High-protein diet induces oxidative stress in rat brain: protective action of high-intensity exercise against lipid peroxidation

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Abstract

Introduction: It is well established that soy protein diets as well as aerobic exercise could promote antioxidant capacity and consequently reduce free radicals overproduction on brain. However, little is known regarding to the high-protein diets and high-intensity exercise (HIE) on brain oxidative stress markers.

Materials and Methods: A total of 40 male Wistar rats were randomly distributed in 4 experimental groups (n=10): normal-protein or high-protein diets with or without HIE for an experimental period of 12 weeks. Main oxidative damage markers in brain such as thiobarbituric acid-reactive substances (TBARs) and protein carbonyl content (PCC) were assessed. In addition, brain manganese superoxide dismutase (Mn-SOD), copper/zinc superoxide dismutase (CuZn-SOD) and catalase (CAT) antioxidant enzymes activity, and protein level of Nuclear factor erythroid 2 related factor 2 (Nrf2) were measured.

Results and discussion: Brain TBARs, PCC, tSOD, Mn-SOD, CuZn-SOD and CAT levels were higher in the high-protein compared to the normal-protein groups (all, p<0.05). In addition, the expression of Nrf2 protein was higher in the high-protein and HIE groups compared to the normal-protein and sedentary groups, respectively (both, p<0.01). A protein amount*HIE interaction was found on brain TBARs content, and tSOD and CuZn-SOD activity derived from a HIE-induced decrease in the high-protein but not in the normal-protein group (p<0.05).

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**Conclusión:** El consumo de una dieta hipertrofica produce altos niveles de peroxidación lipídica en el cerebro, a pesar de los altos niveles de capacidad enzimática antioxidante detectados. Sin embargo, el efecto del EAI podría atenuar los niveles de peroxidación lipídica producidos por el consumo de una dieta hipertrofica.

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**Conclusions:** The high-protein diets consumption produces higher levels of brain lipid peroxidation, in spite of higher levels of antioxidant enzymatic capacity. However, HIE may attenuate the deleterious effect of a high-protein diet on brain lipid peroxidation when both effects are combined.

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Keywords: Superoxide Dismutase. Catalase. Thiobarbituric Acid Reactive Substances. NF-E2-Related Factor 2. Soybean Proteins. Hypertrophy.

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**Abbreviations**

ROS: Reactive oxygen species.
SOD: Superoxide dismutase.
CAT: Catalase.
TBARs: Thiobarbituric acid reactive substances.
Nrf2: Nuclear factor erythroid 2 related factor 2.
HIE: High intensity exercise.
PCC: Protein carbonyl content.
SEM: Standard error of the mean.
ANOVA: Analysis of variance.

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**Introduction**

Reactive oxygen species (ROS) are by-products of aerobic cellular metabolism that can induce oxidative stress. The major antioxidant enzymes in the rat brain are superoxide dismutase (SOD) and catalase (CAT). These enzymes play an important role in order to avoid ROS deleterious effects. Indeed, the imbalance between ROS generation and antioxidant capacity leads to oxidative stress. In addition, the oxidative damage repair systems are important in order to minimize the dangerous effects of high production of ROS. The most common markers to investigate the oxidative damage on lipids and proteins are the production of thiobarbituric acid reactive substances (TBARs) and protein carbonyls, respectively.

Brain is particularly vulnerable to ROS production because it only accounts for a ~2% of total body weight and metabolizes 20% of total body oxygen, with a limited amount of antioxidant capacity. Furthermore, lipid peroxidation leads to the production of toxic compounds such as aldehydes or dienals (e.g., 4-hydroxynonenal), which in turn may cause neuronal apoptosis. In consequence, brain oxidative stress has been suggested to play a role in neurodegenerative disorders such as Parkinson’s disease, Alzheimer’s disease, multiple sclerosis, and amyotrophic lateral sclerosis.

The effects of high-protein diets have been of great interest in the last decade. Supplementation with high-protein diets is often used to improve physical status causing an effective reduction in body weight, fat deposition and improving plasma lipid profile. Some studies have shown the beneficial effects of high-protein diets on rodent brain such as protecting against cerebral ischemia and reducing apoptosis in the ischemic cortex. Nevertheless, little is known regarding the effects of high-protein diet on brain oxidative stress markers. Therefore, it is of importance to clarify the physiological effects of a high-protein diet on brain oxidative stress.

