A 6-week training program increased muscle antioxidant system in elderly diabetic fatty rats

Manuel Rosety-Rodriguez1AB, Ignacio Rosety2CD, Gabriel Fornieles-Gonzalez1AB, Antonio Jesus Diaz-Ordonez1EF, Alejandra Camacho3EF, Miguel Angel Rosety1BF, Antonio Pardo4AB, Manuel Rosety1EF, Ramon Alvero5CD, Francisco Javier Ordonez1DE

1 School of Sport Medicine, University of Cadiz, Cadiz, Spain
2 Department of Human Anatomy, University of Cadiz, Cadiz, Spain
3 Hospital SAS Juan Ramon Jimenez, Huelva, Spain
4 Department of Internal Medicine, Ruber International Hospital, Madrid, Spain
5 School of Sport Medicine, University of Malaga, Malaga, Spain

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Summary

Background: It is widely accepted that oxidative stress is associated with the physiopathology of type 2 diabetes mellitus. In fact, it has been pointed out as a therapeutic target in T2DM. Fortunately, several papers have reported that long-term training programs improved the antioxidant system in young and adult diabetic rats. Accordingly, this study was designed to assess the influence of a shorter training program in elderly diabetic fatty rats.

Material/Methods: Study subjects were 24 male homozygous Zucker diabetic fatty rats (Gmi, fa/fa) aged 18 weeks with an average weight of 370–450 g. After a 2-week period of environmental adaptation, animals were randomly distributed into the Exercised Group (n=12) that performed a 6-week swimming training protocol and the Sedentary Group (n=12). Animals were sacrificed under anesthesia 24 h after the last exercise session. Serum metabolic profile was determined. Total antioxidant status (TAS), MnSOD expression, glutathione status and ROS generation were assayed in gastrocnemius muscle.

Results: When compared with controls, exercised rats significantly improved their metabolic profile. Total antioxidant status (0.19±0.002 vs. 0.13±0.002 µg/mg protein; p<0.001) and MnSOD expression (8471±90 vs. 6258±102 U/µg protein; p=0.003) were also increased in exercised rats.

Conclusions: A 6-week swimming training program improved the antioxidant system in elderly fatty diabetic rats. Fortunately, this improvement was enough to reduce oxidative damage, expressed as protein oxidation. A major finding of this study was that our training protocol lasted just 6 weeks, in contrast to longer protocols previously published.

key words: oxidative stress • exercise • type 2 diabetes mellitus • Zucker rat

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Author’s address: M. Rosety-Rodriguez, School of Sport Medicine, University of Cadiz, Pza. Fragela s/n 11003 Cadiz, Spain, e-mail: manuel.rosetyrodriguez@uca.es
BACKGROUND

Recent studies have reported that oxidative stress may be associated with the multifactorial etiology of insulin resistance, primarily in skeletal muscle tissue, and the subsequent development of T2DM [1]. Accordingly, oxidative stress has been selected as a therapeutic target in T2DM [1,2].

Fortunately, several papers have reported that long-term training programs have improved the antioxidant system in young and adult diabetic rats [3,4]. However, to our knowledge, little attention has been paid to elderly individuals. In addition, it would be of interest to reduce the length of training programs as compared to those previously published. Although extrapolation from animal studies to humans requires caution, shorter training programs may facilitate follow-up, thereby reducing drop-out rates [5,6].

The present study was designed to explore whether a short, 6-week, training program can improve the antioxidant system in skeletal muscle of elderly diabetic fatty rats.

MATERIAL AND METHODS

Animals

The study subjects were 24 male homozygous Zucker diabetic fatty (ZDF) rats (Gmi, fa/fa) aged 18 weeks with an average weight of 370–450 g. Animals were housed in single cages in an environmentally controlled laboratory (temperature 22°C) with a 12:12-h light-dark cycle. A standard rodent chow adjusted to their body weight (100 mg/g of weight) was provided. Tap water was given ad libitum.

Swimming training program

After a 2-week period of environmental adaptation, animals (n=24) were randomly distributed into the Exercised Group (n=12) that performed a 6-week swimming training protocol, and the Sedentary Group (n=12).

