	10
3	9/6	12
4	9/6	14
5	18/12	16
6	18/12	18
7	18/12	20
8	21/15	20
9	21/15	22
10	21/15	22

S: Sham; AS: aortic stenosis; velocity determined by m/min

Clinical and pathological findings of heart failure

The presence of HF in the AS group was confirmed upon sacrifice of the rats 28 weeks after surgery using clinical (tachypnea and laboured respiration) and pathological (ascites, pleural effusion, left atrium thrombi, and right ventricular hypertrophy) (Cicogna et al., 1999; Boluyt et al., 2005; Bregagnollo et al., 2005) signs, as summarised in Table 2. The lung and liver weights are summarised in Table 5.

Laser-scanning confocal microscopy analysis

The animals from each group (n=3) were anaesthetised with an intraperitoneal injection and were intracardially perfused with PBS followed by freshly

Table 2. Clinical and pathological signs in AS and ASTR groups 28 weeks after surgery.

Clinical Signs	Groups	Intensity	Relative Frequency (%)
Tachypnea	AS	↑	33
	AS	↑↑	67
	AS	↑↑↑	-
	ASTR	↑	33
	ASTR	↑↑	-
	ASTR	↑↑↑	-
Ascites	AS	↑	22
	AS	↑↑	78
	AS	↑↑↑	-
	ASTR	↑	55
	ASTR	↑↑	-
	ASTR	↑↑↑	-
Pleural effusion	AS	↑	56
	AS	↑↑	22
	AS	↑↑↑	22
	ASTR	↑	55
	ASTR	↑↑	-
	ASTR	↑↑↑	-
Left atrium thrombi	AS		56
	ASTR		11

Intensity of signs: ↑=slight; ↑↑=moderate; ↑↑↑=Intensity; - not present

prepared cold fixative (2% formaldehyde in PBS). The muscle segments of the diaphragm muscle fibres were reduced at the insertion of the phrenic nerve and incubated with 0.1 M glycine for 30 min. The samples were then washed and incubated with 1% collagenase (Type I, Sigma, C0130) for 30 min. Following incubation, the samples were washed and incubated with 1 μg/ml rhodamine-α-bungarotoxin (Rh-BTX; Molecular Probes) for 40 min at room temperature to distribute the nAChR in the muscle. The muscle segments were then incubated with 1% Triton X-100 (Sigma, T9284) for 1 hour, followed by incubation in blocking solution (125 µl of Triton X-100 and 1 ml of bovine serum albumin diluted in 23.8 ml of PBS) overnight for 18 h. The next day, in the morning, the muscles were incubated with the primary anti-neurofilament antibody (anti-neurofilament 200, Sigma, N5389, 1 µl: 1 ml in blocking solution) at 4°C for 12 h. On the following day, the muscles were incubated with secondary anti-mouse IgG-FITC antibody (Sigma, F0257, 1 µl: 1 ml in blocking solution) for 3 h at 4°C to stain the nerve terminal. Subsequently, each segment was imaged using a laser-scanning confocal system (TCS SPE; Leica, Mannheim, Germany). Images were acquired using a 40× objective (1.15 numeric aperture; ACS APO, Leica) at a constant pinhole setting to preserve the thickness of the confocal plane. For dual colour imaging, the Rh-BTX-conjugated secondary antibody and green fluorescence were sequentially excited using 488-nm and 532-nm diode lasers. The laser intensity and gain settings were adjusted on a per-image basis to compensate intensity losses due to the thickness of the sample and the excitation channels of the krypton/argon laser. For each acquired image, z-sectioning was performed to view more than one junction. The final image was obtained by combining the images acquired every 1 µm along the z-axis into a single projected view. Approximately 95 NMJs per animal were examined using confocal microscopy. All digital images were measured by adjusting the set scale with the scale bar using Image J software for Windows, version 1.43u, National Institutes of Health, USA. Comparisons of the nAChR junctional area were performed using the frequency (%) distributed in each of the following classes: class I: <19 μm^2; class II: 20-30 μm^2; class III: 31-40 μm; class IV: 41-50 μm^2; and class V: >51 μm^2.

Real-time PCR assessment of the gene expression of nAChR subunits in diaphragm muscle fibres

Following anaesthesia, the diaphragm muscle fibres of eight animals per group were immediately frozen in liquid nitrogen and processed to assess gene expression. Total RNA was extracted from the diaphragm muscle fibres using TRIzol® Reagent (Life Technologies, Carlsbad, CA, USA). The frozen muscles were mechanically homogenised on ice in 1 ml of ice-cold TRIzol® Reagent. Total RNA was solubilised in nuclease-free H_2O, incubated with DNase I (Life Technologies, Carlsbad, CA, USA) to remove DNA present in the sample and quantified by measuring the

Table 3. Oligonucleotide primers used for Real-time PCR amplification of reverse transcribed RNA.

Gene	Acession no.	Seqüência of primer (5'-3')	PCR lenght (pb)
Alpha 1	NM_024485	F:TCCCTTCGATGAGCAGAACT R:AGCCGTCATAGGTCCAAGTG	56
Beta 1	NM_012528. 1	F:CATCGAGTCTCTCCGTGTCA R:GGCAACGTCAAAGTTTCCAT	88
Delta	NM_019298. 1	F:ACCACCAAGACGTCACCTTC R:CCGAGGTCTTCTCTCCACAG	144
Epsilon	NM_017194	F:GGCTCAACTTCAGCAAGGAC R:AGCCATACATGTTCGGAAGG	70
Gamma	NM_019145	F:CCACCAGAAGGTGGTGTTCT R:TGAGGAGATGAGCACACAGG	91
ACTB	NM_031144	F:TCAGGTCATCACTATCGGCAATG R:TTTCATGGATGCCACAGGATTC	61
Cyclophilin A	NM_017101.1	F:TATCTGCACTGCCAAGACTGAGTG R:CTTCTTGCTGGTCTTGCCATTCC	127
GAPDH	NM_017008.3	F:CAACTCCCTCAAGATTGTCAGCAA R:GGCATGGACTGTGGTCATGA	118
HPRT	NM_012583	F:CTCATGGACTGATTATGGACAGGAC R:GCAGGTCAGCAAAGAACTTATAGCC	123
TBP	NM_001004198.1	F:GCCACGAACAACTGCGTTGAT R:AGCCCAGCTTCTGCACAACTCTA	132