Since the 1990s, there has been evidence about the benefits of exercise on brain function, which could play an important preventive and therapeutic role on oxidative stress-associated brain disease. Exercise may increase the level, activation, and mRNA expression of endogenous antioxidant systems in the brain thus down-regulating the levels of the oxidative damage. Recent studies have observed that chronic exercise activates the Nuclear factor erythroid 2 related factor 2 (Nrf2) in human skeletal muscle and rat kidney, whereas acute exercise promotes myocardial Nrf2 function. However, the mechanisms of Nrf2 activation have not been investigated in the context of brain after a high intensity exercise (HIE).

Despite the numerous studies that have analyzed the effects of different intensities and types of exercise on brain oxidative stress, the findings are still unclear or inconclusive regarding high-intensity training. To the best of our knowledge, no previous studies have investigated the specific combined effects of high-protein diet and HIE on brain oxidative stress. Therefore, in order to deepen this knowledge, the purpose of the present study was to investigate the effects of high-protein diet and HIE, based on hypertrophy resistance training, on brain oxidative stress markers and antioxidant enzyme defense systems.

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**Materials and methods**

**Animals and experimental design**

A total of forty albino male Wistar rats were randomly distributed in 4 experimental groups derived of 2 interventions: protein amount of the diet (normal-protein vs. high-protein) (n=20) and HIE (seden-
tary vs. HIE) (n=20). Each specific intervention (i.e. normal-protein sedentary, normal-protein exercise, high-protein sedentary, high-protein exercise) was developed in groups of 10 rats and the experimental period lasted 12 weeks.

The animals (aged 8 weeks) had initial body weights of 163±19 g, had free access to type 2 water (>15 MΩ cm) and consumed the diets ad libitum. Food intake and body weight were measured daily and weekly, respectively, for all animals. The rats were located in a well-ventilated thermostatically controlled room (21±2°C). A 12:12 reverse light-dark cycle (08.00-20.00 h) was implemented in order to allow exercise training during the day. At the end of the experimental period, the animals were anesthetized with ketamine-xylazine and sacrificed by cannulation of the abdominal aorta. Brains were extracted, weighed and immediately frozen in liquid N2 and kept at -80°C until further analyses. Carcass weight was recorded. Carcass is the weight of the slaughtered animal’s cold body after being skinned, bled and eviscerated, and after removal of the head, the tail and the feet.

All experiments were performed according to Directional Guides Related to Animal Housing and Care (European Community Council, 1986)24. All procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada.

Experimental diets

Formulation of the experimental diets is presented in table I. All diets were formulated to meet the nutrient requirements of rats25 following the recommendations of the American Institute of Nutrition (AIN-93M)26, with slight modifications. We selected a 45% protein level for the high-protein diet at the expense of carbohydrates (wheat starch) following previously established and similar studies in rats27-29. A 10% protein content was chosen for the normal-protein diet groups. A commercial soy-protein isolate was used as the only protein source since it is widely available.

High-intensity exercise

The experimental groups were trained following a resistance training protocol in a motorized treadmill (Panlab Treadmills for 5 rats, LE 8710R) with bagged weights tied with a cord to the tail. This type of training was chosen in order to reproduce the type of exercise performed by people interested in gaining muscle mass and strength who usually combine high-protein diets with HIE29 (see Table II). Therefore, our training protocol follows the established principles for human strength training, involving weights, repetitions, and sets to maximize muscle gain30.

The training groups exercised on alternate days (3-4 sessions/week) at a constant speed of 35 cm/s during the whole experimental period (12 weeks) in their dark phase. Prior to exercise training, animals were adapted to the treadmill on a daily basis for 1 week, the first three days without weight and the last four days with 20% of their body weight. The training protocol used in the present study had been previously developed and deeply described by Aparicio et al29. The entire training process was designed and controlled by sport scientists in collaboration with experienced researchers trained to work with rats.

