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CONSEQUENCES OF HIGH-PROTEIN DIETS CONSUMPTION, ANABOLIC ANDROGENIC STEROIDS ADMINISTRATION AND HIGH-INTENSITY EXERCISE ON BRAIN AND KIDNEY OXIDATIVE STRESS MARKERS IN WISTAR RATS

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CONSECUENCIAS DEL CONSUMO DE DIETAS HIPERPROTEICAS, DE LA ADMINISTRACIÓN DE ANABOLIZANTES ANDROGÉNICOS ESTEROIDEOS Y DE LA PRÁCTICA DE UN ENTRENAMIENTO DE ALTA INTENSIDAD SOBRE MARCADORES DE ESTRÉS OXIDATIVO EN CEREBRO Y RIÑÓN EN RATAS WISTAR

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Tesis Doctoral con Mención Internacional presentada por:

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Que la Tesis Doctoral Internacional titulada: "**Consequences of high-protein diets consumption, anabolic androgenic steroids administration and high-intensity exercise on brain and kidney oxidative stress markers in Wistar rats/Consecuencias del consumo de dietas hiperproteicas, de la administración de anabolizantes androgénicos esteroideos y de la práctica de un entrenamiento de alta intensidad sobre marcadores de estrés oxidativo en cerebro y riñón en ratas Wistar**", que presenta D. Daniel Camiletti Moirón al superior juicio del Tribunal que designe la Universidad de Granada, ha sido realizada bajo mi dirección durante los años 2011-2015, siendo expresión de la capacidad técnica e interpretativa de su autor en condiciones tan aventajadas que le hacen merecedor al Titulo de Doctor, siempre y cuando así lo considere el citado Tribunal.

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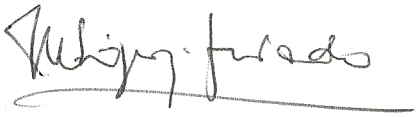

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En Granada, 26 de febrero de 2015

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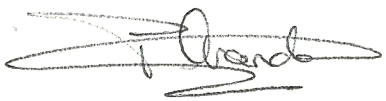

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El doctorando, **D. DANIEL CAMILETTI MOIRÓN**, y los directores de la Tesis, Dra. **MARÍA LÓPEZ-JURADO ROMERO DE LA CRUZ, Dr. JESÚS MARÍA PORRES FOULQUIE** y **Dra. PILAR ARANDA RAMÍREZ**, garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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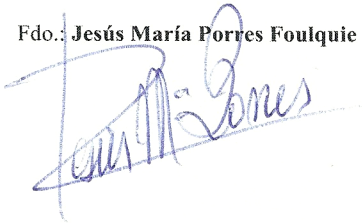

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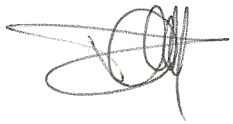

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El doctorando D. **Daniel Camiletti Moirón**, ha realizado esta Tesis Doctoral como beneficiario de una beca-contrato con cargo al programa de Formación de Profesorado Universitario (FPU) del Ministerio de Educación, Cultura y Deporte, por *Resolución de 28 de abril de 2011, Orden EDU//1203/2011*, por la que se concedieron ayudas para becas y contratos del programa FPU, en el marco del Estatuto del personal investigador en formación, del Programa Nacional de Formación de Recursos Humanos de Investigación, del Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica 2008-2011. De acuerdo a la *Orden EDU/3083/2009, de 6 de noviembre de 2009 (Boletín Oficial del Estado del 17 de noviembre)*, por la que se convocaron ayudas para becas y contratos en el marco del Estatuto del personal investigador en formación.

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PROYECTO: Efectos de esteroides anabolizantes y de una dieta basada en suplementos de lactosuero o proteína vegetal sobre parámetros musculares, hepáticos y renales en ratas sometidas a entrenamiento de fuerza. Acrónimo: "NutriHealth". DEP2008-04376.

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Y, así mismo, dichos resultados forman parte de los siguientes congresos y publicaciones científicas:

Congresos

Camiletti-Moirón D, Aparicio VA, Nebot E, Medina G, Martínez R, Kapravelou G, Andrade A, Bernier M, de Cabo R, Porres JM,

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Camiletti-Moirón D, Aparicio VA, Nebot E, Medina G, Martínez R, Kapravelou G, Andrade A, Porres JM, López-Jurado M, Aranda P. High-intensity exercise reduces renal oxidative effects of anabolic androgenic steroids. *XXXVII Congreso de la Sociedad Española de Ciencias Fisiológicas – (SECF).* Granada 24-26 de Septiembre. 2014. Published in Acta Physiologica, September 2014, Vol. 212, Supplement 698, page 31. ISSN: 1748-1716; IF: 4,5; Q1. y en Libro de Actas: Abstracts of the 37th Congress of the Spanish Society of Physiological Sciences (SECF). Spain Granada 24-26 de Septiembre. Págs. 70-71. 2014 ISBN: 978-84-697-1203-0; Depósito Legal: GR 1649-2014

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Nutrición Hospitalaria. 2015; 31(2):866-874. Area: Nutrition & Dietetics (62/78), Q4. IF: 1.250. ISSN: 0212-1611.

Aparicio VA, Tassi M, Nebot E, Camiletti-Moirón D, Ortega E, Porres JM, and Aranda P. High-intensity exercise may compromise renal morphology in rats. *International Journal of Sports Medicine*. 2014. 35(8):639-44. doi: 10.1055/s-0033-1354383. Area: Sport Sciences (18/84), Q1. IF: 2.268. ISSN: 0172-4622.

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A mi madre

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ABBREVIATIONS [ABREVIATURAS]

AAS: Anabolic androgenic steroids

ANOVA: Analysis of variance

CAT: Catalase

CK: creatine kinase

CKD: Chronic kidney disease

CuZn-SOD: Cooper/zinc superoxide dismutase

DDT: Dichlorodiphenyltrichloroethane

DETAPAC: Diethylenetriaminepentaacetic acid

DNPH: 2,4-dinitrophenylhydrazine

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol tetraacetic acid

GFAP: Glial fibrillary acidic protein

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: Reduced glutathione

GSSG: Oxidized glutathione

GST: Glutathione S-transferase

HIE: High intensity exercise

HNE: 4-hydroxynonenal

HO1: Hemoxygenase-1

LDH: Lactate dehydrogenase

LDL: Low-density lipoprotein

MDA: Malondialdehyde

Mn-SOD: Manganese superoxide dismutase

N: Nitrogen

NADPH: nicotine adenine dinucleotide phosphate

NF-κβ: Nuclear factor kappa-β

NQO1: NAD(P)H dehydrogenase, Quinone 1

Nrf2: Nuclear factor erythroid 2 related factor 2

PCC: Protein carbonyl content

RM: Repetition maximum

ROS: Reactive oxygen species

SEM: Standard error of the mean

SOD: Superoxide dismutase

STAT3: Signal transducer and activator of transcription 3

TAC: Total antioxidant capacity

TBARs: Thiobarbituric acid reactive substances

t-SOD: Total superoxide dismutase

γGCLC: γ-glutamyl cysteine ligase-catalytic

SUMMARY [RESUMEN]

SUMMARY

In the last decades, the combination of high-intensity exercise, high-protein diets and the administration of anabolic androgenic steroids by some individuals or sports practitioners have increased in popularity. Likewise, in the last years, soy protein has become in the main source of protein consumed by this type of practitioners, probably due to its antioxidant or healthy properties, rather than other source of protein such as casein or whey.