The Exercised Group swam individually in plastic tanks 50x100x45 cm, once per day (1 h), 3 days per week (Monday; Wednesday; Friday), during the daily dark phase, under red light to enable visual observation. During swimming sessions, rats wore elastic chest bands to which attachable loads could be added. Rats commenced exercising without any additional load for the first week. However, during the second week of treatment rats had 3% body weight added, increasing 1% each week until the end of the study. Loads were adjusted with the body weight every week.

To minimize stress associated with cold or hot water exposure, water temperature was monitored and maintained at 32°C. In order to separate the effects of exercise and the stress associated with the exercise environment, sedentary rats were individually placed in identical swimming tanks, but sat in shallow water at the same temperature, duration and frequency as the exercised rats. Animals were towel-dried and left for 1 h in a heated room to minimize the effects of cold exposure [7,8].

Animals were sacrificed 24-h after the last exercise session, under anesthesia, using 1 ml of ketamine injection (1 g/10 ml), by cervical dislocation.

Metabolic profile

Blood samples were collected by cardiac puncture and were centrifuged for 15 min at 3000 rpm. The serum thus obtained was used for biochemical analysis. Glycemic and lipid profiles were assessed using standard laboratory methods. Glycosylated hemoglobin (HbA1c) was determined using a specific monoclonal antibody with a turbidimetric readout (DCA 200+ Analyzer, Bayer Diagnostics). Insulin levels were assessed using the ultrasensitive rat insulin enzyme-linked immunosorbent assay kit (Mercodia, Sweden). Finally, HOMA-IR, an index of insulin resistance, was calculated using the following formula [9]:

HOMA-IR = [(fasting plasma glucose (mg/dl) × fasting plasma insulin (µU/ml)]/405.

ROS generation, muscle protein oxidation, total antioxidant status, MnSOD expression and GSSG/GSH ratio assessment

Gastrocnemius muscles were excised and immediately frozen in liquid nitrogen and stored at -80°C for subsequent use. Generation of ROS and other pro-oxidants in muscle mitochondria was evaluated by using the rate of dichlorodifluorescein (DCFH) oxidation [10]. Briefly, isolated mitochondria were incubated in the respective buffers at 37°C for 15 min to allow DCFH-diacetate to cross the mitochondrial membrane. The solution was then centrifuged at 12,000 g for 8 min, and the supernatant containing excess DCFH-diacetate not crossing the mitochondrial membrane was discarded. The mitochondrial pellets were resuspended, and 50 µl of the suspension (<2 mg protein) were used for assay. DCF formation was followed at the excitation wavelength of 488 nm and emission wavelength of 525 nm for 30 min by using a Hitachi F-2000 fluorescence spectrometer. The rate of DCFH conversion to DCF was linear for at least 60 min, corrected with the auto-oxidation rate of DCFH without protein. The units were pmol DCF formed per minute per milligram protein.

To determine superoxide-derived changes in protein carbonylation, we performed an assay for carbonylated proteins provided by Chemicon (Oxyblot oxidized protein detection kit; Millipore, Amsterdam, Netherlands). Briefly, carbonyl groups in the protein side chains are derived to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine. The DNP-derived protein samples are subjected to gel electrophoresis and subsequent Western blotting. Densitometry of the 2,4-dinitrophenylhydrazine-derived bands in the gel was performed and normalized to a standard control sample loaded on all gels and expressed as arbitrary units (AU), as previously stated [11].

Total antioxidant status (TAS) was determined by the method of absorbance of ATBS cation [12]. Protein expression for MnSOD and Cu, ZnSOD were determined by Western immunoblot analysis [13]. GSH and glutathione disulfide (GSSG) were analyzed by using HPLC method, as previously described [14].

Ethics and statistic assessment

Our protocol complied with U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in
Testing, Research, and Training, and was approved by our Institutional Ethics Committee.

Results are expressed as mean ±SD. The statistical analysis of data was performed using analysis of variance (one-way ANOVA). The significance of the changes observed was ascertained at p<0.05.