Animals in the control group were managed identically to exercising animals, with the exception of exercise training.

| Table I
<table>
<thead>
<tr>
<th>Formulation of the experimental diets</th>
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<tr>
<td><strong>Nutritional Composition</strong> (g/100g DM)</td>
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<tr>
<td></td>
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<tr>
<td>Soy protein supplement</td>
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<tr>
<td>Mineral mix (AIN-93M-MX)</td>
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<tr>
<td>Vitamin mix (AIN-93-VX)</td>
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<td>Fat (olive oil)</td>
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<td>Choline chloride</td>
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<td>Cellulose</td>
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<tr>
<td>Starch</td>
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<td>Methionine</td>
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<td>Sucrose</td>
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| Table II
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<th>Details of the high-intensity exercise program</th>
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<td><strong>Week</strong></td>
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<td>12</td>
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</tbody>
</table>

RM, repetition maximum.
Chemical analyses

Brain homogenate preparation for oxidative damage markers and antioxidant activity

Brain aliquots (1 g) were collected and processed under anti-oxidative conditions. Samples were homogenized in 50 mM phosphate buffer (pH 7.8) containing 0.1% Triton X-100 and 1.34 mM diethylenetriaminepentaacetic acid (DETAPAC) (1:10w/v) using a Micra D-1 homogenizer (ART moderne labortechnik) at 18,000 rpm during 30 sec followed by treatment with Sonoplus HD 2070 ultrasonic homogenizer (Bandelin) at 50% power for 10 sec. Homogenates were centrifuged at 19,621 g, 4°C for 45 min (BECKMAN, Allegra 64R), and the supernatants were used to determine the oxidative damage markers and the antioxidant enzymes activity.

Oxidative damage markers

Thiobarbituric acid-reactive substances (TBARs)

Thiobarbituric acid reactive substances (TBARs) were used as a marker of lipid peroxidation. Brain supernatants were used to determine lipid peroxidation by measuring TBARs as described by Ohkawa et al.11. The results were expressed as nmol of Malondialdehyde per mg of protein (nmolMDA/mg) from duplicate reactions.

Protein carbonyl content (PCC)

Total carbonyl contents in brain were used as a biomarker of protein oxidation. The contents were determined spectrophotometrically using a protein carbonyl colorimetric assay Kit (Cayman, USA) according to the method of Levine et al.12. Results were expressed as nmol of reactive carbonyl compounds/mg protein of tissue.

Antioxidant enzyme activity

Total SOD activity was measured as described by Ukeda et al.13 adapted to a micro-plate reader. Mn-SOD activity was determined by the same method after treating the samples with 4 mM KCN for 30 min (final concentration of KCN 1 mM was set for all the samples). CuZn-SOD activity was determined by subtracting the Mn-SOD activity from the iSOD activity. One unit of SOD activity was defined as the enzyme needed to inhibit 50% 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction. Catalase activity (CAT) was measured by the method of Aebi14 monitoring the disappearance of H2O2 in the presence of brain homogenate at 240 nm and was expressed as µmol of H2O2 consumption per minute per milligram of protein. Protein concentration was determined by the method of Lowry15.

Western blotting analysis

Brain aliquots (1 g) were collected and processed under anti-oxidative conditions. Samples were homogenized (1:10 w/v) in 20 mM Tris-HCl (pH 8.0) containing 0.1% octylphenoxypolyethoxyethanol (Igepal), 100 mM ethylene glycol tetraacetic acid (EGTA), 100 mM dichlorodiphenyltrichloroethane (DDT), 100 mM sodium orthovanadate, 2 mM AEBSF, 1 mM EDTA, 130 µM Bestatin, 14 µM E-64, 1 µM Leupeptin and 0.3 µM Aprotinin. Samples were homogenized with a Micra D-1 homogenizer (ART moderne labortechnik) at 18,000 rpm for 30 sec followed by treatment with a Sonoplus HD 2070 ultrasonic homogenizer (Bandelin) at 50% power for 10 sec. Homogenates were centrifuged at 19,621 g, 4°C for 45 min (BECKMAN, Allegra 64R), and the supernatants were collected and stored at -80°C until further use. The protein concentration was measured by the method of Lowry et al.15. Samples (40 µg protein) were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electro transferred to reinforced cellulose nitrate membranes (Schleicher & Schuell, Dassel, Germany) using a Mini Trans-Blot cell system (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% non-fat dry powdered milk dissolved in Tris-buffered saline TWEEN-20 (TBS-T) for 2 h at room temperature. After blocking, the membranes were incubated with primary polyclonal rabbit anti-Nrf2 antibody (1:1500, Abcam Cambridge, USA) overnight at 4°C. A goat anti-rabbit immunoglobulin G associated to an enhanced chemiluminescence reagent mixture (Western Lightning, PerkinElmer Inc., Waltham, MA, USA) was used to estimate the amount of protein expressed using a Fujifilm Luminescent Image Analyzer LAS-4000 mini System (Fujifilm, Tokyo, Japan). Equality of protein loading was checked standardizing the bands to β-actin (1:2000, Abcam Cambridge, USA). The optical density of the protein bands was measured and quantified by Image J software. Results were expressed in relative density units.

