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A N   I N T E R N A T I O N A L   J O U R N A L

Oxidative Stress During Rehabilitation from Protein Malnutrition Associated with Aerobic Exercise in Rats

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ABSTRACT

This study was designed to evaluate biomarkers of oxidative stress in rats with or without aerobic exercise during recovery from protein malnutrition. From the 30th to the 90th day of life, male Wistar rats were fed a low protein diet (LP + 6%) followed by a normal protein diet (NP = 17%) until the 120th day and separated in two groups: sedentary (S) and exercise trained (E = swimming 1h/day, 5 days/week, with from the 90th to the 120th day). Rats fed a normal protein diet were used as controls. Results showed that physical exercise had beneficial effects on body weight gain during nutrition rehabilitation. Erythrocytes catalase and glutathione reductase (biomarkers of the antioxidant system) were significantly reduced in all groups in comparison to the sedentary control group. The plasma concentration of TBARs (biomarkers of the oxidative damage) was also lower in the recovered rats, suggesting that the improvement in body growth after nutritional rehabilitation with physical exercise could be related to a decrease in the oxidative stress level.

Key words: Protein malnutrition, nutritional recovery, exercise, oxidative stress

INTRODUCTION

Exercise training has many physiological effects consistent with improved health. Several studies have shown that exercise is associated with increased oxidative capacity of skeletal muscle (Pels et al., 1989) and muscle growth (Watt et al., 1982), improvement in cardiac function (Perez et al., 2003) enhanced bone mineralization (Baker and Griminger, 1983) and increased life span (Goodrick, 1980). Physical exercise may also be a beneficial part of rehabilitation from malnutrition. Torun and Viteri (1984) reported that physically active rats grew more in length and weight than the inactive counterparts also. These authors reported that 24-48 months old children under treatment for severe protein malnutrition were assigned to either an active group (stimulated, but not forced to participate in games and activities that involved walking uphill, climbing a ramp running, and climbing stars) or a control group (with ad libitum pattern of physical activity and rest observed in child care and nutrition rehabilitation centers). The active group grew more in length and lean body mass than the control group (Torun and Viteri, 1984).

Increased energy demand during physical exercise induced increase in oxygen supply to active tissues (Sen, 2001). These resulted in an increased production of reactive oxygen species (ROS), mainly due to elevated rates of mitochondrial respiratory chain (Sjödin et al., 1990; Sen, 2001).

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When ROS production overcomes the capacity of the body antioxidant system, an oxidative stress occurs and many cellular constituents such as lipids, proteins and DNA may suffer oxidation due to ROS attack, compromising cell function. It has also been suggested that kwashiorkor, a severe form of protein malnutrition, may itself be a product of the radical initiated tissue damage in the malnourished child (Golden and Ramdath, 1987; Manary et al., 2000). The present study was designed to evaluate biomarkers of oxidative stress, nutritional status and muscle metabolism in rats submitted or not to exercise during a month recovery from protein malnutrition.

METHODS AND MATERIALS

Animals and diets

Young (30 days) male Wistar rats were fed isocaloric (3948 kcal/kg) normal (NP= 17%) or low (LP=6%) protein diets ad libitum. Table I shows the composition of both diets. The normal protein diet followed the American Institute of Nutrition AIN-93G recommendations for rodents (Reeves et al., 1993). Detailed mineral and vitamin mix composition were described elsewhere (Reeves et al., 1993).

Table 1 - Diet composition (g/kg).

Components	Normal protein (AIN-93)	Low protein
Casein (84% protein)	202	71.5
Cornstarch	397	480
Dextrinized cornstarch	130.5	159
Sucrose	100	121
Soybean oil	70	70
L-cystine	3	1
choline chloridrate	2.5	2.5
mineral mix (AIN-93)	35	35
vitamin mix (AIN-93)	10	10
Fiber (mycrocellulose)	50	50

Reeves et al., (1993).

