Nitrosative Stress in Skeletal Muscle of Diabetic Rats Submitted to Aerobic Exercise

Deyse Yorgos Lima¹, Adelson Marçal Rodrigues¹, Giovana Rita Punaro², Margaret Gori Mouro¹,², Elisa Mieko Suemitsu Higa¹,²,*

¹Translational Medicine, Universidade Federal de Sao Paulo, Sao Paulo, Brazil
²Nephrology Division, Universidade Federal de Sao Paulo, Sao Paulo, Brazil

Email address
emshiga@gmail.com (E. M. S. Higa), deyseyorgos@gmail.com (D. Y. Lima)
*Corresponding author

Citation

Abstract
The aim of this study was to assess the nitrosative stress in muscle tissue of diabetic rats submitted to aerobic training. Diabetes mellitus was induced by streptozotocin in male adult Wistar rats. The animals were submitted to aerobic training on treadmill and after 8 weeks, the gastrocnemius was removed for analysis. Data are shown as mean ±SEM; statistical analysis by One-Way ANOVA, with significance at p<0.05. DM+SE when compared with CTL+SE showed a reduction of body weight followed by other changes as increased chow and water intake, diuresis, glycemia and TBARS in the muscle. There was a significant improvement in all metabolic parameters in the trained diabetic animals when compared to the untrained; moderate exercise in diabetic animals reduced significantly the lipoperoxidation in the muscle. There was also an increase of superoxide anion and antioxidant defenses; this defense neutralized the action of anion, shown by reduction of the nitrotyrosine levels. Therefore, the results show that moderate exercise promotes benefits to the skeletal muscle, reducing the diabetic complications i.e., the oxidative stress, the glutathionylation and the nitrosative stress. It is suggested, that the aerobic training can be an adjuvant treatment, which could enhance the life quality of diabetic patients.

1. Introduction

Diabetes mellitus is a chronic disease that occurs when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin produced. The hyperglycemia is a common effect of uncontrolled diabetes and over time leads to serious damage to many of target tissue, including the skeletal muscle [1]. Hyperglycemia induces the synthesis of reactive oxygen or nitrogen species (ROS, RNS; respectively), such as peroxynitrite, superoxide anion and nitric oxide that attacks macromolecules, as evidenced by oxidation of lipids, proteins and DNA [2; 3].

Vascular endothelium has important regulatory role in the functions of vasculature and inflammatory activity, producing several vasodilator substances, including nitric oxide (NO); this is responsible for maintaining vascular tone (with predominance of vasodilation) and blood flow control [4]. NO is a potent vasodilator, participating in redox equilibrium and in the glucose uptake [5]. This molecule can be produced by nitric
2. Methods

2.1. Chemicals

Streptozotocin, goat anti-catalase and vanadium were purchased from Sigma Chemical (St. Louis, MO, USA). Citric acid for preparation of the citrate buffer and trichloroacetic acid were acquired from LabSynth (Sao Paulo, SP, Brazil). Thiobarbituric acid was purchased from J. T. Baker Chemical (Phillipsburg, NJ, USA). The Dopalen (ketamine chloride) and Anasedan (xylazinechloridrate) anesthetics were obtained from Sespo (Sao Paulo, SP, Brazil). Nitroblue tetrazolium chloride (NBT) was obtained from BioAssay Systems, EnzyChrom (Hayward, CA, USA). Antibody anti-eNOS was obtained from BD Biosciences (Sao Paulo, SP, Brazil), anti-iNOS, and antibody anti-gluthatione were acquired from Millipore (Bedford, MA, USA). Antibody anti-catalase and vanadium were purchased from Sigma Chemical (St. Louis, MO, USA). Anti-iNOS and actin were from Santa Cruz Biotechnology, CA, USA. Catalase Assay Kit (ECAT-100) was acquired from BioAssay Systems, EnzyChrom (Hayward, CA, USA).

2.2. Animals

Male Wistar rats of eight weeks of age, weighing approximately 210 g were obtained from the Central Animal Housing of Escola Paulista de Medicina. The animals were maintained in the Nephrology Division at temperatures of 22±2°C and at a light/dark cycle of 12/12 h, beginning at 06:00 hours. Rats were allocated into four groups: CTL+SE (sedentary control); CTL+EX (control plus exercises); DM+SE (sedentary diabetic); and DM+EX (diabetic plus exercises); n = 4 for each group. All the procedures were approved by the Ethics Committee in Research of Universidade Federal de Sao Paulo (UNIFESP, SP, Brazil), protocol #757778.