Brain is particularly vulnerable to reactive oxygen species 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. Brain is also considered highly sensitive to oxidative damage, because it possesses high amounts of phospholipids and polyunsaturated fatty acids both of which are highly susceptible to oxidants. On the other hand, kidney is one of the most imperative tissues in the organism due to its straight relation with other systems (e.g. cardiovascular system) making its impeccable functioning highly valuable. Oxidative stress and inflammation play a critical role in the

pathogenesis and progression of chronic kidney disease. Thus, an improper or maladaptive activation of oxidative processes may be chronically present in pathological situations, such as uraemia, contributing to chronic cell and kidney injury.

The overall objective of this PhD Thesis was to analyse the brain and kidney antioxidant defence system and oxidative damage as well as the renal morphology effects of high-protein diet, high-intensity exercise, and anabolic androgenic steroids administration in Wistar rats.

The main findings from this Thesis suggest that: 1) High-protein diets may cause oxidative damage to the brain by means of lipid and protein oxidation, which could explain the induction of the endogenous antioxidant defence system. 2) High-intensity exercise protocol 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. 3) High-protein diets led to a prooxidant status at kidney level. However, the beneficial effect of high-intensity exercise observed on brain, did not appear at kidney level. 4) The high-intensity exercise protocol displayed a worse renal morphological profile, which might be associated with a higher risk for incidence of kidney disease in the long-term. The stress induced by the type of exercise performed in the present Thesis could be

related to this worse morphological renal status. 5) Under our experimental conditions, the present results suggest that high-intensity exercise reduce the negative effects of anabolic androgenic steroids on brain redox status. High-intensity exercise also improved the harmful effects caused by the anabolic androgenic steroids administration on kidney lipid and protein oxidation.

The results of the current Thesis underline that high-protein diets intake and the anabolic androgenic steroids administration instigated brain and kidney damage by means of the induction of lipid and protein oxidation. Despite the apparently beneficial effect of high-intensity exercise among the others two interventions studied, cautiousness should be taken with this protocol regarding to brain and kidney overproduction of their antioxidant defence systems.

RESUMEN

En las últimas décadas, la combinación de un ejercicio de alta intensidad y la administración de anabolizantes androgénicos esteroideos ha aumentado en popularidad en algunos individuos o deportistas. Del mismo modo, en los últimos años, la proteína de soja se ha convertido en la principal fuente de proteína consumida por este tipo de practicantes, probablemente por sus propiedades antioxidantes y saludables, en lugar de otro tipos de fuentes proteicas como la caseína o la proteína de lactosuero.

El cerebro es particularmente vulnerable a la producción de especies reactivas de oxígeno ya que sólo representa un ~2% del peso corporal total y metaboliza el 20% del oxígeno corporal total, con una cantidad limitada de la capacidad antioxidante. El cerebro también se considera altamente sensible al daño oxidativo porque posee altas cantidades de fosfolípidos y ácidos grasos poliinsaturados, los cuales son altamente susceptibles a oxidarse. Por otra parte, el riñón es uno de los tejidos más imperativos del organismo debido a su relación directa con otros sistemas (ej. sistema cardiovascular) que hacen de gran valor su

impecable funcionamiento. El estrés oxidativo y la inflamación desempeñan un papel crítico en la patogénesis y progresión de la enfermedad renal crónica. Por lo tanto, una activación inadecuada o una mala adaptación de los procesos oxidativos puede hacer crónicas ciertas situaciones patológicas, tales como uremia, contribuyendo al daño crónico celular y renal.

El objetivo general de esta Tesis Doctoral ha sido analizar los efectos de las dietas hiperproteicas de soja, del ejercicio de alta intensidad y de la administración de anabolizantes androgénicos esteroideos sobre el sistema de defensa antioxidante y daño oxidativo cerebral y renal, así como la morfología renal en ratas Wistar.

Los principales resultados de esta Tesis sugieren que: 1) Las dietas altas en proteínas pueden causar daño oxidativo en el cerebro por medio de la oxidación de lípidos y proteínas, lo que podría explicar la inducción del sistema de defensa antioxidante endógeno. 2) El protocolo de ejercicio de alta intensidad mejoró los efectos nocivos provocados por la dieta alta en proteínas, y puede ser un medio eficaz para proteger el cerebro contra la agresividad producida por dicha dieta. 3) Las dietas altas en proteínas conducen a un estado prooxidante a nivel renal. Por otra parte, el efecto beneficioso del ejercicio de alta intensidad observado

en el cerebro, no se mostró a nivel del riñón. 4) El protocolo de ejercicio de alta intensidad mostró un peor perfil morfológico renal, lo que podría estar asociado con un mayor riesgo de incidencia de enfermedades renales a largo plazo. El estrés inducido por el tipo de ejercicio realizado en la presente Tesis podría estar relacionado con este peor estado morfológico renal. 5) Bajo nuestras condiciones experimentales, los resultados sugieren que el ejercicio de alta intensidad reduce el efecto negativo de los anabolizantes androgénicos esteroideos sobre el estado redox del cerebro. El ejercicio de alta intensidad también mejoró el daño producido por la administración de anabolizantes androgénicos esteroideos en la oxidación de lípidos y proteínas del riñón.

Los resultados de la presente Tesis doctoral subrayan que el consumo de dietas ricas en proteínas y la administración de anabolizantes androgénicos esteroideos desencadenan daño cerebral y renal a través de la inducción de la oxidación de lípidos y proteínas. A pesar del aparente efecto beneficioso del ejercicio de alta intensidad frente a las otras dos intervenciones ensayadas, se debe tener cautela con este protocolo respecto a la estimulación del sistema de defensa antioxidante tanto del cerebro como del riñón.

INTRODUCTION [INTRODUCCIÓN]

INTRODUCTION [INTRODUCCIÓN]

Oxidative stress

Free radical

Electrons within atoms and molecules occupy regions of space known as orbitals. Each orbital can hold a maximum of two electrons. For example, the two electrons that form a covalent bond occupy the same orbital, but have opposite spins. If an orbital contains only one electron, that electron is said to be unpaired, and therefore may be seen as having one or more "dangling" covalent bonds. Thus, a free radical is defined as any species capable of independent existence (hence the term ‘free’) that contains one or more unpaired electrons (1). However, when 2 free radicals share their unpaired electrons, non-radical forms are created. With some exceptions, these “dangling” bonds make free radicals highly chemically reactive towards other substances, or even towards themselves: their molecules will often spontaneously dimerize or polymerize if they come in contact with each other. Most radicals are reasonably stable only at very low concentrations in inert media or in a vacuum.