Experimental groups

Control
• Normal protein sedentary [NP-S]: rats fed the normal protein diet for 90 days and kept sedentary (n = 15);
• Normal protein exercised [NP-E]: rats fed the normal protein diet for 90 days and submitted to swimming for one hour/day, 5 days a week, supporting a load equivalent to 5% of body weight, in a tank filled with water at 30 ± 2°C, during the last 30 days (n = 10);

Recovered
• Low protein/normal protein sedentary [LP/NP-S]: rats fed the low protein diet for 60 days and the normal protein diet for 30 days , kept sedentary (n = 15);
• Low protein/normal protein exercised [LP/NP-E]: rats fed the low protein diet for 60 days and the normal protein diet for 30 days associated with swimming exercise (n = 10).

Tissue Samples

At the 90th day of experiment, rats from all groups were anesthetized without previous fasting with an intraperitoneal injection of chloral hydrate 10% (wt/vol), 0.3 mL for each 100 g of body weight for tissue and blood extraction. They were used in experiments as soon as anesthesia was assured by loss of pedal and corneal reflexes. Heparinized blood samples were obtained from portal vein, for preparation of a hemolysate and for plasma separation. After blood collection, the animals were sacrificed for other biological material obtainment. At the 60th day of treatment rats of the NP-S and of the LP/NP-S groups were sacrificed for biochemical analysis related to nutritional status evaluation.

Soleus Muscle Glucose Metabolism

For measurements of muscle glucose uptake and oxidation, glycogen synthesis and lactate production, longitudinal soleus muscle strips (25-

30 mg) were placed in glass vials containing d [U14 C] glucose, (0.25 µCi/µmol glucose), [3H]-2-deoxyglucose (0.1 µCi/µmol glucose) and non radioactive glucose to a final concentration of 5.5 mmol/L plus insulin (100miU/mL). Glucose uptake and oxidation as well as glycogen synthesis and lactate production were determined as previously described (Prada et al., 2003; Nunes et al., 2005).

Enzyme Assays
The hemolysate was diluted 1:20 in 0.1M phosphate buffer, pH 7.0 before assaying. All enzyme assays were performed in triplicate, according to the following methods. Catalase (CAT) activity was measured in 50 mM phosphate buffer (pH 7.0) by monitoring the decrease in absorbance at 240 nm for 30 sec after the addition of 10 mM hydrogen peroxide (Aebi, 1984). One unit of catalase activity is the amount of enzyme present that decomposes 1 µM H_2O_2/min at 25°C (Aebi, 1984). The erythrocyte CAT activity was expressed as IU/g Hb.min. Glutathione Reductase (GR) activity was measured in a 0.2 M phosphate buffer (pH 7.0) containing 2 mM EDTA, 0.1 mM NADPH and 0.75 mM DTNB. GSSG (1mM) was added into the cuvette to start the reaction and the absorbance was followed at 412 nm for 3 min (Smith, et al., 1988). The erythrocyte GR activity was expressed as IU/g Hb (Beutler, 1975).

Plasma Reactive Carbonyl Derivatives
The plasma reactive carbonyl derivatives (RCD) was measured by carbonyl reagent 2.4-dinitropheylhydrazine (DNPH) using a spectrophotometric method (Eston Finney et al., 1996). Plasma (200 µL) was mixed with 1 mL H_2O and 2 mL of 20% tricloroacetic acid (TCA) and centrifuged at 1,000 g for 10 min. The pellet was suspended in 1 mL of 10 mM DNPH and incubated for 60 min at 37°C. For blank 1 mL of 1M HCl was used instead DNPH. Subsequently, 1mL of 20% TCA was added and the sample was centrifuged at 1,000 g for 10 min. The pellet was washed with 1:1 ethanol/ethyl acetate solution and centrifuged at 1,000 g for 10 min. The pellet was mixed with 1 mL of 6 M guanidine (diluted in 20 mM H_2PO_4, pH 2.3). Finally the sample was incubated for 40 min at 37°C. The absorbance was measured at 380 nm. The results were expressed as µmols RCD/mL of plasma The analyses were done

in triplicate and the mean was used to statistical analysis.