2.3. Diabetes Induction

Animals received a single intravenous administration of 60 mg/kg BW of streptozotocin (Sigma Chemical Co, St Louis, MO, USA) dissolved in 0.1 mol/L of citrate buffer, pH 4.5 [16]. Two days later, blood sample was collected from tail vein and diabetes was defined as fasting blood glucose >200 mg/dL; animals failing in this criterion were excluded.

Animals of all groups were placed in individual metabolic cages with water and food ad libitum for 24-h urine collection, prior and after the 8 weeks of protocol. The animals were removed from the metabolic cages and a blood sample from the retro-orbital plexus was collected under anesthesia (ketamine and xylazine) after three hours fasting (same day). All samples were placed at – 20°C. At the end of the exercise protocol, the animals were euthanized with a high dose of anesthetic (ketamine chloridrate at 90 mg/kg and xylazine chloridrate at 18 mg/kg, both intraperitoneally), followed by a small incision of the diaphragm [15].

2.4. Physical Training

The exercise protocol started on the fifth day after induction of DM; it consisted of a moderate running on a motor driven treadmill during 60 min/day at 16 m/min, 5 days/week, during 8 weeks at no inclination (0%). The training program was preceded by one week (period of adaptation) to the aerobic exercise; this adaptation was made in periods of 10, 20 and 30 minutes with a speed of 10 m/min, with 2 min of interval for each time. After the adaptation week, every day the running speed was increased gradually, until the rats ran at the standard speed of 16 m/min, which was estimated by others, through lactate test, and classified as moderate exercise [17].

2.5. Tissue Preparation

The gastrocnemius muscle was removed and immediately transferred to ice-cold containers with 0.9% NaCl and homogenized in 0.1 mol/L Tris-HCl buffer (pH 7.4). After centrifugation at 3000 rpm at 4°C for 10 min, the supernatant was collected and stored at -80°C to be used for lipid peroxidation, superoxide anion and NO assays. The protein content was analyzed by Bradford assay [18].

2.6. Oxidative Stress

The lipid peroxidation was estimated by thiobarbituric acid reactive substance (TBARS) method, using a molar extinction coefficient of the malondialdehyde (1.56 x 105
mol\(^{-1}\) cm\(^{-1}\)) and the superoxide anion level in muscle gastrocnemius homogenate at the end of the 8 weeks exercise was detected indirectly according to the adapted nitroblue tetrazolium (NBT) protocol and the optical density (OD) was read in microplate reader at 560 nm [19].

NO was measured to evaluate the magnitude of vascular damage and the oxidative stress equilibrium in diabetic rats. NO is extremely unstable; however, used a method in which nitrite and nitrate present in the gastrocnemius muscle homogenate were re-converted to NO through reaction with vanadium. This NO was quantified by a chemiluminescence method using a Nitric Oxide Analyzer (Sievers Instruments, Inc, Boulder, CO, USA), a high-sensitive detector for measuring NO (~1 pmol) [20].

### 2.7. Catalase (CAT) Activity

The CAT in the muscle tissue was determined according to the instructions of a commercial kit Catalase Assay (ECAT-100) from BioAssay Systems (Hayward, USA) and read at 570 nm on microplate reader (Synergy HT, Biotek, Winooski, USA).

### 2.8. Immunoblotting in the Gastrocnemius

To determine the protein expression, 55 µg of total protein of each sample were separated on 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The blots were then incubated with antibody anti eNOS (1:500), iNOS (1:200), catalase (CAT) (1:1,000), glutathione (1:1,000) and nitrotyrosine (NT) (1:500). The bands were visualized by chemiluminescence reagent (Millipore, Sao Paulo, Brazil) and analyzed by gel documentation (Alliance 4.7 Uvitec, Cambridge, United Kingdom). The relative expression of each protein was normalized by actin [21].

### 2.9. Statistical Analysis

The results were expressed as mean ±SEM and the values were compared using one-way analysis of variance (ANOVA) followed by Newman-Keuls Multiple Comparison post test with statistical significant when p <0.05.

### 3. Results

The metabolic parameters of sedentary diabetic animals (DM+SE) demonstrated the complications of the disease, such as increased chow and water intake, diuresis and glycemia, followed by reduction of the body mass, in relation to its control (CTL+SE). The diabetic animals submitted to exercise (DM+EX) showed a significant improvement of all metabolic variables, mainly blood glucose lowering when compared to DM+SE (Table 1).