Free radicals play an important role in a number of biological processes. Many of these are necessary for life, such as the intracellular killing of bacteria by phagocytic cells such as granulocytes and macrophages. Researchers have also implicated free radicals in certain cell signalling processes, (2) known as redox signalling. The three most important oxygen-centred free radicals are superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (HO•) and hydrogen peroxide (H_2O_2). These free radical are more reactive than O_2 and grouped are known as reactive oxygen species (ROS) (1). They derive from molecular oxygen under reducing conditions. A notable example of a these free radical is the hydroxyl radical (HO•), a molecule that is one hydrogen atom short of a water molecule and thus has one bond "dangling" from the oxygen. Two other examples are the carbene molecule ($:CH_2$), which has two dangling bonds; and the superoxide anion ($O_2^{\bullet -}$), the oxygen molecule O_2 with one extra electron, which has one dangling bond. However, because of their reactivity, these same free radicals may participate in undesirable side reactions resulting in cell damage. In contrast, the hydroxyl anion (HO^-), the oxide anion (O^{2-}) and the carbenium cation (CH_{3+}) are not radicals, since the bonds that may appear to be dangling are in fact resolved by the addition or removal of electrons (3).

Excessive amounts of these free radicals can lead to cell injury and death, which may contribute to many diseases such as cancer, stroke, myocardial infarction, diabetes and major disorders. Many forms of cancer are thought to be the result of reactions between free radicals and DNA, potentially resulting in mutations that can adversely affect the cell cycle and potentially lead to malignancy (4). Thus, the condition in which the delicate balance existing between free radicals production and their subsequent amelioration via the antioxidant defence system becomes skewed in favour of free radical expression is named oxidative stress (5). Therefore, oxidative damage repair systems are important in order to minimize the dangerous effects of pro-oxidant ROS (6). This imbalance occurs due to two reasons; either by the overproduction of ROS such as the superoxide radical ($O_2^{\bullet -}$) or hydroxyl radical (HO•), or by the diminution in the elimination of ROS by oxidant defence mechanisms (7). Thus, in order to struggle oxidative stress, cells possess their own antioxidant defence machinery that includes three major endogenous antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidases (GPx), and catalase (CAT) (8,9), and a number of other non-enzyme molecules, such as reduced and oxidized glutathione (GSH/GSSG). Among these three enzymes, SOD catalyses

the conversion of O_2^- to H_2O_2, while CAT converts H_2O_2 into H_2O and O_2. Using GSH, GPx, catalyses the reduction of two molecules of peroxide to produce GSSG and water (10). Besides these enzymes, glutathione S-transferase (GST), glutathione reductase (GR), as well as non-enzymatic glutathione (GSH) and glutathione disulphide (GSSG), in combination play various important roles in the series of antioxidant defence activities. Because free radicals are necessary for life, the organism has a number of mechanisms to minimize free-radical-induced damage and to repair damage that occurs, such as the enzymes superoxide dismutase, catalase, and glutathione peroxidase and glutathione reductase. Additionally, an enhanced level of ROS molecules has deleterious cellular impact and results in the damage of vital cellular macromolecules such as lipids (11), proteins (12) and nucleic acids (13).

Oxidative stress markers

Protein carbonyl

The most general indicator and by far the most commonly used marker of protein oxidation is protein carbonyl content (14). Redox cycling cations such as Fe^{2+} or Cu^{2+} can bind to cation binding locations on proteins and with the aid of further attack by H_2O_2 or O_2 can transform side-chain

amine groups on several amino acids (i.e. lysine, arginine, proline, or histidine) into carbonyls. Several approaches have been taken to detect and quantitate the carbonyl content in protein preparations. The most convenient procedure is the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazine, which can be analysed spectrophotometrically (15).

Lipid peroxidation

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It most often affects polyunsaturated fatty acids, because they contain multiple double bonds in between which lie methylene bridges (-CH_2-) that possess especially reactive hydrogen's. As with any radical reaction, the reaction consists of three major steps: initiation, propagation, and termination.

Initiation is the step in which a fatty acid radical is produced. The most notable initiators in living cells are ROS, such as OH· and HO_2, which combines with a hydrogen atom to make water and a fatty acid radical.

The fatty acid radical is not a very stable molecule, so it reacts readily with molecular oxygen, thereby creating a peroxyl-fatty acid radical. This radical is also an unstable species that reacts with another free fatty acid, producing a different fatty acid radical and lipid peroxide, or cyclic peroxide if it had reacted with itself. This cycle continues, as the new fatty acid radical reacts in the same way.

When a radical reacts with a non-radical, it always produces another radical, who is why the process is called a "chain reaction mechanism". The radical reaction stops when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough for them to be a high probability of collision of two radicals. Living organisms have different molecules that speed up termination by catching free radicals and, therefore, protecting the cell membrane. One important such anti-oxidants made within the body include the enzymes SOD, CAT, and GPx.

The end products of lipid peroxidation are reactive aldehydes, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), the second one being known also as "second messenger of free radicals" and major bioactive marker of lipid peroxidation, due to its numerous biological activities resembling activities of reactive oxygen species.

Certain diagnostic tests are available for the quantification of the end-products of lipid peroxidation, to be specific, MDA (16). The most commonly used test is called a thiobarbituric acid reactive substances (TBARS) assay. Thiobarbituric acid reacts with MDA to yield a fluorescent product. However, recent studies have demonstrated that there are other sources of MDA, so this test is not completely specific for lipid peroxidation (17). In recent years development of immunochemical detection of HNE-histidine adducts opened more advanced methodological possibilities for qualitative and quantitative detection of lipid peroxidation in various human and animal tissues as well as in body fluids, including human serum and plasma samples (18).

Antioxidant enzymes

Superoxide dismutase

The SODs are enzymes that alternately catalyse the dismutation (or partitioning) of the toxic superoxide (O_2^-) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is produced as a by-product of oxygen metabolism and causes many types of cell damage. Thus, SOD is an important antioxidant defence in nearly all-living cells exposed to oxygen.

Catalase

Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. Catalase is a common enzyme found in nearly all-living organisms exposed to oxygen (such as vegetables, fruit or animals). This enzyme catalyses the decomposition of hydrogen peroxide to water and oxygen (19) and protects the cell from oxidative damage. Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert approximately 5 million molecules of hydrogen peroxide to water and oxygen each second.

Glutathione peroxidase

GPx is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. The biochemical function of glutathione peroxidase is to reduce hydrogen peroxide, organic hydroperoxide and lipid hydroperoxides to

their corresponding alcohols and to reduce free hydrogen peroxide to water.

Others anti-inflammatory and oxidative stress markers

Nuclear factor erythroid 2 related factor 2

Nuclear factor erythroid 2 related factor 2 (Nrf2) is a transcription factor that in humans is encoded by the *NFE2L2* gene (20). Nrf2 is a basic leucine zipper protein that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation (21).

Under normal or unstressed conditions, Nrf2 is kept in the cytoplasm by a cluster of proteins that degrade it quickly. Under oxidative stress, Nrf2 is not degraded, but instead travels to the nucleus where it binds to a DNA promoter and initiates transcription of antioxidative genes and their proteins. Nrf2 is ubiquitously expressed with the highest concentrations (in descending order) in the kidney, muscle, lung, heart, liver, and brain (20).

NAD(P)H dehydrogenase, Quinone 1

NAD(P)H dehydrogenase, Quinone 1 (NQO1) plays an important role in neuroprotection through its anti-oxidative properties (22). NQO1 is an enzyme that in humans is encoded by the *NQO1* gene (23). NQO1 gene is a member of the NAD(P)H dehydrogenase (quinone) family encoding a cytoplasmic 2-electron reductase and this FAD-binding protein forms homodimers and reduces quinones to hydroquinones. This protein enzymatic activity prevents the one electron reduction of quinones that results in the production of radical species. Altered expression of this protein has been seen in many tumours and is also associated with Alzheimer's disease (24).