Plasma Thiobarbituric Acid Reactive Substances
Plasma Thiobarbituric Acid Reactive Substances (TBARs) were measured by fluorimetry, according YAGI (Yagi, 1994). Plasma (20 µL) was mixed with 4.0 mL of N/12 H_2SO_4. Phosphotungstic acid 10% (0.5 mL) was added to this mixture, which was centrifuged at 3,000 rpm for 10 min. The pellet was mixed with 2.0 mL of N/12 H_2SO_4 and 0.3 mL of 10% phosphotungstic acid and centrifuged again at 3,000 rpm for 10 min. Distilled (4.0 mL) water and 1.0 mL of thiobarbituric acid (TBA) (1:1 0.67 % TBA aqueous solution and glacial acetic acid) suspended the pellet. This mixture was heated at 95°C for 60 min. After cooling, 5.0 mL of n-butanol was added, and the mixture, shaken vigorously. After centrifugation at 3,000 rpm for 15 min, the n-butanol layer was measured at 553 nm with excitation at 515 nm.

Nutritional Status Evaluation
For nutritional status evaluation, plasma glucose, total protein, albumin and free fatty acids (FFA) levels were measured by colorimetric methods, as described by Nogueira et al (1990). Liver lipids were extracted with ethanol (Prada et al., 2003) and measured by a colorimetric method (Nogueira et al., 1990).

Statistical Analysis
The results were expressed as means ± SD for the number of rats (n) indicated. When working with muscle strips, n refered to the number of strips. The data were analyzed by Student´s unpaired t-test or ANOVA, where adequate, and P < 0.05 values indicated significant difference.

RESULTS

Effects of the low protein diet on nutritional status
Table 2 shows the results on plasma markers of nutritional status after 60 days feeding the LP diet compared to NP group. The LP group showed significantly reduced body weight gain, hypoproteinemia, hypoglycemia, hypoalbuminemia, high circulating FFA and liver lipid levels. Despite

of the low body weight gain observed in the LP animals, no difference in food intake was seen between LP and NP.

Table 2 - Body weight gain (g), food intake (g/100g body weight), plasma glucose (mg/dL), plasma protein, plasma albumin (g/dL), plasma FFA (µEq/L) and liver lipid levels after 60 days of malnutrition

Groups	NP	LP
Body weight gain	149.6 ± 4.94	55.4 ± 3.94 [*]
Food intake	20.5 ± 3.12	22.9 ± 4.50
Plasma glucose	122 ± 5.0	100 ± 4.2 [*]
Plasma total protein	6.4 ± 0.9	5.5 ± 0.10 [*]
Plasma albumin	5.8 ± 0.11	4.7 ± 0.13 [*]
Plasma FFA	259.2 ± 11.7	322.2 ± 45.4 [*]
Liver lipid	2.6 ± 0.11	7.2 ± 0.44 [*]

Results are mean $\pm$ SD from 5 rats in the each group, except for body weight gain and food intake where n = 15.
NP = Normal protein diet, LP = Low protein diet
([*]) significantly from NP ($P < 0.05$, t-test).

Effects of one month of re-feeding associated or not to exercise on nutritional status
Table 3 shows the data for both groups (LP and NP) after 30 days of recovery feeding a NP diet (LP/NP-S and NP-S). No significant difference in proteinemia, albuminemia, circulating FFA levels and liver lipid content between the groups. Body weight gain was twice when compared to that observed in the group one month before (LP group, Table 2), but significantly lower in the protein restricted (LP/NP-S) than in normal protein (NP-S) group.
Table 3 also showed that there was a significant increase in body weight gain in LP/NP-E compared to LP/NP-S group. The results were completely different for NP groups, with a significant decrease in body weight gain in NP-E compared to NP-S group despite the food intake was higher in trained (NP-E and LP/NP-E) than in sedentary (NP-S and LP/NP-S) groups. Plasma glucose was elevated in the LP/NP-E and LP/NP-S groups in relation to the control (NP-S and NP-E) animals. Plasma albumin was lower in both exercised groups, significantly for the LP/NP-E group. On the other hand, proteinemia, circulating FFA levels and liver lipid content did not change with exercise even in LP/NP-E group when compared to the others.