Accession no., GenBank accession number, bp, base pairs

optical density (OD) at 260 nm. Total RNA was determined by measuring the sample absorbance at 260 nm using a NanoVue spectrophotometer (GE Healthcare, USA), and RNA purity was assessed by determination of the A_{260}/A_{280} and A_{260}/A_{230} ratios (acceptable when both ratios were >1.8). RNA integrity was confirmed by obtaining an RNA integrity number (RIN)>8 using an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). For each sample, cDNA was synthesised from 2 μg of total RNA using components from the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The reaction contained 10 μl of 10× Reverse Transcription Buffer, 4 μl of 25× dNTPs, 10 μl of 10× random primers, 100 units of RNase inhibitor (Life Technologies, Carlsbad, CA, USA) and 250 units of MultiScribe™ Reverse Transcriptase. The final volume was adjusted to 100 μl with nuclease-free H_2O. The primers were allowed to anneal for 10 min at 25°C before the reaction proceeded for 2 h at 37°C. Control ''No RT'' reactions were performed by omitting the RT enzyme. These reactions were PCR amplified to ensure the absence of DNA contamination. The resultant cDNA samples were aliquoted and stored at -20°C. Two microlitres of cDNA from the RT reaction, corresponding to 40 ng of total RNA, was used as the template in the subsequent real-time PCR analysis. Real-time PCR was performed in a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using the following conditions: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min.

The reactions were performed in duplicate in a final volume of 25 μl containing each primer at a final concentration of 0.4 μM and 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Gene sequences were selected from the accession numbers in the National Center for Biotechnology Information Database and are summarised in Table 3. The primer sequences were designed using the primer design function of the software Primer Express® v3.0 (Applied Biosystems, Foster City, CA, USA). Melting dissociation curves were plotted to confirm that only a single product was amplified. Control reactions lacking the cDNA template were performed to check for reagent contamination. Gene expression was compared between individual samples using the ΔΔCq method, as previously described (Livak and Schmittgen, 2001) The expression levels of the following genes were assessed using the DataAssist™ software (Life Technologies, Carlsbad, CA, USA) and used as reference genes for normalisation of the data (Vandesompele et al., 2002): β-actin protein (ACTB), hypoxanthine-guanine phosphoribosyltransferase (HPRT), TATA box-binding protein (TBP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin A (CypA).

Statistical analysis

The echocardiographic data obtained from the control and aortic stenosis groups 18 weeks after surgery were analysed using Student's t-test for independent samples (the data are expressed as the means ± standard deviation). To analyse the effect of exercise on the cardiac parameters before and after aerobic training, the echocardiographic and anatomical parameters obtained from the four groups 28 weeks after surgery were analysed using two-way ANOVA, followed by the Student-Newman-Keuls test.

The nAChR area expressed as a frequency distribution (%) was evaluated using the Goodman test (Goodman 1964, 1965)

The mRNA expression of subunits of nAChR processed by Real-time PCR are expressed as the

Table 4. Echocardiographical evaluation of the control (Sham, n=11) and aortic stenosis (AS, n=11) groups 18 weeks after surgery.

Parameters	Groups	
	Sham	AS
Heart Rate (bpm)	293±34	284±34
LVDD (mm)	8.01±0.46	8.23±0.75
LVRWT (mm)	1.44±0.08	2.06±0.21*
LA/Ao ratio	1.47±0.16	1.91±0.27*
LVEFS (%)	55.29±5.89	54.48±9.58
LVPWSV (mm/s)	39.41±4.47	27.34±5.63*
E/A ratio	1.46±0.28	5.06±2.61*

LV, left ventricular; LVDD, LV diastolic dimension; LVRWT, LV relative thickness in diastole; LA/Ao ratio, left atrium/aorta; LVEFS, LV endocardial fractional shortening; LVPWSV, LV posterior wall shortening velocity; E/A ratio, E wave mitral flow; A wave mitral flow; Values represent mean ± SD; *, P<0.05 compared to Sham. Student's t-test.

Table 5. Echocardiographical evaluation of the control (Sham, n=11), control training (ShamTR, n=11), aortic stenosis (AS, n=11) and aortic stenosis training (ASTR, n=11) groups 28 weeks after surgery.

Parameters	Groups			
	Sham	ShamTR	AS	ASTR
Body Weight (g)	489.4±29.3	475.3±47.9	464.3±35.6	440±45.2
LVDD (mm)	8.40±0.65	8.42±0.38	9.14±0.32*	8.19±0.77#
LVRWT (mm)	1.46±0.05	1.43±0.07	2.27±0.20*	2.15±0.25†
LA/Ao ratio	1.44±0.09	1.56±0.18	2.21±0.30*	2.08±0.24†
LVEFS (%)	55.3±4.40	54.4±4.02	44.8±7.20*	58.8±10.72#
LVPWSV (mm/s)	37.72±4.81	37.17±3.51	23.39±3.72*	29.18±5.17†#
E/A ratio	1.33±0.31	1.36±0.23	7.93±1.75*	4.24±2.70†#
Lung (g)	1.92±0.44	4,02±0,04	3.25±0.62*	2.52±0.59
Liver (wet/dry) (g)	3,19±0,02	3,15±0,07	3,37±0,11	3,26±0,04

LV, left ventricular; LVDD, LV diastolic dimension; LVRWT, LV relative thickness in diastole; LA/Ao ratio, left atrium/aorta; LVEFS, LV endocardial fractional shortening; LVPWSV, LV posterior wall shortening velocity; E/A ratio, E wave mitral flow; A wave mitral flow; Values represent mean±SD; *, P<0.05 compared to Sham; †, P<0.05 compared to ShamTR; #, P<0.05 compared to AS. Two-way ANOVA followed by the Student-Newman-Keuls test.

relative expression (RQ) observed using the DataAssist™ software and are expressed as the means ± standard deviation (SD).The comparisons among groups were performed using one-way analysis of variance (ANOVA), followed by a posteriori Tukey's multiple comparison test.