Glial fibrillary acidic protein

Glial fibrillary acidic protein (GFAP) is a protein that is encoded by the GFAP gene in humans (25). GFAP is an intermediate filament protein that is expressed by numerous cell types of the central nervous system including astrocytes (26), and ependymal cells (27). GFAP is also expressed in glomeruli and peritubular fibroblasts taken from kidneys (28), stellate cells of the pancreas and liver in rats (29). First described in

1971 (30), GFAP is thought to help to maintain astrocyte mechanical strength (31), as well as the shape of cells but its exact function remains poorly understood, despite the number of studies using it as a cell marker. GFAP is expressed in the central nervous system in astrocyte cells (26,32). It is involved in many important central nervous system processes, including cell communication and the functioning of the blood brain barrier.

Nuclear factor kappa-β

Nuclear factor kappa-β (NF-κβ) is a protein complex that controls transcription of DNA. NF-κβ is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low-density lipoprotein (LDL), and bacterial or viral antigens (33–37). NF-κβ plays a key role in regulating the immune response to infection (κ light chains are critical components of immunoglobulin). Incorrect regulation of NF-κβ has been linked to cancer, inflammatory, and autoimmune diseases, septic shock, viral infection, and improper immune development. NF-κβ has also been implicated in processes of synaptic plasticity and memory (38–42). In

brief, NF-κβ can be understood to be a protein responsible for cytokine production and cell survival.

Signal transducer and activator of transcription 3

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor, which in humans is encoded by the STAT3 gene. The protein encoded by this gene is a member of the STAT protein family. In response to cytokines and growth factors, STAT family members are phosphorylated by receptor-associated kinases and then form homo- or heterodimers that translocate to the cell nucleus, where they act as transcription activators. STAT3 mediates the expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis. Moreover, most of the available evidence comes from studies that examined the effect of specific interventions, e.g. focus on just exercise or just protein source in the diet. However, until date, the combined effect and interactions taking place between the dietary protein amount, protein source, resistance training and AAS-administration is unknown.

Oxidative stress on Brain and Kidney

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 (5). Furthermore, lipid peroxidation leads to the production of toxic compounds such aldehydes or dienals (e.g., 4-hydroxynonenal), which in turn may cause neuronal apoptosis (43). 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 (1,44,45). The brain readily suffers oxidative damage due to its higher metabolic rate, lipid content and lower levels of CAT and GPx (46–48). Additionally, most of the studies have showed an increase on brain lipid peroxidation markers such as TBARs, MDA (49,50), HNE (51) and some isoprostanes (52) in Alzheimer's disease patients.

The kidney is one of the most imperative tissues in the organism due to its straight relation with other systems (e.g. cardiovascular system) making its impeccable functioning highly valuable (53,54). Oxidative stress and inflammation play a critical role in the pathogenesis and progression of chronic kidney disease (55–58). Thus, markers of kidney

disease wasting (closely related to oxidative stress) such as hypoalbuminemia, anorexia, body weight and fat loss, rather than traditional cardiovascular risk factors, appear to be the strongest predictors of early death in maintenance haemodialysis patients (54). Thus, an improper or maladaptive activation of oxidative processes may be chronically present in pathological situations, such as uraemia, contributing to chronic cell and kidney injury (59,60). Blood levels of several lipid and protein oxidation products such as F2-isoprostanes are increased in maintenance haemodialysis patients (61,62). The kidney also plays an essential role in the long-term regulation of arterial pressure and a vital role in the initiation, development and maintenance of chronic hypertension.

High-intensity exercise and 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 (63,64). Exercise may increase the level, activation, and Messenger RNA expression of endogenous antioxidant systems in the brain thus down-regulating the levels of the oxidative damage (65,66).

Experimental evidence has shown that ROS play a key role in the pathophysiological pathways of a wide variety of clinical and experimental renal diseases (67–73). Chronic kidney disease (CKD) is characterized by progressive loss of nephrons caused by increased intraglomerular pressure and hyperfiltration. CKD is associated with a high prevalence of several other diseases, and has become a worldwide health issue due to the high economic costs involved in CKD diagnosis and treatment (74). Previous studies have associated CKD with oxidative stress. Physical training is an important component in the treatment of CKD (68). According to (75) and (76), exercise conditioning has been shown to have a positive influence on physical capacity, hypertension, left ventricular function, lipid and glucose metabolism, oxidative status, anaemia, and quality of life in CKD patients and patients on renal replacement therapy. Moreover, exercise exerted a positive influence on oxidative stress parameters, especially on the reduction in superoxide production and oxidative damage, as well as an improvement in the antioxidant defence system, like SOD and GPX (77).

Recent studies have observed that chronic exercise activates the Nrf2 in human skeletal muscle and rat kidney (78,79), 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). Consequently, it is well established that regular exercise plays an important preventive and therapeutic role on oxidative stress-associated brain diseases such as Alzheimer's and Parkinson's (63). However, the benefits of HIE on brain function are under debate due to the overproduction in ROS that this type of exercise can induce (80). Likewise, the effect of exercise, and more specifically its type, dose and intensity, on renal status is rather unknown (81). Thus, the overproduction of ROS can alter the concentrations of different early biomarkers of oxidative stress such as plasma total antioxidant capacity (TAC) or erythrocyte GSH and CAT activity, suggesting modifications in blood redox status (82). Hence, given that hypertrophy resistance training is the main exercise modality practiced by high-protein users (83) and the effect of high-protein diets in combination with HIE on brain and renal redox status has been inconclusive or scarcely investigated, one of the aims of this present Thesis was to investigate the effects of an hypertrophy resistance training protocol (i.e. HIE) on brain and renal redox status.

High soy-protein diets and oxidative stress

Soy as main source of protein has been preferred instead of others source of protein such as casein or whey because of their beneficial effects such as reducing cardiovascular risk (84,85), promoting a more alkaline plasma and urinary profile, with their consequent renal benefits (86), encouraging a better bone health in weak populations (e.g. elderly, or perimenopausal women) (87) and decreasing plasma TBARs concentrations (88).

Likewise, the effects of high-protein diets have been of great interest in the last decade, specially among athletes and people interested in gaining muscle mass (89,90). However, the scientific literature on whether ingestion of high protein might cause adverse effects to the healthy population is controversial (90,91). Thus, 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 as well as to better maintain bone properties (87,90,92–95).

Similarly, 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 (96,97). Nevertheless, others authors reported that high protein diets may produce harmful renal

effects such as increased urinary N excretion, glomerular filtration rate, kidney hypertrophy, renal hemodynamic and eicosanoid production in renal tubules (98–100).

In regard to the effects of high-protein diets and brain and kidney oxidative status, the literature is mainly scarce (83). In fact, to the best of our knowledge, only the study performed by Camiletti-Moirón et al. (83) showed that high-soy protein intake produced higher levels of brain lipid and protein oxidation. Therefore, in order to deepen this knowledge, it is of importance to clarify the physiological effects of high-soy protein diets on brain and kidney redox status.