Effects of low protein diet and re-feeding associated or not to exercise on skeletal muscle glucose metabolism
Skeletal muscle glucose uptake and glycogen synthesis were significantly higher only in trained (NP-E and LP/NP-E) rat isolated muscle than in the corresponding sedentary (NP-S and LP/NP-S) ones. However, the LP/NP-E group exhibited a significant lower glycogen synthesis when compared to NP-E group. No difference in glucose oxidation was observed among the four groups. The LP/NP-E rats showed higher muscle lactate production than LP/NP-S rats (Fig. 1).

Effects of low protein diet and re-feeding associated or not to exercise on biomarkers of oxidative damage and antioxidant system
Fig. 2 shows the effects of re-feeding on oxidative stress markers. Plasma TBARs concentrations were higher in the NP-E and in LP/NP-E than in the corresponding sedentary groups, but it appeared significantly decreased in the recovered (LP/NP-S) rats compared to the corresponding control rats (NP-S) (Fig. 2A). Plasma RCD concentrations were significantly increased only in the exercised control rats (NP-E) compared to the other groups (Fig. 2B).
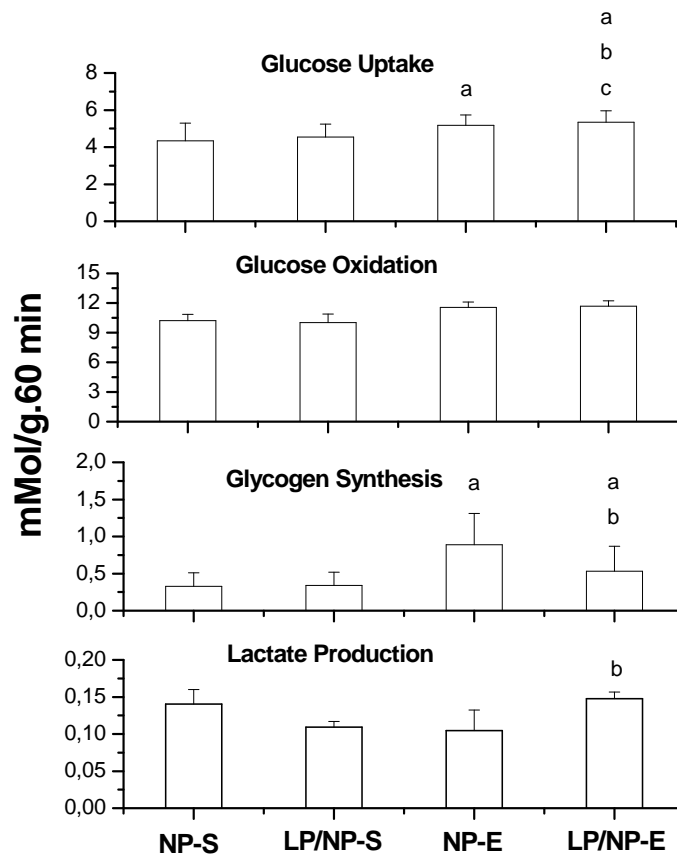
Fig. 1 - Skeletal muscle glucose uptake, glucose oxidation, glycogen synthesis and lactate production by soleus muscle after 60 days of protein restriction followed by nutritional recovery associated or not to exercise for 30 days. Results are mean ± S.D. of the 10 muscles strips per group. NP = normal protein; LP = Low protein diet; S = Sedentary; E = Exercise trained.

([a]) Significant differences (P < 0.05, ANOVA) in comparison to the NP-S rats.
([b]) Significant differences (P < 0.05, ANOVA) in comparison to the LP/NP-S rats.
([c]) Significant differences (P < 0.05, ANOVA) in comparison to the NP-E rats.

Table 3 - Body weight gain (g), food intake (g/100g body weight), plasma glucose (mg/dL), plasma protein (g/dL), plasma albumin (g/dL), plasma FFA (μEq/L) and liver lipid (mg/100mg) levels after 30 days of malnutrition and nutritional recovery associated or not exercise for 30 days.