Statistical calculations were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant at a level of $P<0.05$.

Results

Echocardiography evaluation

Eighteen weeks following the induction of aortic stenosis, the AS group exhibited increases in the echocardiographic parameters LVRWT, the LA/Ao ratio, LVPWSV and the E/A ratio (Table 5). Twenty-eight weeks after the surgery, the AS group exhibited changes in cardiac structure and function in the LVDD, LVRWT,

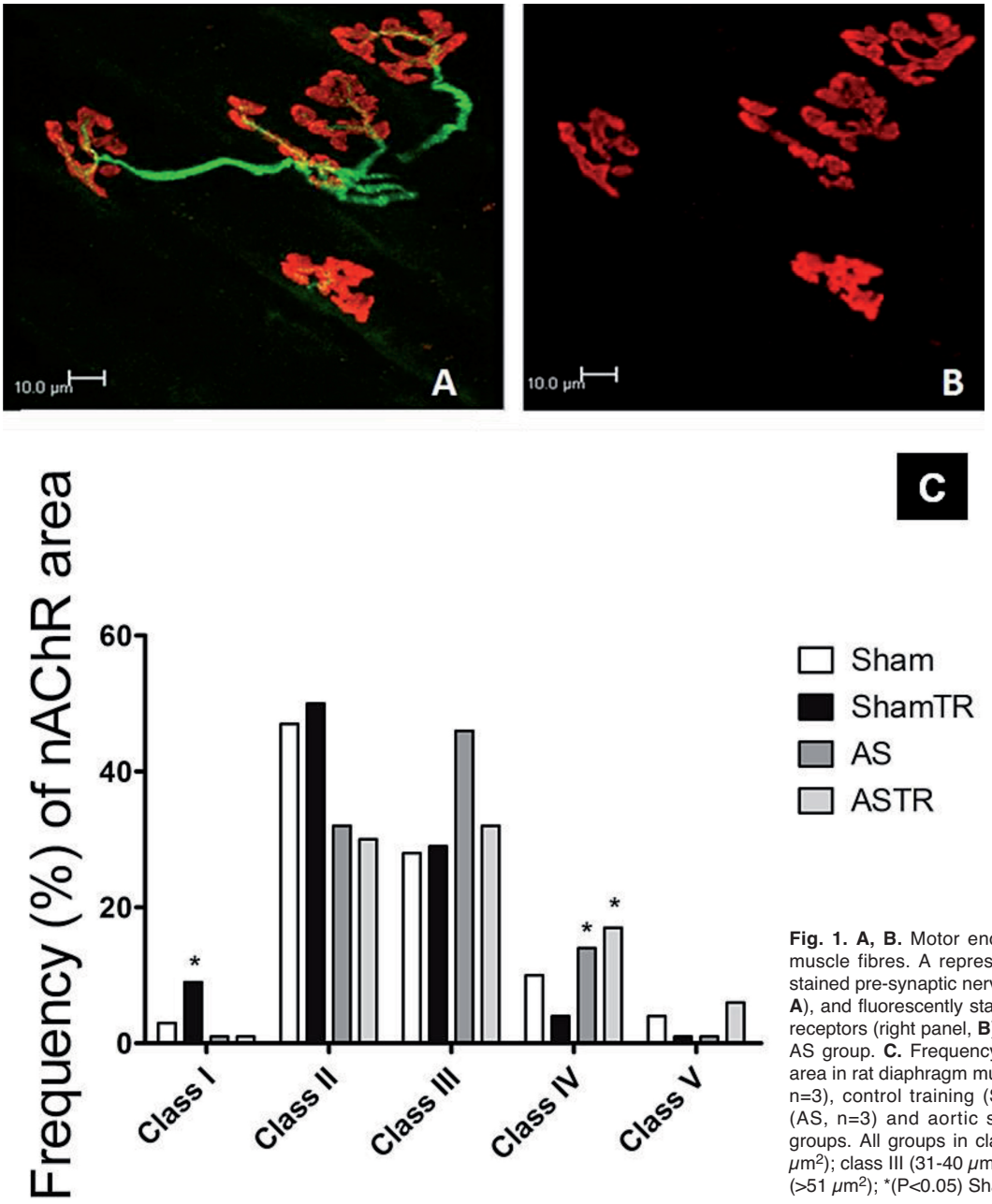

Fig. 1. A, B. Motor endplates showing rat diaphragm muscle fibres. A representative image of fluorescently stained pre-synaptic nerve terminal branching (left panel, **A**), and fluorescently stained post-synaptic acetylcholine receptors (right panel, **B**) of the same NMJs linked at the AS group. **C.** Frequency distribution (%) of the nAChR area in rat diaphragm muscle fibres in the control (Sham, n=3), control training (ShamTR, n=3), aortic stenosis (AS, n=3) and aortic stenosis training (ASTR, n=3) groups. All groups in class I (<19 μm^2); class II (20-30 μm^2); class III (31-40 μm^2); class IV (41-50 μm^2); class V (>51 μm^2); *($P<0.05$) Sham versus AS group.