Serum metabolic profile in both exercised and unexercised ZDF rats is listed in Table 1.

Regarding oxidant production in muscle mitochondria as revealed by DCFH oxidation rate, we found no significant differences between the Exercised and Sedentary groups (96.4±7.7 vs. 93.0±7.2 pmol DCF/min/mg; p=0.64).

Total antioxidant status (TAS) in gastrocnemius tissue was significantly increased in exercised rats as compared with sedentary counterparts (0.19±0.002 vs. 0.13±0.002 µg/mg protein; p<0.001*). The expression of antioxidant enzyme MnSOD was also increased in ZDF rats that performed our 6-week training program as compared with sedentary controls (8471±90 vs. 6258±102 U/µg protein; p=0.003*). In contrast, no significant differences were found in Zn, CuSOD expression between exercised rats and sedentary controls (101.6±7.3 vs. 97.6±8.1 U/µg protein; p=0.87).

Finally, the improvement of antioxidant system was enough to reduce carbonylated proteins in exercised diabetic fatty rats as compared with sedentary animals (1.37±0.33 vs. 1.62±0.58 UA; p=0.011*). These results are summarized in Table 2.

**Table 1.** Comparative analysis of metabolic profile in exercised (n=12) and sedentary ZDF rats (n=12) at the end of the study.

|                    | Exercised       | Sedentary       | p value |
|--------------------|-----------------|-----------------|---------|
| Body weight (g)    | 433.2±3.1       | 405.8±3.7       | 0.22    |
| Glucose (mmol/l)   | 29.4±1.7        | 33.2±1.9        | <0.001* |
| Hb1Ac (%)          | 10.6±0.7        | 11.1±1.9        | 0.31    |
| Insulin (pmol/l)   | 1206.7±11.9     | 1342.5±12.4     | 0.018*  |
| HOMA-IR (U)        | 34.2±6.6        | 38.8±6.9        | 0.027*  |
| Total-Cholesterol (mmol/l) | 4.11±0.6 | 5.25±0.7 | 0.042* |
| Triacylglycerols (mmol/l) | 3.03±0.4 | 4.26±0.6 | 0.006* |

**Table 2.** Comparative analysis of muscle antioxidant system, reactive oxidant species (ROS) production and protein oxidation in exercised (n=12) and sedentary ZDF rats (n=12) at the end of the study.

|                    | Exercised       | Sedentary       | p Value  |
|--------------------|-----------------|-----------------|----------|
| TAS (µg/mg protein)| 0.19±0.002      | 0.13±0.002      | <0.001*  |
| MnSOD (U/µg protein) | 8471±90       | 6258±102        | 0.003*   |
| ZnCuSOD (U/µg protein) | 101.6±7.3   | 97.6±8.1        | 0.87     |
| GSSG/GSH ratio     | 0.34±0.006      | 0.40±0.004      | 0.032*   |
| ROS (pmol DCFH/min/mg) | 96.4±7.7     | 93.0±7.2        | 0.64     |
| Carbonylated protein (UA) | 1.37±0.33     | 1.62±0.58       | 0.011*   |

TAS – total antioxidant status; GSSG – oxidized glutathione; GSH – reduced glutathione; DCFH – dichlorofluorescin * p value <0.05.

**RESULTS**

**DISCUSSION**

The present study has generated some interesting and significant data. But firstly, it should be pointed out that the Zucker Diabetic Fatty (ZDF) rat is a good animal model, not only for studying T2DM physiopathology, but also for assessing the effects of therapeutic options such as intervention programs based on exercise [15]. In fact, the development of diabetes is quite similar to what is seen in obese humans.
since it results from hyperphagic behavior due to a leptin receptor mutation (fa gene) [16].

And secondly, we chose swimming because it is widely used to identify biochemical and molecular responses to exercise. Swimming is a uniform type of activity that is less traumatic to animals than other forms of exercise. Further, we did not find significant changes in body weight, in agreement with previous studies [8]. Interestingly, a study that employed forced running as the exercise regime showed that body weight increased in exercised animals despite a lack of differences in muscle mass or fat mass. Although we did not measure glycosuria in our study, it has been reported that 25–30% of the caloric intake is excreted in untreated ZDF rats [17].