Anabolic androgenic steroids and oxidative stress

Anabolic androgenic steroids (AAS) have both protein synthesizing (anabolic) and masculinizing (androgenic) effects on the body (101). Although AAS may be prescribed for patients with pathological conditions (e.g. hypogonadism or sarcopenia) (102), they are widely used among professional athletes, competitive and recreational body builders or even non-athletic adolescents because AAS are some of the most powerful performance enhancing substances (103). Severe effects such as adverse plasma and hepatic lipid profile can emerge with prolonged use

or high doses of AAS (104). Regarding brain function, AAS may adversely affect neural activity in the hypothalamus and forebrain (105), by promoting neurodegenerative and apoptotic effects (106). Otherwise, due to the kidney is the major organ involved in the drug excretion, this organ is generally affected by high doses of AAS by means of mesangial matrix accumulation and increased heat shock proteins in this tissue (107).

Concerning oxidative status, Tugyan et al. (106) observed that Nandrolone administration displayed excessive MDA levels as well as a diminution in GPx activity in prefrontal cortex and hippocampus. Likewise, Frankenfeld et al. (108) revealed a harmful increase on kidney protein carbonyl content, a decrease of kidney total reduced thiol residues, and a diminished renal CAT activity affected by AAS treatment.

Consequently, some studies have already demonstrated the combined effects of HIE and AAS in other tissues. For example, concerning muscle mass, the combination of these effects induced comparable hypertrophy in the size of all major fibre types on soleus, tibialis anterior and gastrocnemius muscle (109,110). Additionally, the beneficial effects provided by HIE on hippocampal cell proliferation and

apoptotic signalling as well as the improved heart antioxidant capacity were impaired by Nandrolone (111,112). Conversely, Stanozolol treatment protected rat skeletal muscle mitochondria against oxidative damage of proteins and changes in membrane fatty acid composition induced by acute exercise (113). Therefore, the involvement of specific molecular mediators on the biological effects of HIE and/or AAS depend on numerous factors such as the training protocol designed, animal model investigated, age, sex, AAS dose, metabolism or treatment regimen (114,115).

Several oxidative stress brain and kidney markers and antioxidant enzymes have been used to evaluate brain and renal damage. The astrocytes play a key role in brain physiology and diverse neurodegenerative diseases (116). The GFAP is a specific astrocyte marker, which increases as a sign of astrogliosis, associated with conditions of brain injury (117). Glial activation, in response to injury stimuli, commonly involves changes in GFAP and antioxidant defence (118). The Nrf2 plays a central role in the regulation of phase 2 enzymes, such as GPx, GST and NQO1 (119,120). Recent studies have observed that chronic exercise activates the Nrf2 in human skeletal muscle and rat

kidney whereas acute exercise promotes myocardial Nrf2 function (79,121).

STAT3 is activated by cytokines, growth factors, and receptor- or nonreceptor-tyrosine kinases (122,123). A previous study has demonstrated that manganese superoxide dismutase (Mn-SOD), a primary cellular defence enzyme involved in protecting cells from oxidative stress (124), is a direct target of STAT3 in ischemia reperfusion-induced neuronal cell death. Hence, the loss of STAT3 activity reduces Mn-SOD expression after cerebral ischemia (125).

Given that hypertrophy resistance training is the main exercise modality practiced by AAS abusers (126) and the effect of androgens in combination with HIE on brain and kidney redox status has been scarcely investigated, some of the issues of the present Thesis was to investigate the effects of an hypertrophy resistance training protocol (i.e. HIE) and AAS administration on brain and kidney redox status.

AIMS [OBJETIVOS]

AIMS

General:

The overall objective of this PhD Thesis was to analyse the brain and kidney antioxidant defence system and oxidative damage as well as the renal morphology related to high-protein diet, high-intensity exercise, and anabolic androgenic steroids administration in rats.

Specifics:

- To systematic review recent studies analysing the influence of the type of exercise performed and its volume intensity on brain oxidative stress markers. (Paper I).
- To examine the potentially protective action of high-intensity exercise against the brain oxidative damage induced by the intake of a high-protein diet (Paper II).
- To study the alterations of high-intensity exercise and anabolic androgenic steroids administration on brain oxidative stress in rats (supplementary files).
- To examine the effects of high-protein diets, high-intensity exercise and anabolic-androgenic steroids administration on

kidney oxidative stress markers, as well as, plasma, urinary and morphological renal parameters in rats (Paper III and supplementary files).

OBJETIVOS

Generales:

El objetivo general de esta memoria de Tesis Doctoral fue analizar el sistema de defensa antioxidante y el daño oxidativo a nivel cerebral y renal, así como la morfología renal relacionados con las dietas hiperproteicas, el ejercicio de alta intensidad y la administración de anabolizantes androgénicos esteroideos en ratas.

Específicos:

- Revisar sistemáticamente los últimos estudios que analizaron la influencia del tipo de ejercicio realizado y su intensidad sobre marcadores de estrés oxidativo en cerebro (Artículo I).
- Examinar el potencial acción protector del ejercicio de alta intensidad contra el daño oxidativo cerebral producido por la ingesta de una dieta hiperproteica (Artículo II).
- Estudiar las alteraciones del ejercicio de alta intensidad y la administración de anabolizantes androgénicos esteroideos sobre el estrés oxidativo cerebral en ratas (archivos suplementarios).
- Examinar los efectos de una dieta hiperproteica, del ejercicio de

alta intensidad y de la administración de anabolizantes androgénicos esteroideos sobre marcadores de estrés oxidativo renal, así como sobre parámetros plasmáticos, urinarios y morfológicos renales en ratas (Artículo III y archivos suplementarios).

MATERIAL AND METHODS [MATERIAL Y MÉTODOS]

MATERIAL AND METHODS [MATERIAL Y MÉTODOS]

Systematic Review

1. Search strategy

A systematic review of the literature was conducted up to November 2012 across the following electronic databases: PUBMED, SCOPUS, SPORTS DISCUS, Web Of Science and The Cochrane Library. In addition, manual searching of the reference lists was carried out and results were combined in Endnote (EndNote X3 for Mac OS X, Dakota State University, Thomson Reuters). The date of the first published article related to brain oxidative stress and exercise was chosen as the initial date of the search.

The search strategy used in the mentioned electronic databases was established as: (swim* OR exercise OR training) AND ("oxidative stress" AND brain) for each database.

2. Inclusion and exclusion criteria

Studies proposed to be included in the review were checked for the following criteria: (1) the study was a full report published in a peer-reviewed journal; (2) only studies developed in healthy humans or rodents were included in the review; (3) one or more exercise programs were carried out; (4) keywords combination referred to exercise, oxidative stress or brain were included as a deeper and exhaustive search process.

Articles were included only if they met all of these four criteria and therefore articles were excluded if (1) they were published after November 2012; (2) full-text of the articles was not found; (3) studies were published as an abstract; (4) exercise was not performed; (5) articles were not written in English, Spanish or Portuguese, and (6) the studies were not performed in healthy "humans" or "rodents". Finally, (7) studies that used drugs administration before or after exercise were also excluded.

3. Identification of eligible studies

Eligible studies were longitudinal and cross-sectional observational studies developed in healthy humans or rodents that analysed the

association between exercise and oxidative stress on brain and which did not administer any drug.