LVEFS and the E/A ratio and promoted of cardiac remodelling with systolic and diastolic ventricular dysfunction. When comparing the ASTR and AS groups, we observed that aerobic training improved the cardiac structure and function as indicated by differences between the LVDD, LVEFS, LVPWSV, the E/A ratio, and the lung and liver weights of the two groups (Table 6).

Confocal microscopy analysis

In all evaluated groups, the terminal axon was labelled with an anti-neurofilament antibody (green), and the nAChR were labelled with rhodamine-conjugated α-bungarotoxin (red). Two-dimensional images of labelled motor endplates (red) exhibited distribution in round or oval clusters forming continuous branches that were elongated along the muscle fibres in the AS group (Fig. 1A,B). Regarding the nAChR area, we observed differences in class I (<19 μm^2) and in class IV (41-50 μm^2). The ShamTR group exhibited a lower area represented by class I nAChR compared with the Sham group. The AS and ASTR groups exhibited an increased class IV nAChR area compared with the Sham group.

Real-time PCR assessment of the gene expression of nAChR subunits in diaphragm muscle fibres

All evaluated groups exhibited similar patterns of mRNA expression of the γ, α1 and β1 nAChR subunits: the AS group exhibited higher mRNA expression of all nAChR subunits compared with the Sham group, and the ASTR group exhibited a decrease in the mRNA expression of γ, α1, and β1 nAChR subunits compared with the AS group. However, no significant change was found in the mRNA expression of the nAChRδ subunit. We observed a continuous increase in the mRNA expression of the nAChRε subunit in the AS and ASTR groups compared with the Sham group (Fig. 2).

Discussion

This study demonstrates for the first time that aerobic training attenuated the alterations in the gene expression of nAChR subunits and in the structure of diaphragm NMJs during heart failure induced by aortic stenosis. Based on morphological analysis, our study demonstrates that nAChR in NMJs of rat diaphragm muscle were distributed in a pattern referred to as "continuous branches" or "pretzels" in all of the evaluated groups. The distribution of the nerve terminal was normal and followed the arrangement of the receptors, with their branches covering the nerve terminal in a continuous extension. We observed an increase in the nAChR area in the AS and ASTR groups compared with the Sham group. Aerobic training did not modify the nAChR morphology or the nAChR area in the ASTR group.

Alterations in the NMJs can occur under several

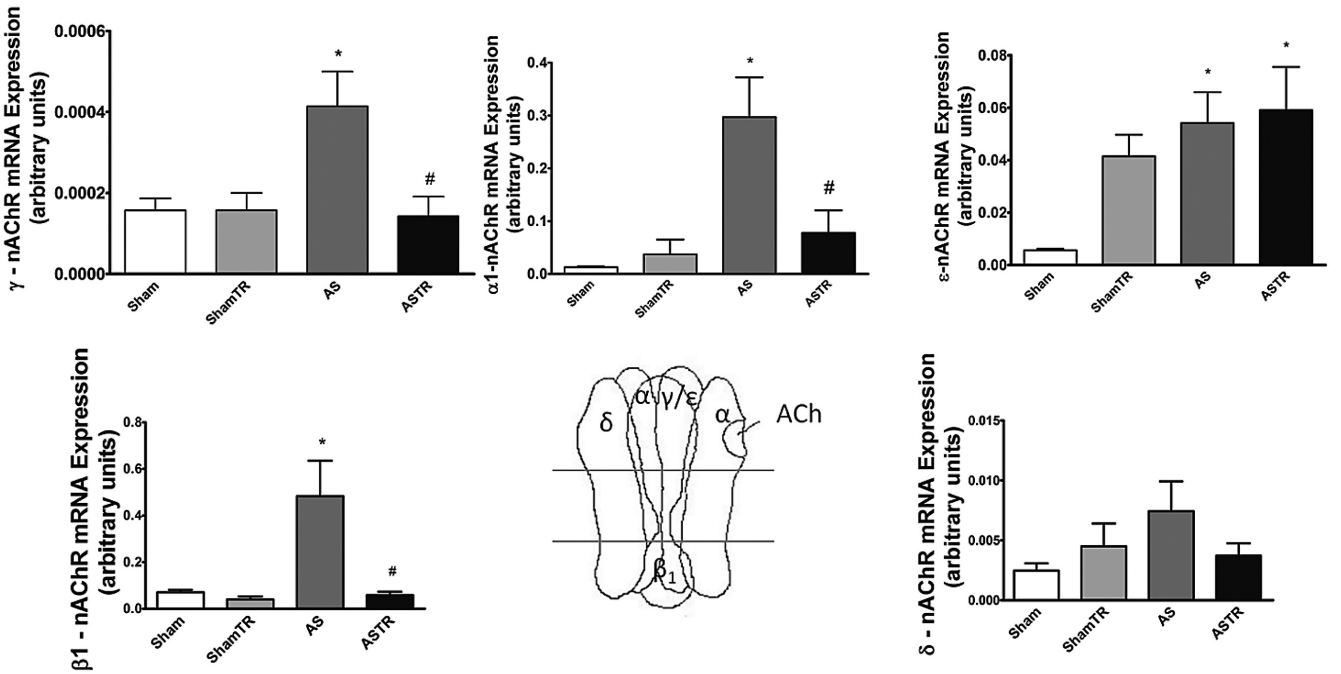

Fig. 2. mRNA expression of nAChR subunits in diaphragm muscle fibres of the control (Sham), control training (ShamTR), aortic stenosis (AS) and aortic stenosis training (ASTR) groups. Values are expressed as the means ± SD. n=7 rats per group. * Significant difference compared with the respective Sham at P<0.05 (ANOVA + Tukey's test); # Significant difference, AS compared with the respective ASTR at P<0.05 (ANOVA + Tukey's test).