#### Table 1. Metabolic profile of the animals at the 8th week protocol.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CTL+SE</th>
<th>CTL+EX</th>
<th>DM+SE</th>
<th>DM+EX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow intake (mg/24h)</td>
<td>16.6 ±0.4</td>
<td>16.7 ±0.4</td>
<td>38.4 ±1.9*</td>
<td>27.5 ±1.8*</td>
</tr>
<tr>
<td>Water intake (mL/24h)</td>
<td>23.7 ±2.2</td>
<td>23.5 ±0.9</td>
<td>174.3 ±14.3*</td>
<td>106.3 ±6.3*</td>
</tr>
<tr>
<td>Diuresis (mL/24h)</td>
<td>14.4 ±0.7</td>
<td>14.1 ±1.1</td>
<td>163.1 ±11.4*</td>
<td>84.5 ±8.2*</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>437.2±4.4</td>
<td>438.6 ±5.4</td>
<td>170.0 ±12.6*</td>
<td>294.2 ±17.3*</td>
</tr>
<tr>
<td>Glycemia (mg/dL)</td>
<td>103.0 ±0.4</td>
<td>101.0 ±3.5</td>
<td>540.6 ±13.9*</td>
<td>337.6 ±5.7*</td>
</tr>
</tbody>
</table>

Data represented as mean ±SEM. CTL+SE: sedentary control; CTL+EX: exercise control; DM+SE: sedentary diabetic; DM+EX: exercise diabetic; n = 4 for all groups. One-way ANOVA with Newman-Keuls post-test; p<0.05: * vs. CTL+SE; Φ vs. DM+SE.

The lipid peroxidation analysis showed that DM+SE group presented higher TBARS levels when compared to CTL+SE. In DM+EX vs. DM+SE, the TBARS levels were decreased and superoxide anion increased (Table 2). NO was not different among the groups; eNOS or iNOS isoforms did not present any difference between the groups, as demonstrated in Figure 1.

#### Table 2. Redox balance and catalase activity in the gastrocnemius at the 8th week protocol.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CTL+SE</th>
<th>CTL+EX</th>
<th>DM+SE</th>
<th>DM+EX</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS (nmol/mg protein)</td>
<td>0.14 ±0.01</td>
<td>0.15 ±0.01</td>
<td>0.20 ±0.01*</td>
<td>0.15 ±0.01*</td>
</tr>
<tr>
<td>O₂⁻ (OD/mg protein)</td>
<td>0.08±0.01</td>
<td>0.09 ±0.00</td>
<td>0.08 ±0.00</td>
<td>0.11 ±0.00*</td>
</tr>
<tr>
<td>NO (mmol/mg protein)</td>
<td>1.83±0.26</td>
<td>1.50 ±0.29</td>
<td>2.21 ±0.08</td>
<td>2.07 ±0.28</td>
</tr>
<tr>
<td>Catalase activity (nM/min)</td>
<td>0.003±0.0</td>
<td>0.003±0.0</td>
<td>0.003±0.0</td>
<td>0.004±0.0*</td>
</tr>
</tbody>
</table>

Data represented as mean ±SEM. CTL+SE: sedentary control; CTL+EX: exercise control; DM+SE: sedentary diabetic; DM+EX: exercise diabetic; n = 4 for all groups. TBARS: thiobarbituric acid reactives substances; O₂⁻: superoxide anion; NO: nitric oxide. One-way ANOVA with Newman-Keuls post-test; p<0.05: * vs. CTL+SE; Φ vs. DM+SE.
When the antioxidant profile was analyzed, the CAT expression was significantly increased in DM+SE (1.175±0.03) and DM+EX (1.150±0.06) compared to CTL+SE (0.800±0.0) and CTL+EX (0.850±0.02) respectively (Figure 2). Although there was no difference in expression, in the Table 2, the CAT activity was significantly augmented in DM+EX as compared to DM+SE. Besides, DM+EX vs. DM+SE was significantly reduced in the glutathione expression (1.283 ±0.09 vs. 1.619 ±0.07) and in CTL+EX (1.695±0.05); likewise the nitrotyrosine expression in the group DM+EX (0.955 ±0.06) as compared to DM+SE and CTL+EX (1.343 ±0.10 and 0.984 ±0.09, respectively), as seen in Figure 2.

4. Discussion

The main findings of this study were that the moderate exercise can downregulate all the metabolic parameters of diabetes, such as hyperglycemia, polyuria, polydipsia and polyphagia, which were present in the model used. Besides, exercises resulted in normalization of lipid peroxidation, increase in the dismutation of superoxide anion and reduced nitrosative/oxidative stress. Since the 18th century, it has been shown that exercise can act improving the glucose
uptake and gluconeogenesis [22]. Glucose has great importance on skeletal muscle functionality mainly in muscle contraction, being vital to human health. This tissue is responsible for the uptake, transport and absorption of glucose by the glucose transporter GLUT4 [23].