With respect to biochemical indices, diabetic fatty rats that performed our training protocol had significantly improved glycemic and lipid profiles. Similar results were found after a 12-week training program in diabetic fatty rats [18]. In addition, a 7-week training program improved biochemical abnormalities associated to diabetes [19]. It should be emphasized that our protocol induced similar effects and lasted just 6 weeks. We also found hyperinsulinemia was significantly reduced in exercised rats when compared to sedentary counterparts. However, our results suggest that exercised rats were still insulin resistant on a systemic level, as indicated by their elevated plasma insulin concentrations. This hyperinsulinemia could be a reflection of insulin resistance in the liver that was not ameliorated by swimming training protocol. Further long-term studies on this topic are required to clarify this issue.

Several studies have reported that reactive oxygen species (ROS) are produced in various tissues under diabetic conditions by several mechanisms, such as non-enzymatic glycosylation reactions, electron transport chain in the mitochondria, and membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [20]. Fortunately, we found no significant differences in oxidant production in muscle mitochondria between Exercised and Sedentary groups. On the contrary, several studies have demonstrated unequivocally that a single session of strenuous exercise significantly enhanced free radical generation in working muscle and oxidative damage in aging rats [21].

Regarding the antioxidant system, we found total antioxidant status (TAS) was significantly higher in exercised rats when compared with controls. Similarly, a 12-week swimming training increased TAS in ZDF rats [18]. Furthermore, a 13-week swimming protocol significantly improved antioxidant enzyme activities in the soleus muscle of exercised rats [22]. Rats used in our study were older and consequently would be expected to have higher oxidative damage, as well as different antioxidant capacities, when compared with younger animals. In any case, the improvements in insulin sensitivity induced by our 6-week training program appear to be related, at least in part, to changes in these pro-oxidants and antioxidants balance.

As hypothesized, the expression of antioxidant enzyme MnSOD was also increased in ZDF rats that performed our 6-week training program when compared with sedentary controls. Previous data have demonstrated that the major antioxidant enzyme, MnSOD, is most consistently upregulated by exercise training. In this respect, a 12-week training program resulted in upregulation of MnSOD in hearts from old rats [23]. The ability of exercise training to upregulate protective MnSOD protein may explain, at least in part, the improvement of total antioxidant status (TAS) that we also found in diabetic exercised rats. In contrast, no significant differences were found in Zn,CuSOD expression. This may be explained, at least in part, since transcriptional response to oxidative stress is impaired in skeletal muscle from aged animals, as previously reported [24].

We have also found significant differences when assessing GSSG/GSH ratio, suggesting that sedentary animals are more vulnerable to oxidative stress. Because mitochondria lack the enzymes in the γ-glutamyl cycle, and GSH is transported into the mitochondria via a membrane-borne energy-dependent system, our results suggest that mitochondrial GSH transport in skeletal muscle may be enhanced in exercised animals, as was reported previously [4].

It should be emphasized that this improvement of the antioxidant system was able to reduce the presence of carbonylated proteins in exercised diabetic fatty rats, when compared with sedentary controls. This finding is of particular interest, since protein oxidation has been involved in the pathogenesis of insulin resistance and complications associated with T2DM [25,26]. Finally, although there are differences between animal and human conditions, our results suggest that regular exercise is an attractive and healthy non-pharmacological therapeutic measure in T2DM.

**Conclusions**

A short swimming program improved metabolic profile and the antioxidant system in Zucker diabetic fatty (ZDF) rats. In addition, our training protocol significantly increased total antioxidant status, glutathione system and MnSOD expression. Fortunately, the improvement in the antioxidant system was enough to reduce oxidative damage, expressed as protein oxidation. A major finding of this study was that our training protocol lasted just 6 weeks, in contrast to longer protocols based on exercise that were previously published in the literature.

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