conditions: (1) during aging, when a significant expansion in the area of the NMJs on type IIx and IIb fibres in rat diaphragm muscle fibres occurs (Prakash and Sieck, 1998; Wang et al., 2004); (2) following muscle denervation with an increased expression of embryonic-type nAChRγ in the rat flexor digitorum brevis muscle (Adams et al., 1995); and (3) during metabolic alterations in the muscle of diabetic mice, in which the nAChR exhibited a branching distribution pattern into patches or islands in the sternocleidomastoid muscle (Marques and Santo Neto, 2002). In this study, the enlargement of the surface area of the nAChR clusters in the AS and ASTR groups may be related to the increased ability to effectively sustain the neuromuscular transmission that is required to generate diaphragm muscle contractions during HF (Ermilov et al., 2007). Aerobic training promotes morphological and physiological adaptations of the NMJs (Deschenes et al., 2011), improving the ability to release neurotransmitters and endurance exercise performance (Andonian and Fahim, 1987; Dorlochter et al., 1991). However, in this study, the low training intensity used may have been insufficient to alter the nAChR morphology and the nAChR area in the ASTR group, although the ShamTR group exhibited an increase in the nAChR class I area compared with the Sham group.

In the diaphragm muscle, the myopathy that occurs during HF is partially due to the shift in muscle phenotype to a more oxidative and slow pattern (Lipkin et al., 1988; Sullivan et al., 1990; Mancini et al., 1992). Slow and oxidative muscle fibres are more resistant to fatigue and can maintain their contractile performance for a longer time (Polla et al., 2004). Accordingly, the proportion of type I and IIa fibres in the diaphragm muscle is also scaled to match the fractional motor unit recruitment necessary to accomplish sustained ventilator behaviours (Mantilla and Sieck, 2011). Therefore, we can infer that the modulation of diaphragm NMJs in the AS group in this study may be in response to the increased heart failure diaphragm muscle work (Tikunov et al., 1997), as demonstrated by the clinical sign of tachypnea.

In this study, aortic stenosis-induced HF promoted an increase in the gene expression of nAChR subunits (γ (foetal), α1, ε (adult), β1 and δ) in the AS group compared with the Sham group. Aerobic training resulted in a slight to moderate decrease in the gene expression of the nAChR subunits in the ASTR group compared with the AS group. The AS group exhibited higher mRNA expression of the γ subunit. This subunit is expressed during the foetal period and is substituted by the adult ε subunit throughout organism development (Koenen et al., 2005). The subunit transition from the γ-type (foetal) to the ε-type (adult) nAChR during development occurs synchronously at all endplates, suggesting that neural activity results in the suppression of the γ subunit gene through transcriptional activation of the ε subunit gene (Missias et al., 1996; Yumoto et al., 2005). Re-expression of the nAChRγ also occurs in many chronic neurogenic and some myogenic disorders, primarily in type I muscle fibres (Gattenlohner et al., 2002). According to Maggs et al. (2008), following denervation, slow MyHC is expressed in addition to other MyHC isoforms, which is consistent with the prevailing view that denervated muscles adopt embryonic characteristics.

Therefore, we can infer that the increased foetal-type nAChR subunit expression in the diaphragm muscle during HF can be, in part, related to the shift in muscle phenotype to a more oxidative and slow pattern that occurs during this syndrome, a fact that is related to the laboured respiration during HF (Tikunov et al., 1996; De Sousa et al., 2001; Lopes et al., 2008). Aerobic training was effective in attenuating the expression of the nAChRγ subunit. A similar behaviour was observed for the structural β1 and δ subunits, although the change in δ levels was not significant.

The α1 subunit of the nAChR contains the binding site for the ACh neurotransmitter. Ach binding to the αγ and αδ sites induces conformational changes, predominantly in the α1 subunit, and promotes channel opening and muscle contraction (Unwin et al., 2002; Kalamida et al., 2007; Souza et al., 2011). In this sense, an increase in the mRNA expression of the α1 subunit, as observed in the AS group, may result in an increase in the number nAChR, which may modulate NMJs properties and muscle contraction. The γ/ε, δ and α1 subunits are involved in shaping the ligand binding sites and in maintaining cooperative interactions between the α1 and other subunits (Sine and Claudio, 1991). The β1 subunit is important for nAChR clustering, as demonstrated by studies on hybrid muscle nAChR, in which the β1 subunit was substituted by its neuronal counterpart (Wheeler et al., 1994).

The decrease in the mRNA expression of γ, α1, ε and β1 subunits in the ASTR group may indicate that aerobic training can adjust the overall activity of the nAChR to changing physiological demand without significantly altering the morphology and size of endplates in both the AS and ASTR groups.

We observed a continuous increase in the mRNA expression of the nAChRε subunit in the ShamTR, AS and ASTR groups compared with the Sham group. This subunit is essential for normal synaptic development and maintenance of NMJs structure and function (Kalamida et al., 2007). Thus, the potential increase in the mRNA expression of the nAChRε subunit in the ShamTR, AS and ASTR groups may aid in stabilising neurotransmission in the diaphragm muscle during heart failure and under conditions of increased physical exercise.

We conclude that aortic stenosis-induced HF altered the mRNA expression of nAChR subunits and the structural characteristics of diaphragm NMJs. The increase in the mRNA expression of the γ, α1, ε and β1 nAChR subunits during HF may account for the enlargement of the NMJs area and may lead to morphofunctional changes in the diaphragm during this

syndrome. In addition, aerobic training did not alter NMJs morphology but attenuated the alterations in heart structure and function and in mRNA expression of nAChR subunits. Our findings demonstrate the beneficial effects of aerobic exercise training to maintain the integrity of the neuromuscular system in the diaphragm muscle during HF and may be critical for non-pharmacological therapy to improve the quality of life for patients with this syndrome.