The abstracts of all articles identified through the search were read by two independent researchers (DCM and VAA), who selected the potentially eligible articles. In the next step, the two independent researchers (DCM and VAA) carefully read and evaluated these articles. A consensus meeting was arranged to sort out differences between DCM and VAA and finally decide if the potentially eligible articles were included or not. The reference list of every selected article was carefully checked to identify other potentially eligible studies.

4. Methodological Quality Assessment

The final sample of studies for review was subsequently analysed by a Methodological Quality Assessment (MQA), according to a modified version of the Downs and Black Quality Index (127) with the Ainge et al. (128) modification for animal models (Table 1, *see paper I).* This modified version consists of a total of 10 questions; 7 of them assess the quality of reporting (including animal-specific questions), two of them assess the internal validity (one each on bias and confounding) and one question assesses the power of each study. MQA was conducted

separately by two researchers (DCM and VAA). For each study a "yes" or "no" was recorded for each question as either one or zero, respectively. Responses were summed to give a total out of 10, which was then expressed as a percentage. Finally, to identify general strengths and weakness across the group of studies, responses for each question were summed to give a total out of 5 questions. For all studies, a total quality score was calculated by counting up the number of positive items (a total score between 0 and 10), which was then expressed as a percentage. Studies were defined as high quality if they had a total score of 7 or higher. A total score of 5 and 6 were defined as low quality, and a score of less than 4 was defined as very low quality (129) (Table 2, *see paper I*). Two reviewers (DCM and VAA) separately evaluated the quality of the studies. A consensus meeting was arranged to sort out differences between both reviewers.

5. Levels of evidence

Three levels of evidence were constructed: (1) strong evidence: consistent findings in three or more high-quality studies; (2) moderate evidence: consistent findings in two high-quality studies; (3) limited or conflicting evidence: consistent findings in multiple low-quality studies,

inconsistent results found in multiple high-quality studies, or results based on one single study (129).

6. Data extraction

For all studies that met the eligibility criteria, all relevant data was extracted: Characteristics of the sample, random or non-randomized experimental designs, exercise protocols performed, chosen enzymes and its measurement methodology, methodology employed during the animals sacrifice and samples saving, brain protein concentration and oxidation estimation methodology, brain area selected, and statistical analysis carried out.

In vivo experiments

1. Animals and experimental design

A total of 80 albino male Wistar rats were randomly distributed into 8 experimental groups derived of 3 interventions: protein amount of the diet (normal-protein vs. high-protein), HIE (untrained vs. HIE) and AAS-administration (non-AAS vs. AAS). Each specific intervention (e.g. HP diet, with HIE and with AAS) was developed in groups of 10 rats and the

experimental period lasted 12 weeks (**Figure 1**).

The animals (aged 8 weeks) with an initial body weight of 170±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 the animals. The rats were located in a well-ventilated thermostatically controlled room (21±2ºC), with a relative humidity ranging from 40 to 60%. 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 N_2 and kept at -80ºC 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.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada (2011-343).

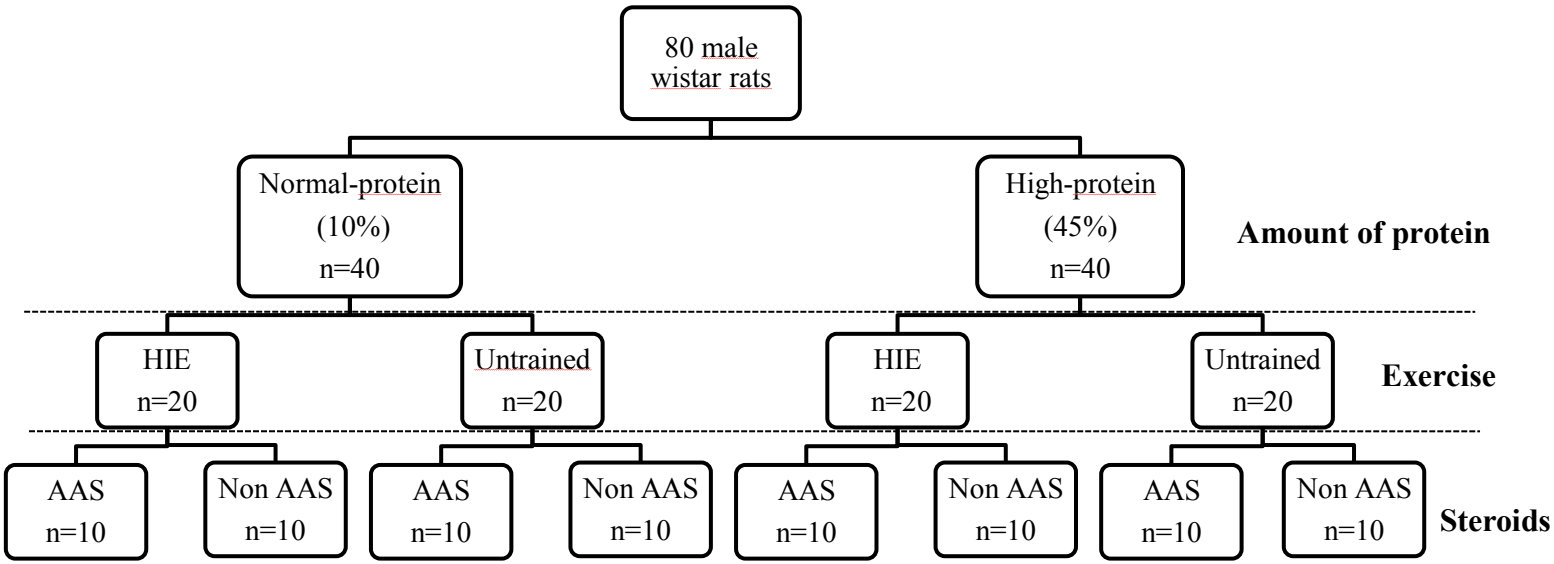

Figure 1. Study design showing the three different interventions: dietary protein amount (Normal-protein vs. High-protein), exercise (untrained vs. High-Intensity Exercise) and anabolic-androgenic steroids (non AAS-administration vs. AAS-administration).

2. Experimental diets

Formulation of the experimental diets is presented in T**able 1**. All diets were formulated to meet the nutrient requirements of rats (130) following the recommendations of the American Institute of Nutrition (AIN-93M) (131), 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 rats (89,94,95). 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.

Table 1. Formulation of the experimental diets.

Nutritional Composition (g/100g DM)	Protein diet	
	Normal-protein	High-protein
Soy protein supplement	13.1	57.4
Mineral mix (AIN-93M-MX)	3.5	3.5
Vitamin mix (AIN-93-VX)	1	1
Fat (olive oil)	4	4
Choline chloride	0.25	0.25
Cellulose	5	5
Starch	62.4	28.6
Methionine	0.5	-
Sucrose	10	-

2.1. Total nitrogen content and total protein concentration

Prior to the diet preparation, total protein concentration of the commercial soy hydrolyzate and its distribution among the protein or non-protein fractions was measured. Total nitrogen (N) content of the commercial soy-protein hydrolyzate was 12.4±0.7g/100g of dry matter, which corresponds to a 77.5% of richness.

Total protein concentration of the experimental diets was also assayed, with values of 44.1±2.2% and 9.8±0.4% for the soy high-protein and normal-protein diets, respectively. These values are adequate for our experimental design.

3. High-intensity exercise

3.1. Training protocol

The experimental groups were trained following a resistance training protocol on 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 (89). 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). Experimentation took place in the 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 has been previously developed and deeply described by Aparicio et al. (89).