Statistical analyses

Results are presented as mean and standard error of the mean (SEM), unless otherwise indicated. The effects of the dietary protein amount (normal-protein vs. high-protein) and the HIE (sedentary vs. HIE) on food intake, carcass weight, final body weight, brain weight, and oxidative stress markers including their two-way interactions, were analyzed by two-way factorial analysis of variance (ANOVA), with the protein...
amount and the exercise as fixed factors. Two-ways interactions terms were introduced into the models to test interactions between both interventions (i.e. protein amount*HIE). A significant $p$ value indicates that there are differences at least between two of the groups. In addition, multiple comparisons between groups were made considering Bonferroni’s adjustment in order to identify which groups the differences were significant (e.g. normal-protein sedentary vs. high-protein and exercise).

All analyses were performed using the Statistical Package for Social Sciences (IBM-SPSS for Mac, version 22.0, Amonk, NY), and the level of significance was set at 0.05.

Results

Final body weight, carcass weight, brain weight and food intake

The effects of the high-protein diet and HIE on final body weight, carcass weight, brain weight and food intake are shown in table III.

Both high-protein and HIE groups significantly decreased food intake when compared to the normal-protein and sedentary groups, respectively (both, $p<0.001$).

No significant differences between groups were observed on final body weight, carcass weight, and brain wet mass weight as expressed in absolute value and brain wet mass weight when referred to the final carcass weight.

Oxidative stress markers

The effects of the high-protein diet and HIE on brain oxidative stress markers are shown in table IV.

High-protein groups significantly increased brain TBARs content and brain protein carbonyl content (PCC) when compared to the normal-protein groups ($p=0.042$ and $p=0.006$, respectively).

High-protein groups significantly augmented brain tSOD, Mn-SOD, CuZn-SOD and CAT activity when compared to the normal-protein groups (all, $p<0.01$).

Significant protein amount*HIE interactions were found for brain TBARs content and CuZn-SOD derived from a HIE-induced decrease in lipid peroxidation and antioxidant activity in the high-protein group that was not observed in the normal-protein group ($p=0.018$ and $p=0.007$, respectively).

Figure 1 shows the effects of the protein amount and HIE on the expression of Nrf2 protein in rat brain.

Both high-protein and HIE groups significantly increased the expression of brain Nrf2 protein when compared to the normal-protein and the sedentary groups, respectively ($p<0.001$ and $p=0.004$, respectively).

Discussion

The purpose of the present study was to analyze the influence of high-protein diet and HIE on brain oxidative stress markers. The main findings of this study were: 1) high-protein diet increased TBARs and PCC concentrations, CuZn-SOD and CAT activity and the expression of Nrf2 protein, and 2) HIE increased CAT activity and the expression of Nrf2 protein. Overall, our findings displayed controversial effects in terms of high-protein diets on brain oxidative stress. The high-protein, low carbohydrate, unbalanced diet, groups appear to promote antioxidant capacity, although this may be in response to higher oxidative damage when compared to the normal-protein groups.

<table>
<thead>
<tr>
<th>Table III</th>
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<tbody>
<tr>
<td>Effects of the dietary protein amount and high-intensity exercise on final body weight, carcass weight, food intake and brain weight</td>
</tr>
<tr>
<td>Normal protein</td>
</tr>
<tr>
<td>Sedentary</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
</tr>
<tr>
<td>Final body weight (g)</td>
</tr>
<tr>
<td>Carcass weight (g)</td>
</tr>
<tr>
<td>Brain (g)</td>
</tr>
<tr>
<td>Brain (g/100g body weight)</td>
</tr>
<tr>
<td>Brain (g/100g carcass weight)</td>
</tr>
</tbody>
</table>

SEM, standard error of the mean. Values expressed as mean of 10 rats. The same letter in the same row indicates no significant difference between groups ($p>0.05$).
High-protein diet induces oxidative stress in rat brain: Protective action of high-intensity exercise against lipid peroxidation

**Table IV**

Effects of the dietary protein amount and high-intensity exercise on brain oxidative stress markers.