Acknowledgements. This study was supported by CNPq, Process number 133932/2010-9, and by FAPESP, Process number 2009/12760-4. This work is part of the M.Sc. Thesis presented by Paula Aiello Tomé de Souza to São Paulo State University, UNESP, Bioscience Institute in 2012.

References

Adams L., Carlson B.M., Henderson L. and Godman P. (1995). Adaptation of nicotinic acetylcholine receptor, myogenin, and MRF4 gene expression to long-term muscle denervation. J. Cell Biol. 131, 1341-1349.

Andonian M.H. and Fahim M.A. (1987). Effects of endurance exercise on the morphology of mouse neuromuscular junctions during ageing. J. Neurocytol. 16, 589-599.

Apel P.J., Alton T., Northam C., Ma J., Callahan M., Sonntag W.E. and Li Z. (2009). How age impairs the response of the neuromuscular junction to nerve transection and repair: an experimental study in rats. J. Orthop. Res. 27, 385-393.

Boluyt M.O., Robinson K.G., Meredith A.L., Sen S., Lakatta E.G., Crow M.T., Brooks W.W., Conrad C.H. and Bing O.H. (2005). Heart failure after long-term supra valvular aortic constriction in rats. Am. J. Hypertension 18, 202-212.

Bregagnollo E.A., Okoshi K., Bregagnollo I.F., Padovani C.R., Okoshi M.P. and Cicogna A.C. (2005). Effects of the prolonged inhibition of the angiotensin-converting enzyme on the morphological and functional characteristics of left ventricular hypertrophy in rats with persistent pressure overload. Arq. Bras. Cardiol. 84, 225-232.

Brenner H.R and Sakmann B. (1978). Gating properties of acetylcholine receptor in newly formed neuromuscular synapses. Nature 271, 366-368.

Carvalho R.F., Cicogna A.C., Campos G.E.R., De Assis J.M.F., Padovani C.R., Okoshi M.P. and Pai-Silva M.D. (2003). Myosin heavy chain expression and atrophy in rat skeletal muscle during transition from cardiac hypertrophy to heart failure. Int. J. Exp. Pathol. 84, 201-206.

Charbonnier F., Della G.B., Armand A.S., Lécolle S., Launay T., Gallien C.L. and Chanoine C. (2003). Specific activation of the acetylcholine receptor subunit genes by MyoD family proteins. J. Biol. Chem. 278, 33169-33174.

Cicogna A.C., Robinson K.G., Conrad C.H., Singh K., Squire R., Okoshi M.P. and Bing O.H. (1999). Direct effects of colchicine on myocardial function studies in hypertrophied and failing spontaneously hypertensive rats. Hypertension 30, 60-65.

Cicogna A.C., Matsubara B.B., Matsubara L.S., Okoshi K., Gut A.L., Padovani C.R., Meyer M.M. and Okoshi M.P. (2002). Volume Overload influence on hypertrophied myocardium function. Jpn. Heart J. 43, 689-695.

De Paiva S.A., Zornoff L.A., Okoshi M.P., Okoshi K., Matsubara L.S., Matsubara B.B., Cicogna A.C. and Campana A.O. (2003). Ventricular remodeling induced by retinoic acid supplementation in adult rats. Am. J. Physiol. Heart Circ. Physiol. 284, H2242-2246.

De Sousa E., Veksler V., Bigard X., Mateo P., Serrurier B. and Ventura-Clapier R. (2001). Dual influence of disease and increased load on diaphragm muscle in heart failure. J. Mol. Cell. Cardiol. 33, 699-710.

De Sousa E., Lechêne P., Fortin D., N'Guessan B., Belmadani S., Bigard X., Veksler V. and Ventura-Clapier R. (2002). Cardiac and skeletal muscle energy metabolism in heart failure: beneficial effects of voluntary activity. Cardiovasc. Res. 56, 260-268.

Deschenes M.R., Judelson D.A., Kraemer W.J., Meskaitis V.J.,Volek J.S., Nindl B.C., Harman F.S. and Deaver D.R. (2000). Effects of resistance training on neuromuscular junction morphology. Muscle Nerve 10, 1576-1581.

Deschenes M.R., Roby M.A., Glass E.K. (2011). Aging influences adaptations of the neuromuscular junction to endurance training. Neuroscience 190, 56-66.

Ding B., Price R.L., Borg T.K., Weinberg E.O., Halloran P.F. and Lorell B.H. (1999). Pressure overload induces severe hypertrophy in mice treated with cyclosporine an inhibitor of calcineurin. Circ. Res. 84, 729-734.

Dorlochter M., Irintchev A., Brinkers M. and Wernig A. (1991). Effects of enhanced activity on synaptic transmission in mouse extensor digitorumlongus muscle. J. Physiol..436, 283-292.

Erbs S., Höllriegel R., Linke A., Beck E.B., Adams V., Gielen S., Möbius-Winkler S., Sandri M., Kränkel N., Hambrecht R. and Schuler G. (2010). Exercise training in patients with advanced chronic heart failure (NYHA IIIb) promotes restoration of peripheral vasomotor function, induction of endogenous regeneration, and improvement of left ventricular function. Circ. Heart Fail. 3, 486-494.

Ermilov L.G., Mantilla C.B., Rowley K.L. and Sieck G.C. (2007). Safety factor for neuromuscular transmission at type-identified diaphragm fibers. Muscle Nerve 35, 800-803.

Ferreira J.C.B., Rolim N.P.L., Bartholomeu J.B., Gobatto C.A., Kokubun E. and Brum P.C. (2007). Maximal lactate steady state in running mice: effect of exercise training. Clin. Exp. Pharmacol. Physiol. 34, 760-765.