Groups	NP-S	NP-E	LP/NP-S	LP/NP-E
Body weight gain	199.5 ± 4.0	146,9 [a] ± 6,7	106.8 [ac] ± 3.2	116.9 [ac] ± 4.8
Food Intake	18.1 ± 4.5	22.1 [a] ± 5.0	21.7 ± 4.7	25.0 [b] ± 4.1
Plasma glucose	111.2 ± 4.0	128.1 ± 10.3	138.1 [a] ± 6.7	152.5 [ac] ± 1.8
Plasma protein	6.9 ± 0.1	7.5 ± 0.5	6.9 ± 0.1	6.8 ± 0.05
Plasma albumin	4.4 ± 0.1	4.1 ± 0.1	4.6 [c] ± 0.1	4.0 [b] ± 0.1
Plasma FFA	283.3 ± 72.7	284.4 ± 12.9	266.6 ± 30.6	244.4 ± 19.8
Liver lipid levels	4.0 ± 0.2	3.9 ± 0.1	4.2 ± 0.2	3.8 ± 0.1

Results are mean ± S.D. from 10 animals per group. NP = normal protein; LP = Low protein diet; S = Sedentary; E = Exercise trained.
([a]) Significant differences in comparison to the NP-S rats ($P < 0.05$, ANOVA).
([b]) Significant differences in comparison to the LP/NP-S rats ($P < 0.05$, ANOVA).
([c]) Significant differences in comparison to the NP-E rats ($P < 0.05$, ANOVA).

These data indicated that the NP-E group was submitted to a higher stress level than the other groups. Fig. 3 showed that erythrocyte CAT and GR activities were significantly reduced in all experimental groups (NP-E, LP/NP-S and LP/NP-E) in comparison to the NP-S group.

DISCUSSION

The rats fed the LP diet exhibited features typical of protein malnutrition such as low body weight gain, hypoglycemia, hypoalbuminemia, high plasma free fatty acid and high lipid levels (Torun and Chew, 1994; Moura and Passos, 2002). The results showed that after one month of recovery with a normal protein diet these metabolic alterations were reversed. Daily food intake and body weight gain were improved when nutritional recovery was associated to physical exercise. These results corroborated the hypothesis that physical exercise caused a beneficial effect on body growth during nutritional recovery (Torun and Viteri, 1984).

The increase in muscular work induces several biochemical reactions essential to muscle growth. Physical training has the ability to revert the alterations in mitochondrial protein induced by catabolic states in rat vastolateral and gastrocnemius muscle (Midaoui et al., 1996). These aspects may be related to growth hormone (GH) levels. GH has many biological effects on body growth affecting protein, carbohydrate and lipid metabolism and stimulating cartilage and bone growth. It has been already demonstrated that administration of GH restored body weight and length in protein deficient young rats (Muaku et al., 1997). GH levels can be influenced by physical activity, with tendency to elevation especially in exercises above the lactate threshold (Felsing et al., 1992).

In the present study, physical training improved glucose uptake by skeletal muscle both in control (NP-E) and recovered (LP/NP-E) rats. This is not surprising, since physical exercise is known to improve glucose tolerance and sensitivity to insulin (Dela, 1990; Nagasawa et al., 1990). Glucose transport into skeletal muscle is acutely regulated by both insulin and by contractile activity (Hendriksen et al., 1996), through the translocation of a glucose transporter isoform, the GLUT-4 (Ezaki, 1997). The capacity for glucose transport is associated with the muscle content of GLUT-4 protein (Hedriksen et al., 1996; Ezaki, 1997). Both insulin stimulated glucose transport (Hedriksen et al., 1996; Bonen et al., 1986) and GLUT-4 protein (Bonen et al., 1986; Rodnik et al., 1992; Houmad et al., 1995) increase in the exercise trained skeletal muscle. Despite the increased glucose uptake of skeletal muscle in LP/NP-E rats and normal uptake in LP/NP-S rats, blood glucose concentration was elevated in both recovered groups, when compared to their control counterparts. This may be indicative of insulin resistance in tissues other than skeletal muscle, induced by the early protein restriction.