<table>
<thead>
<tr>
<th></th>
<th>Normal protein</th>
<th>High protein</th>
<th>SEM</th>
<th>Protein amount</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sedentary</td>
<td>Exercise</td>
<td>Sedentary</td>
<td>Exercise</td>
<td>Sedentary</td>
</tr>
<tr>
<td>TBARs (nmol MDA/mg protein)</td>
<td>19.684a</td>
<td>23.553a,b</td>
<td>27.768b</td>
<td>22.875a,b</td>
<td>0.880</td>
</tr>
<tr>
<td>PCC (nmol/mg protein)</td>
<td>3.496a</td>
<td>2.440a</td>
<td>4.878a</td>
<td>4.948a</td>
<td>0.332</td>
</tr>
<tr>
<td>tSOD (U/mg protein)</td>
<td>137.474a</td>
<td>154.578a,b</td>
<td>192.837c</td>
<td>173.469b,c</td>
<td>3.551</td>
</tr>
<tr>
<td>Mn-SOD (U/mg protein)</td>
<td>63.990a</td>
<td>71.828a,b</td>
<td>79.299b</td>
<td>80.359b</td>
<td>1.912</td>
</tr>
<tr>
<td>CuZn-SOD (U/mg protein)</td>
<td>73.484a</td>
<td>82.750a</td>
<td>113.538b</td>
<td>93.110a</td>
<td>2.578</td>
</tr>
<tr>
<td>CAT (µmolH2O2/min/mg protein)</td>
<td>2.852a</td>
<td>4.274b</td>
<td>3.526a,b</td>
<td>5.640c</td>
<td>0.126</td>
</tr>
</tbody>
</table>

SEM, standard error of the mean; TBARs, thiobarbituric acid-reactive substances; PCC, protein carbonyl content; tSOD, total superoxide dismutase; Mn-SOD, manganese superoxide dismutase; CuZn-SOD, copper and zinc superoxide dismutase; CAT, catalase. Values expressed as mean of ten rats. The same letter in the same row indicates no significant difference between groups (p > 0.05).

**Fig. 1.**—Effects of the high-protein diet and high-intensity exercise on brain Nrf2 protein levels, n=8. The representative western blots show the Nrf2 bands (left lines) and the β-actin bands used as a loading control (right lines). Annotation indicates significant effect of a = exercise, b = anabolic androgenic steroids. p < 0.05.

NS, Normal-protein and Sedentary; NE, Normal-protein and Exercise; HS, High-protein and Sedentary; HE, High-protein and Exercise; Nrf2, Nuclear factor erythroid 2 related factor 2.

**Food consumption, body weight and body composition**

Food intake is markedly affected by diet composition and physical activity. Few studies in animals as well as in humans have illustrated that high-protein diets provide higher satiety levels than others macronutrients, thus leading to a decrease of food intake. Likewise, the decreased food intake may be attributed to the HIE protocol carried out, which led to a high stress situation resulting in the higher levels of corticosterone. These assertions are in agreement with our findings that the high-protein and the HIE groups displayed a reduced food intake when com-
pared to the normal-protein and the sedentary groups, respectively.

High-protein and brain oxidative stress

Despite the beneficial effects of high-protein diets on rodent brain, little is known about its effects on brain oxidative stress. However, there have been some studies on other organs that have shown the oxidative effects of high-protein diet consumption. In a study performed in Zucker obese rats, an increased dietary protein intake induced oxidative stress in the kidney and aorta, at least partially due to increased expression of NAD(P)H oxidase components. Others have suggested that high-protein diet intake may cause an imbalance between ROS generation and the capacity of the antioxidant defense system in digestive organs of mice such as duodenum, liver and pancreas, which leads to an induction of oxidative stress. This imbalance is reflected with a diminished antioxidant defense system and increased concentration of malondialdehyde (MDA), a superoxide anion and the precursor of most ROS and mediator in oxidative chain reactions. Additionally, in a study performed by Sophia et al., high-protein diet consumption caused a significant alteration in the antioxidant status of pancreas by increasing lipid peroxidation and decreasing the content of reduced glutathione, vitamin C, the activity of SOD, CAT and glutathione peroxidase. In the present study, high-protein diets appeared to increase antioxidant activity as well as the overexpression of Nrf2, although this may be attributed to the production of higher levels of brain lipids and protein oxidation. The higher the brain lipid peroxidation levels observed in the high-protein groups, the higher the antioxidant enzyme activity produced by a high-protein diet consumption.