Gattenlohner S., Schneider C., Thamer C., Klein R., Roggendorf W., Gohlke F., Niethammer C., Czub S., Vincent A. and Muller-Hermelink H.K. (2002) Expression of fetal type acetylcholine receptor to type I muscle fibres in human neuromuscular disorders. Brain 125, 1309-1319.

Giannuzzi P., Temporelli P.L., Corrà U. and Tavazzi L. (2003). Antiremodeling effect of long-term exercise training in patients with stable chronic heart failure: results of the exercise in left ventricular dysfunction and chronic heart failure (ELVD-CHF) trial. Circulation 108, 554-559.

Goodman L.A. (1964). Simultaneous confidence intervals for contrasts among multinomial populations. Ann. Math. Stat. 35, 716-25.

Goodman L. (1965). Simultaneous confidence intervals for multinomial proportions. Technometrics 7, 247-54.

Hambrecht R., Gielen S., Linke A., Fiehn. E., Yu J., Walther C., Schoene N. and Schuler G. (2000). Effects of exercise training on left ventricular function and peripheral resistance in patients with chronic heart failure: A randomized trial. JAMA 283, 3095-3101.

Hernsbergen E. and Kernell D. (1993). Daily duration of activity in ankle muscles of cats. Glasgow. In: International Congress of Physiological Sciences. 18, 32.

Higuchi O. and Yamanashi Y. (2011). Molecular mechanisms underlying the formation of neuromuscular junction. Brain Nerve 63, 649-655.

Jin T.E., Wernig A. and Witzemann V. (2008). Changes in acetylcholine receptor function induce shifts in muscle fiber type composition. FEBS J. 275, 2042-2054.

Kalamida D., Poulas K., Avramopoulou V., Fostieri E., Lagoumintzis G., Lazaridis K., Sideri A., Zouridakis M. and Tzartos S.J. (2007).Muscle and neuronal nicotinic acetylcholine receptors. Structure, function and pathogenicity. FEBS J. 274, 3799-3845.

Kang J.R., Zamorano D.P. and Gupta R. (2011) Limb salvage with major nerve injury: current management and future directions. J. Am. Ac. Orthop. Surg. 19, S28-34.

Koenen, M., Peter, C., Villarroel, A., Witzemann, V., Sakmann, B. (2005). Acetylcholine receptor channel subtype directs the innervation pattern of skeletal muscle. EMBO Rep. 6, 570-576.

Linke A., Adams V., Schulze P.C., Erbs S., Gielen S., Fiehn E., Möbius-Winkler S., Schubert A., Schuler G. and Hambrecht R. (2005). Antioxidative effects of exercise training in patients with chronic heart failure: increase in radical scavenger enzyme activity in skeletal muscle. Circulation 111, 1763-1770.

Lipkin D.P., Jones D.A., Round J.M. and Poole-Wilson P.A. (1988). Abnormalities of skeletal muscle in patients with chronic heart failure. Int. J. Cardiol. 18, 187-195.

Litwin S.E., Katz S.E., Weinberg E.O., Lorell B.H., Aurigemma G.P. and Douglas P.S. (1995). Serial echocardiographic-doppler assessment of left ventricular geometry and function in rats with pressure-overload hypertrophy: chronic angiotensin-converting enzyme inhibition attenuates the transition to heart failure. Circulation 91, 2642-2654.

Livak K.J. and Schmittgen T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402-408.

Lopes F.S, Carvalho R.F., Campos G.E., Sugizaki M.M., Padovani C.R., Nogueira C.R., Cicogna A.C. and Pai-Silva M.D. (2008). Down-regulation of MyoD gene expression in rat diaphragm muscle with heart failure. Int. J. Exp. Path 89, 216-222.

Maggs A.M., Huxley C. and Hughes S.M. (2008). Nerve-dependent changes in skeletal muscle myosin heavy chain after experimental denervation, cross-reinnervation and in a demyelinating mouse model of Charcot-Marie-Tooth disease type 1A. Muscle Nerve 6, 1572-1584.

Magnusson G., Gordon A., Kaijser L., Sylvén C., Isberg B., Karpakka J. and Saltin B. (1996). High intensity knee extensor training, in patients with chronic heart failure: major skeletal muscle improvement. Eur. Heart J. 17, 1048-1055.

Mancini D.M., Walter G.,Reichek N., Lenkinski R., McCully K.K., Mullen J.L. and Wilson J.R. (1992). Contribution of skeletal muscle atrophy to exercise intolerance and altered muscle metabolism in heart failure. Circulation 85, 1364-1373.

Mantilla C.B, and Sieck G.C. (2011). Phrenic motor unit recruitment during ventilatory and non-ventilatory behaviors. Respir. Physiol. Neurobiol. 179, 57-63.

Marques M.J. and Santo Neto H. (2002). Acetylcholine receptors and nerve terminal distribution at the neuromuscular junction of non-obese diabetic mice. Anat. Rec. 267, 112-119.

Martyn J.A.J., Fagerlund M.J. and Eriksson L.I. (2009). Basic principles of neuromuscular transmission. Anaesthesia 64, 1-9.

Mishina M., Takai T., Imoto K., Noda M., Takahashi T., Numa S., Methfessel C. and Sakmann B. (1986). Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. Nature 321, 406-411.

Missias A.C., Chu G.C., Klocke B.J., Sanes J.R. and Merlie J.P. (1996). Maturation of the acetylcholine receptor in skeletal muscle: regulation of the AChR γ-to-ε switch. Dev. Biol. 179, 223-238.

Piña I.L., Apstein C.S., Balady G.J., Belardinelli R., Chaitman B.R., Duscha B.D., Fletcher B.J., Fleg J.L., Myers J.N. and Sullivan M.J. (2003). Exercise and heart failure: A statement from the American Heart Association Committee on exercise, rehabilitation, and prevention. Circulation 107, 1210-1225.