Oxidative stress is the imbalance between oxidants and antioxidants, being a major mechanism of lipid peroxidation [24]. Although some studies demonstrate that oxidative stress plays an important role in the development of diabetes complications, the contribution of oxidative stress on the skeletal muscle in diabetes is not yet well known [25; 26]. According to Halliwel and Gutteridge (2007), the ROS are generated during the normal metabolism and increase as the raise in oxygen consumption. The mitochondrial respiratory chain, ischemia and reperfusion, inflammatory response, NADPH oxidase, heme proteins and xanthine oxidase are considered the main sources of ROS production, during and after exercise [27]. Some studies assert that the skeletal muscle, in addition to blood, is one of the main generators of ROS, and this increase during the exercise, occurs especially in the muscle fibers [28].

The increased lipid peroxidation observed in this study, in diabetic group indirectly characterizes the oxidative stress, which was reduced after aerobic exercise. Sertuk et al. (2005) also demonstrated its effectiveness preventing the raising of the lipid peroxidation marker in trained diabetic subjects. Some studies demonstrate that physical training can promote a decrease in the oxidative stress status, diminishing the levels of lipid peroxidation and ROS, thereby reducing the deleterious effects caused by them [29] and increasing the antioxidant system, improving the redox state [30; 31].

Furthermore, it is known that NO can act in cellular signaling and can easily react with superoxide anion (O$_2^-$) to form the peroxynitrite (ONOO$^-$), strong oxidizing agent which leads to lipid peroxidation of cell membranes, reducing the bioavailability of both, O$_2^-$ and NO [32]. The peroxynitrite produced in this reaction participates on nitration process of the tyrosine residues of cellular proteins, generating the nitrotyrosine; the latter is considered a marker of nitrative stress and it is NO dependent, since it is directly related to the endogenous formation of peroxynitrite (ONOO$^-$) [33]. In this study, there was no change in the NO levels between control and treated groups perhaps because a reaction with O$_2^-$

Usually the skeletal muscle expresses two isozymes of NOS (eNOS and nNOS). However, iNOS can also be expressed in skeletal muscle during inflammatory conditions. There was no statistical significance in the expression of eNOS or iNOS in skeletal muscle. McConnellet al. (2007) also showed similar data relative to eNOS, failing to detect iNOS in human skeletal muscle, but only an increase in the nNOS isoform [21]. The expression of iNOS is very variable in skeletal muscle and it depends on the disease state and on the investigated species, being increased in skeletal muscle cells of patients with chronic heart failure [34], animals and cells culture after exposure to lipopolysaccharide (LPS) or inflammatory cytokines [35].

For the maintenance of redox balance in the body, the enzymatic antioxidant defense system plays an important role in the decomposition of superoxide anion, hydrogen peroxide (H$_2$O$_2$) and lipoperoxide [36]. The oxygenation of the muscle during exercise leads to highest production of superoxide anion; therefore, this may not work in the degradation of the cells, since this is physiologic, resulting in amelioration of the antioxidant status in diabetic animals [24]. The SOD enzyme has the function of dismutate the superoxide radical into H$_2$O$_2$, which can be subsequently catalyzed by the enzymes CAT and the glutathione reduced - glutathione peroxidase system (GSH-GPx), being converted in water and molecular oxygen [37]. Data from this study showed that the increase of superoxide anion in DM+EX group was able to trigger the process of dismutation and activate the antioxidant action due to increase of the CAT activity in this group, which reflected in the significant reduction of the nitrotyrosine.

As CAT acts together with SOD, it is believed that SOD and CAT system may have been activated so efficiently that reduced the glutathionylation in the trained diabetic group, since this process occurs when there is a link between GSH and proteins, demonstrating protective action of GSH against oxidative/nitrosative stress deleterious effects, preventing the irreversible oxidation of the thiols groups [38]; indicating that physic exercise can modulate the balance between pro-oxidants and antioxidants depending on the frequency and intensity of activity as shown by others [13].

In summary, in the experimental model of diabetes in rats, moderate aerobic exercise promotes several benefits, reducing the hyperglycemia, the glutathionylation and the nitrosative stress, moreover, promoting the restoring the redox balance in the skeletal muscle, by increasing the expression of the antioxidant levels. It is suggested that the aerobic training as an adjuvant treatment in diabetic patients, helping to improve quality of life in these individuals.

Acknowledgements

The authors acknowledge the support provided by “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Apoio a Pesquisa da UNIFESP (FAP)”.

Disclosure

All the authors declared that they have no conflict of interests. The authors alone are responsible for the content and writing of the article.

Funding

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo a Pesquisa do Estado de Sao Paulo (FAPESP).
References