High intensity exercise and brain oxidative stress

Controversial findings in the literature have been observed regarding HIE on brain oxidative stress. On one hand, some authors suggest that intermittent anaerobic exercise and acute exhausted exercise (HIE) increases brain antioxidant capacity and does not induce lipid peroxidation. On the other hand, ROS production may be strongly and persistently increased under HIE, and the antioxidant response may not be effective to reset the system to the original level of brain redox homeostasis.

In the present study, CAT activity levels increased after 12 weeks of HIE. However, HIE did not alter Mn-SOD and CuZn-SOD brain activity. In a previous study carried out in human plasma, CAT activity did not change in response to resistance training until the participants showed symptoms of overtraining. In addition, Margonis et al. observed that in a 12-week human resistance-training program involving 3-weeks training (4 times a week) periods and a 3-week recovery period, up-regulation of CAT activity coincided with the maximum training load and performance decrement. The training protocol carried out in this study was found to induce overtraining and may explain our findings related to the increased CAT activity. Nevertheless, it should be taken into consideration that such activity may not represent a significant proportion of brain total antioxidant activity due to its low values.

In spite of the controversial findings regarding the HIE, acute exercise promotes free radicals and ROS generation, which may lead to lipid peroxidation. In the present study, the HIE protocol induced lower lipid peroxidation when a high-rather than a normal-protein diet was consumed by the animals. Therefore, the magnitude of ROS generation and lipid peroxidation was not only a result the exercise mode, intensity and duration, but also high-protein levels in the diet.

A recent study has reported that acute exercise induces ROS production and activates Nrf2 in the myocardial tissue. Furthermore, Nrf2 might be a potential target in order to protect heart tissue from diseases such as ischemia/reperfusion injury and myocardial infarction induced by high levels of ROS in the myocardium. These results concur with the present study in that Nrf2 levels were higher in the HIE compared to the sedentary group. Thus, Nrf2 may develop a neuroprotective effect after HIE in rat brain.

The present study has several limitations that need to be mentioned. First, it may be beneficial to compare our results with different sources of protein for the interpretations of the present findings. Second, the protein carbonyl assay could suffer confounding factors. However, it is important to highlight that this is the first study to analyze the effects of a high-protein diet and a HIE, based on a hypertrophy resistance training protocol, on brain oxidative stress.

Conclusions

Overall, our results suggest that consumption of high-protein diets cause oxidative damage to the brain by means of lipid and protein oxidation. Such increased oxidative damage may in turn induce the endogenous antioxidant defense system. HIE did not worsen the deleterious effects caused by high-protein diet and may be an efficient way to protect the brain against high dietary protein aggression.

Acknowledgments

This study was supported by the project DEP2008-04376 from the Ministry of Science and Innovation and grants from the Spanish Ministry of Education (D.C.M. grant number AP2009-3173), (E.N. grant number AP2009-5033), and is part of the PhD Thesis
of Daniel Camilletti Moirón “Effects of high-protein diets, high-intensity exercise and anabolic androgenic steroids on brain and kidney oxidative stress markers”. The authors want to gratefully all the researchers of the Department of Physiology for their collaboration.

Conflict of interest

The authors declare no conflict of interests.

Authorship

The contributions of the authors were as follow: V.A., J.M.P., M.L. and P.A. designed the trial; D.C.M., V.A., E.N. and G.K. conducted the feeding and exercise experiments; D.C.M., G.M., R.M. and A.A. were responsible for the laboratory analysis; D.C.M. analyzed the data and wrote the manuscript; V.A., E.N., G.M., R.M., G.K., A.A., J.M.P., M.L. and P.A. revised the manuscript.

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