Besides increased glucose uptake, other skeletal muscle adaptations to aerobic training, concerning glucose metabolism are present as an increased glycogen synthesis (Houmad et al., 1995; Bagby et al., 1978) and improved lactate release from muscle to blood through an increased plasma membrane transport system (Pilegaard et al., 1993). In the present study, both increased muscle glycogen synthesis and increased lactate release to the culture medium in the skeletal muscle isolated from protein restrict trained rats (LP/NP-E) were observed. Taken together, these results indicated that protein restriction early in life did not impair muscle glucose metabolism responses to exercise training.

Protein restriction seemed to accentuate lactate release from the trained skeletal muscle. Peroxidation of unsaturated fatty acid residues of phospholipids in cell membranes might result in significant loss in membrane integrity, which was one of the most striking effects of oxidative damage (Tappel, 1973), leading to generation of potentially harmful aldehydes and alkanes that reacted with thiobarbituric acid. Therefore, the nonspecific test of thiobarbituric acid reactive substances (TBARs) was often applied to assess the degree of oxidative stress (Tappel, 1973). In addition, oxidative alteration of aminoacids has significant effects on cellular function as oxidatively modified proteins modify their physiological activity and tend to be very sensitive to proteolytic degradation (Stadtman, and Oliver, 1991).
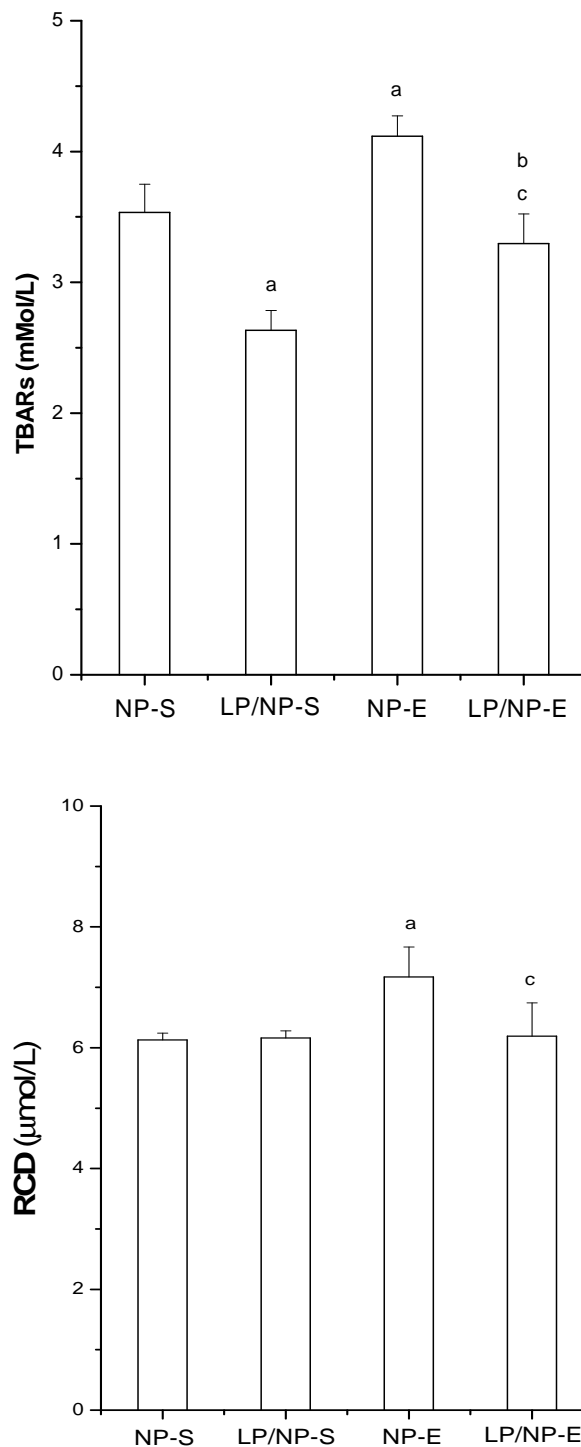
Figure 2 - Plasma thiobarbituric acid reactive substances (TBARs) and Reactive Carbonyl Derivative (RCD) after 60 days of protein restriction followed by nutritional recovery associated or not to exercise for 30 days. Results are mean ± S.D. of the 10 animals per group. NP = normal protein; LP = Low protein diet; S = Sedentary; E = Exercise trained.
([a]) Significant differences (P < 0,05, ANOVA) in comparison to the NP-S rats.
([b]) Significant differences (P < 0,05, ANOVA) in comparison to the LP/NP-S rats.
([c]) Significant differences (P < 0,05, ANOVA) in comparison to the NP-E rats.
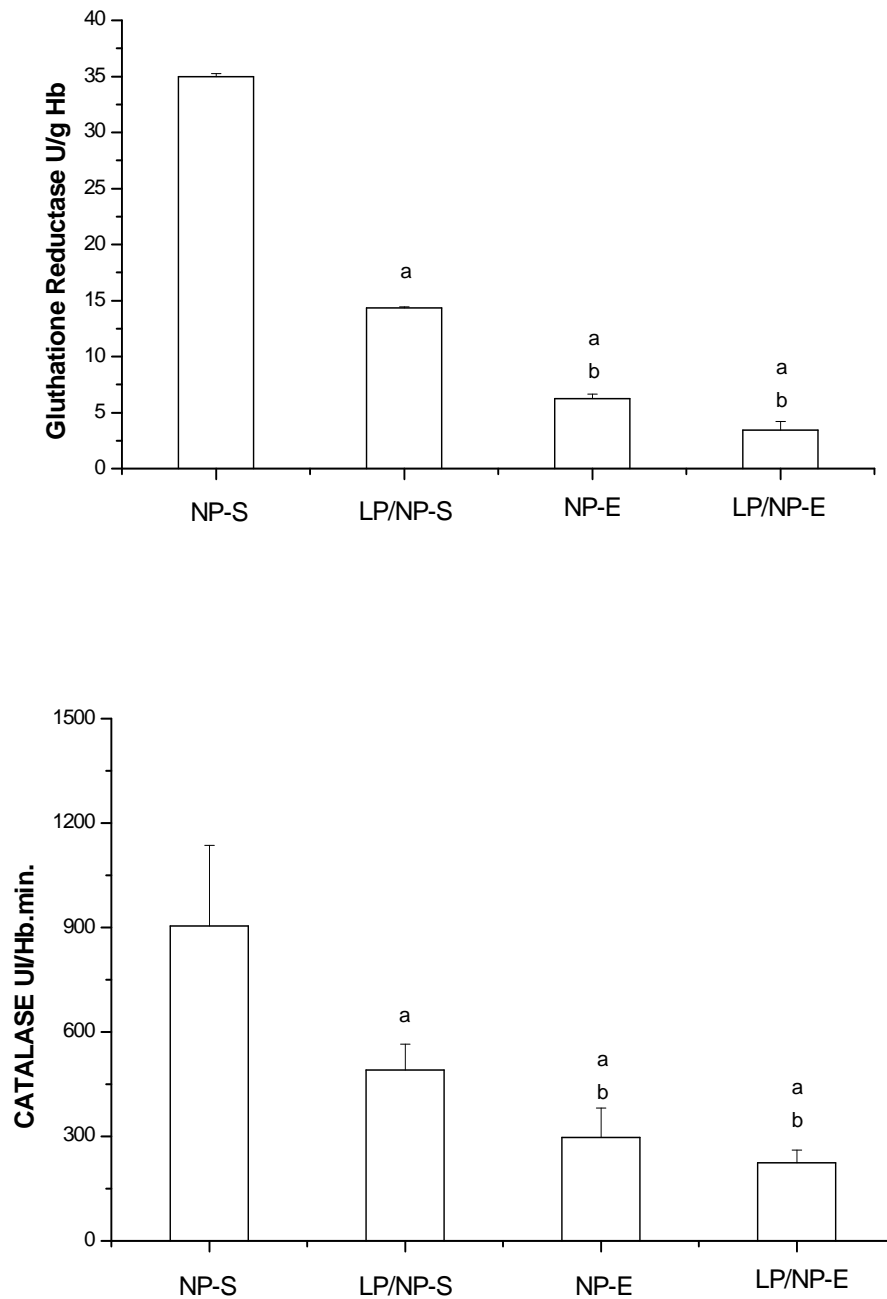
Figure 3 - Blood Glutathione Reductase and Catalase activity after 60 days of protein restriction followed by nutritional recovery associated or not to exercise for 30 days. Results are mean ± S.D. of the 10 animals per group. NP = normal protein; LP = Low protein diet; S = Sedentary; E = Exercise trained.
([a]) Significant differences (P < 0,05, ANOVA) in comparison to the NP-S rats.
([b]) Significant differences (P < 0,05, ANOVA) in comparison to the LP/NP-S rats.