During the exercise period, the training weights (loads) were progressively increased and individually adjusted once per week to the percentage of one repetition maximum (1 RM), defined as the maximum load that each rat could carry in the bag. The entire training process was designed and controlled by sport scientists in collaboration with experienced researchers trained to work with rats. The number of sessions performed each week, the number of sets per session, the time spent in each set, and the load carried by the animals is shown in **Table 2.**

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

Table 2. Details of the high-intensity exercise protocol.

Week	Work time (min)	Sets	Time between sets (min)	Weight (% 1 RM)
1	2	10	1	55
2	2	10	1	60
3	2	10	1	65
4	2	10	1.5	70
5	2	10	1.5	70
6	2.5	10	1.5	75
7	2.5	12	1.5	75
8	2	12	2	80
9	2.5	12	2	80
10	1.5	12	2	85
11	2	12	2.5	85
12	1	12	2.5	85

RM, repetition maximum.

3.2. Repetition maximum test

The 1 RM test was conducted as follows: the rat was placed in a flat, horizontal and non-slippery surface with a specific loaded bag that was

tied to its tail. The rat was acoustically stimulated and immediately reacted by moving forward. This procedure was repeated several times, increasing the load every time, until the rat could not move forward, yet actively stimulated. The load achieved at this point was considered the 1 RM and was weekly measured in all animals to adapt the percentage of 1 RM load during the training period.

4. Anabolic androgenic steroids administration

Following similar studies performed in rats, the animals received 10 mg/kg body weight of Stanozolol once a week by intramuscular injection in the gluteus (alternating the lateral side each week) for 12 weeks. This dose is comparable to the dose that has been reported as being frequently used by athletes (600 mg/week or approximately 8 mg/Kg/week) (132,133). We used a commercially available Stanozolol solution of 50 mg/ml (Winstrol Depot, Desma Pharma group) that was diluted with saline solution to appropriate concentrations for the lower doses to keep the volume of injection constant. The non-AAS administered group was injected with saline solution as placebo.

5. Chemical analyses

5.1. Plasmatic parameters

The plasma urea, total protein, creatinine and albumin concentrations were measured using an autoanalyzer (Hitachi-Roche p800, F. Hoffmann-La Roche Ltd. Switzerland).

5.2. Brain and kidney homogenate preparation for oxidative damage markers and antioxidant activity

Brain (1 g) and kidney (0.5 g) 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 for 30 sec followed by treatment with Sonoplus HD 2070 ultrasonic homogenizer (Bandelin) at 50% power for 10 sec. Homogenates were centrifuged at 19,921 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.

6. Oxidative damage markers

6.1. Thiobarbituric acid-reactive substances

Thiobarbituric acid reactive substances (TBARs) were used as a marker of lipid peroxidation. Brain and kidney supernatants were used to determine lipid peroxidation by measuring TBARs as described by Ohkawa et al. (134). The results were expressed as nmol of MDA per mg of protein (nmolMDA/mg) from duplicate reactions.

6.2. Protein carbonyl content

Total carbonyl content in brain and kidney were used as a biomarker of protein oxidation. The content was determined by spectrophotometry using a protein carbonyl colorimetric assay kit (Cayman, USA) according to Levine et al. (15). Results were expressed as nmol of reactive carbonyl compounds/mg protein of tissue.

7. Antioxidant enzyme activity

7.1. Superoxide dismutase

Total superoxide dismutase (t-SOD) activity was measured as described by Ukeda et al. (135) and adapted to a micro-plate reader. Manganese

superoxide dismutase (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). Cooper/zinc superoxide dismutase (CuZn-SOD) activity was determined by subtracting the Mn-SOD activity from the t-SOD 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. Protein concentration was determined by the method of Lowry (136).

7.2. Catalase

Catalase activity was measured as described by Aebi (137) by monitoring the disappearance of H_2O_2 in the presence of brain or kidney homogenate at λ=240 nm and was expressed as μmol of H_2O_2 consumption per minute per milligram of protein. Protein concentration was determined by the method of Lowry (136).

7.3. Glutathione peroxidase

This method is based in the oxidation of nicotine adenine dinucleotide phosphate (NADPH) induced by glutathione reductase,

coupled with the reduction of glutathione previously oxidized by glutathione peroxidase. The activities were spectrophotometrically determined at λ=340 nm, 37^{o}C during 4 min, repeating the measurements every 15 sec according to the protocol previously described by Lawrence et al. (138,139) with slight modifications. Catalysed and non-catalysed reactions were simultaneously performed. Regarding the non-catalysed reactions, 240μL of 2 mM NADPH/1 mM ethylenediaminetetraacetic acid (EDTA) in 50 mM phosphate buffer, pH 7.4, were mixed with 15 μL of kidney homogenate and 10 μL of 22 mM cumene hydroperoxide in each well. As for the catalysed reaction, 240 μL 2mM NADPH/1 mM EDTA in 50 mM phosphate buffer, pH 7.4, were mixed with 15 μL kidney homogenate, 4,5 μL glutathione reductase (0.04 mU/mL), and 10 μL 22 mM cumene hydroperoxide. The results were expressed as nmol NADPH/min/mg of protein.

8. Western blotting analysis

8.1. Brain homogenate preparation for Western blotting analysis

Brain samples (1 g) were homogenized (1:10 w/v) in 20 mM Tris·HCl (pH 8.0) containing 0.1% octylphenoxypolyethoxyethanol (lgepal), 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 Aproptinin. Samples were homogenized with a Micra D-1 homogenizer (ART moderne labortechnik) at 18,000 rpm for 30 seconds followed by treatment with Sonoplus HD 2070 ultrasonic homogenizer (Bandelin) at 50% power for 10 seconds. Homogenates were centrifuged at 19,621 g and 4°C for 45 min (BECKMAN, Allegra 64R), supernatants were collected and stored at -80ºC until use. The concentration of protein was measured by the method of Lowry et al. (136). Samples (40 µg protein) were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, proteins were 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 hours at room temperature. After blocking, the membranes were incubated with primary polyclonal rabbit anti-Nrf2 antibody (1:1500, Abcam Cambridge, USA) overnight at 4ºC. 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.

8.2. Western blotting

Brain aliquots (1 g) were homogenized (1:10 w/v) in 20 mM Tris·HCl (pH 8.0) containing 0.1% octylphenoxypolyethoxyethanol (lgepal), 100 mM EGTA, 100 mM 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 Aproptinin. Samples were homogenized with a Micra D-1 homogenizer (ART moderne labortechnik) at 18,000 rpm for 30 seconds followed by treatment with Sonoplus HD 2070 ultrasonic homogenizer (Bandelin) at 50% power for 10 seconds. Homogenates were centrifuged at 19,621 g and 4°C for 45 min (BECKMAN, Allegra 64R), supernatants were collected and stored at -80ºC until use. Protein concentration was quantified using the Bradford assay method (Bio-Rad, Hercules, CA).