Polla B., D'Antona G., Bottinelli R. and Reggiani C. (2004). Respiratory muscle fibres: specialisation and plasticity. Thorax 59, 808-817.

Prakash Y.S. and Sieck G.C. (1998). Age-related remodeling of neuromuscular junctions on type-identified diaphragm fibers. Muscle Nerve 21, 887-895.

Pu C.T., Johnson M.T., Forman D.E., Hausdorff J.M., Roubenoff R., Foldvari M., Fielding R.A. and Singh M.A. (2012). Randomized trial of progressive resistance training to counteract the myopathy of chronic heart failure randomized trial of progressive resistance training to counteract the myopathy of chronic heart failure. J. Appl. Physiol. 90, 2341-2350.

Sahn D.J., De Maria A., Kisslo J. and Weyman A. (1978). Recommendations regarding quantitation in M-mode echocardiography: results of a survey of echocardiographic measurements. Circulation 58, 1072-1083.

Selig S.E., Carey M.F., Menzies D.G., Patterson J., Geerling R.H., Williams A.D., Bamroongsuk V., Toia D., Krum H. and Hare D.L. (2004). Moderate-intensity resistance exercise training in patients with chronic heart failure improves strength, endurance, heart rate variability, and forearm blood flow. J. Card. Fail. 10, 21-30.

Sieck G.C., Zhan W.Z., Prakash Y.S., Daood M.J. and Watchko J.F. (1995). SDH and actomyosin ATPase activities of different fiber types in rat diaphragm muscle. J. Appl. Physiol. 79, 1629-1639.

Sieck G.C., Fournier M. and Enad J.G. (1989). Fiber type composition of muscle units in the cat diaphragm. Neurosci. Lett. 97, 29-34.

Sine S.M. and Claudio T. (1991). Gamma- and delta-subunits regulate the affinity and the cooperativity of ligand binding to the acetylcholine receptor. J. Biol. Chem. 266, 19369-19377.

Simonini A., Massie B.M., Long C.S., Qi M. and Samarel A.M. (1996). Alterations in skeletal muscle gene expression in the rat with chronic congestive heart failure. J. Mol. Cell. Cardiol. 28, 1683-1691.

Siu P.M., Donley D.A., Bryner R.W. and Alway S.E. (2004). Myogenin and oxidative enzyme gene expression levels are elevated in rat soleus muscles after endurance training. J. Appl. Physiol. 97, 277-285.

Smart N. (2011). Exercise training for heart failure patients with and without systolic dysfunction: an evidence-based analysis of how patients benefit. Cardiol. Res. Pract. 1-7.

Souza P.A., Matheus S.M., Castan E.P., Campos D.H., Cicogna A.C., Carvalho R.F. and Dal-Pai-Silva M. (2011). Morphological aspects of neuromuscular junctions and gene expression of nicotinic acetylcholine receptors (nAChRs) in skeletal muscle of rats with heart failure. J. Mol. Hist. 42, 557-565.

Sullivan M.J., Green H.J. and Cobb F.R. (1990). Skeletal muscle biochemistry and histology in ambulatory patients with long-term heart failure. Circulation 81, 518-527.

Tikunov B.A., Mancini D., Levine S. (1996) Changes in myofibrillar protein composition of human diaphragm elicited by congestive heart failure. J. Mol. Cell. Cardiol. 28, 2537-2541.

Tikunov B.A., Mancini D. and Levine S. (1997). Chronic congestive heart failure elicits adaptations of endurance exercise in diaphragmatic muscle. Circulation 95, 910 - 916.

Unwin N., Miyazawa A., Li J. and Fujiyoshi Y. (2002). Activation of the nicotinic acetylcholine receptor involves a switch in conformation of α subunits. J. Mol. Biol. 319, 1165-1176.

Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A. and Speleman F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, 1-12.

Vescovo G., Ceconi C., Bernocchi P., Ferrari R., Carraro U., Ambrosio G.B. and Libera L.D. (1998). Skeletal muscle myosin heavy chain expression in rats with monocrotaline-induced cardiac hypertrophy and failure. Relation to blood flow and degree of muscle atrophy. Cardiovasc. Res. 39, 233-241.

Wang T.J., Larson M.G., Levy D., Benjamin E.J., Leip E.P., Omland T., Wolf P.A. and Vasan R.S. (2004). Plasma natriuretic peptide levels and the risk of cardiovascular events and death. N. Engl. J. Med. 350, 655-63.

Wheeler S.V., Jane S.D., Cross K.M., Chad J.E. and Foreman R.C. (1994). Membrane clustering and bungarotoxin binding by the nicotinic acetylcholine receptor: role of the beta subunit. J Neurochem. 63, 1891-1899.

Williams A.D., Carey M.F., Selig S., Hayes A., Krum H., Patterson J., Toia D. and Hare D.L. (2007). Circuit resistance training in chronic heart failure improves skeletal muscle mitochondrial ATP production rate a randomized controlled trial. J. Card. Fail.13, 79-85.

Willmann R. and Fuhrer C. (2002). Neuromuscular synaptogenesis: clustering of acetylcholine receptors revisited. Cell. Mol. Life. Sci. 59, 1296- 1316.

Witzemann V., Brenner H.R. and Sakmann B. (1991). Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. J. Cell Biol. 114, 125-141.

Wood S.J. and Slater C.R. (1997). The contribution of postsynaptic folds to the safety factor for neuromuscular transmission in rat fast- and slow-twitch muscles. J. Phys. 500, 165-176.

Wu H., Xiong W.C. and Mei L. (2010). To build a synapse: signaling pathways in neuromuscular junction assembly. Development 137, 1017-1033.

Yumoto N., Wakatsuki S. and Sehara-Fujisawa A. (2005).The acetylcholine γ to ε switch occurs in individual endplates. Biochem. Biophys. Res. Commun. 331, 1522-1527.

Accepted December 30, 2014