Oxidative modifications of amino acids residues include transformation of amino acid residues such as proline, arginine and lysine to reactive carbonyl derivatives (RCD) and links have been established between the degree of RCD accumulation and a variety of physiopathological conditions (Stadtman, 1992).

Results showed that plasma TBARs concentration decreased significantly in the protein restricted recovered groups (LP/NP-S) in relation to the corresponding control group (NP-S). This suggested a low oxidative damage to plasma or tissue lipids in the first group, which could result from a lower ROS production or a higher removal capacity in protein restricted than in control rats. This apparent protection of the recovered animals could be better explained by an efficient plasma antioxidant defense, reflected in this study by a decrease in the plasma albumin concentrations than by an increase in efficiency in the enzymatic antioxidant system, as the enzymes GR and CAT are significantly decreased in this group (LP/NP-S) compared to control group (NP-S).

Exercise trained rats (NP-E and LP/NP-E) showed increase in plasma TBARs concentrations when compared to their sedentary counterparts, indicating that training led to an increase in the oxidative stress level, apparently higher for control trained rats (NP-E). Single bouts of exercise may lead to increase in the content of TBARs (Alessio and Goldfarb, 1988) and RCD (Radák et al., 1998). Aerobic exercise training, in turn may reduce TBARs (Alessio and Goldfarb, 1988) and RCD (Radák et al., 1999) after single bouts of exercise, but may be ineffective in changing significantly these biomarkers of oxidative damage at rest (Pereira et al., 1994; Radák et al., 1999). These discrepancies between the present and previous results (Pereira et al., 1994; Radák et al., 1999) concerning the effects of exercise training on biomarkers of oxidative damage measured of rest could be attributed to differences in the swimming training protocol performed by the rats.

In summary, the results presented here suggested that the improvement in body growth and muscle glucose metabolism after nutritional rehabilitation with physical exercise can be related with a decrease in the oxidative stress level, although the enzymatic antioxidant activity is also affected after four weeks of training.

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RESUMO

O presente estudo foi delineado para avaliar biomarcadores de estresse oxidativo em ratos submetidos ou não ao exercício físico durante a recuperação da desnutrição protéica. Para tanto, ratos Wistar alimentados com dieta hipoprotéica (LP = 6%) dos 30 dias até 90 dias, e então com dieta normoprotéica (NP = 17%) até 120 dias, foram separados em dois grupos: sedentário (S) e exercitado (E = natação 1h/dia, 5 dia/semana, dos 90 até os 120 dias de vida). Ratos alimentados com dieta normoprotéica foram usados como controles. Nossos resultados mostraram que o exercício físico durante recuperação nutricional, teve efeitos benéficos no ganho de peso corporal e no metabolismo glicídico muscular. Catalase e glutationa reductase (biomarcadores do sistema de antioxidante) mostraram-se significativamente reduzidas em todos os grupos quando comparadas ao grupo controle sedentário. A concentração plasmática de TBARs (biomarcadores do ataque oxidativo) também foi mais baixa nos ratos recuperados, sugerindo que a melhoria no crescimento corporal e no metabolismo de glicose do músculo após reabilitação nutricional com exercício físico pode estar relacionada com uma diminuição no nível de estresse oxidativo.

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