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis under reducing conditions and then transferred to nitrocellulose membranes. Western blots were performed according to standard methods. Membranes were blocked in 5% skimmed milk, and then incubated (overnight at 4ºC or room temperature for 3-4 h) with the antibody of interest, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The visualization of immunoreactive bands was performed using the ECL Plus Western blotting detection system (GE Healthcare, Pascataway, NJ). The primary antibodies were directed against NF-κβ p65 (Epitomics, Burlingame, CA; 1:1000); GFAP (Cell signalling Technology, Danvers, MA; 1:1000); GPx (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200); NQO1 (Abcam, Inc., Cambridge, MA; 1:2500); STAT3 (Cell Signalling Technology, Danvers, MA; 1:1000) and Nrf2 (Abcam, Inc., Cambridge, MA; 1:1500). The quantification was performed by volume densitometry using Image J software (NIH, Bethesda, MD) and normalization to ponceau reagent with the exception of Nrf2 that was normalized to β-actin.

9. Histological analysis

The left-kidney samples were fixed in 4% buffered formalin and

embedded in paraffin. Subsequently, 4-micrometer-thick sections were obtained and stained with 1% Picro-sirius red F3BA (Gurr, BDH Chemicals Ltd, Poole, United Kingdom) (140). This technique facilitates the visualization of connective fibres as deep red stains on a pale yellow background (140). The sections were assessed by optical microscopy. Forty images per sample were captured: 20 of the glomerulus to determine the morphometry and the intraglomerular connective tissue and 20 of the tubulointerstitial area to measure the interstitial connective tissue.

All images were acquired using the 20 ^{x} lens and analysed with the Fibrosis HR® software (141). This image analysis application allowed us to automatically quantify morphometric parameters by using various image-processing algorithms (141).

We estimated the following 8 morphological variables that we describe for the better understanding of the present results: a) Percentage of interstitial connective tissue in relation to the image area, excluding the glomerular area (the connective tissue that is in the gap over the Bowman's capsule). b) The area of interstitial connective tissue (including Bowman's capsule). The Fibrosis HR® software divides glomerular tufts into 2 categories: "glomerular tuft I" and "glomerular

tuft II". The variable "glomerular tuft I" corresponds to the renal corpuscle excluding the Bowman's capsule. The variable "glomerular tuft II" corresponds to the renal corpuscle excluding the Bowman's capsule and considering the area of the capillary lumens and urinary spaces in the glomerulus. c) Glomerular tuft I area. d) Glomerular tuft II area. e) Glomerular tuft I percentage (percentage of glomerular tuft I related to the glomerular area). f) Glomerular tuft II percentage (percentage of glomerular tuft II related to the glomerular area). g) Mesangial area. h) Glomerular area.

10. Statistical analyses

Results are presented as mean and standard error of the mean (SEM), unless otherwise indicated. The effects of the HIE (untrained vs. HIE), the AAS administration (non-AAS vs. AAS) and the dietary protein amount (normal-protein vs. high-protein) on food intake, final body weight, carcass weight, brain and kidney weight, oxidative stress markers as well as plasma, urinary, and renal morphology parameters were analysed by three-way factorial analysis of variance (ANOVA), with HIE, AAS and dietary protein amount as fixed factors. Two-ways interactions terms were introduced into the models to test interactions

between the following factors: dietary protein amount*HIE and AAS*HIE. A significant *p* value indicates that there are differences in at least two of the groups. In addition, multiple comparisons between groups were made considering Bonferroni's adjustment in order to identify between which groups the differences were significant (e.g. untrained without AAS vs. HIE with dietary protein amount).

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 [RESULTADOS]

RESULTS

The results of the present Doctoral Thesis are shown as a compilation of scientific papers combined with unpublished data. They are enclosed in the form they have been published complemented with results not published yet. Rigorously, each scientific paper is composed by an introduction, a description of the material and method, results, discussion, conclusion and bibliography.

Los resultados obtenidos en la presente Tesis Doctoral se presentan a continuación en la forma en que han sido publicados combinados con resultados aún sin publicar. Como es de rigor, cada artículo cuenta con su introducción correspondiente, descripción de la metodología utilizada, exposición de los resultados, discusión de los mismos y bibliografía.

1. OXIDATIVE EFFECTS OF HIGH-PROTEIN DIETS AND HIGH-INTENSITY EXERCISE

1.1. BRAIN

Does exercise reduce brain oxidative stress? A systematic review

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Review

Does exercise reduce brain oxidative stress? A systematic review

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The aim of the present systematic review was to investigate the influence of different exercise programs on brain oxidative stress. A search of the literature was conducted up to 1 December 2012 across five databases: PUBMED, SCOPUS, SPORTS DISCUS, Web of Science, and The Cochrane Library. The search strategy used in the electronic databases mentioned was established as: (swim* OR exercise OR training) AND ("oxidative stress" AND brain) for each database. A methodological quality assessment valuation/estimation was additionally carried out in the final sample of studies. Of 1553 potentially eligible papers, 19 were included after inclusion and exclusion criteria. The methodological quality assessment showed a total score in the Quality Index between 40% and 80%, with a mean quality of 56.8%. Overall, regular moderate aerobic exercise appears to promote antioxidant capacity on brain. In contrast, anaerobic or high-intensity exercise, aerobic-exhausted exercise, or the combination of both types of training could deteriorate the antioxidant response. Future investigations should be focused on establishing a standardized exercise protocol, depending on the exercise metabolism wanted to test, which could enhance the objective knowledge in this topic.

Exercise could increase the resistance against oxidative stress, providing enhanced protection (Alessio, 1993; Radak et al., 2000a, 2002, 2008a). According to the original stress theory developed by Selye (1956), for a chronic stressor the body replies with a decreased (alarm reaction), and then with an increased resistance (stage of resistance), which is followed by exhaustion of the body (stage of exhaustion). Therefore, chronic stressors could be very dangerous because the resting period, which is obligatory for recovery and efficient stress response, is missing (Radak et al., 2008b). However, many unanswered questions remain concerning the intensity and duration of the exercise to be prescribed (Daniels et al., 2012). For instance, in extremely long-duration exercise, such as 18–24 consecutive hours of running or swimming, even in superbly trained individuals, the body can suffer serious "exhaustion" that could endanger the health of the individuals (Radak et al., 2008b). On the other hand, under normal conditions, exercise bouts are followed by rest periods where the body has the capability to cope with the exercise "stressor" and as a result, adaptation takes place. Indeed, the adaptive effects of regular exercise are systemic and, depending on the characteristics of exercise, the effects are specific (Radak et al., 2001a).

Since the 90s, there is evidence about the benefits of regular exercise on brain function, which could play an important preventive and therapeutic role on oxidative stress-associated brain diseases (Mattson et al., 2004; Mattson & Magnus, 2006; Radak et al., 2008a). Brain is considered highly sensitive to oxidative damage because it possesses high amounts of phospholipids and polyunsaturated fatty acids, both of which are highly susceptible to oxidants, have high oxygen consumption, and low levels of antioxidant enzymes (Jenner, 2003; Tuon et al., 2012). Exercise may increase the level, activation, and mRNA expression of endogenous antioxidant systems in the brain, and it down-regulates the levels of the oxidative damage (Um et al., 2008; Aguiar et al., 2008a, 2010, 2011; Tuon et al., 2012) that have been implicated in reducing the risk of brain oxidative damage, but this response depends on the type of exercise used (Tuon et al., 2012).

Until date, no review has been deeply explored the relationship between exercise intensity and type and oxidative stress on brain in order to better understand the dose and type of exercise more beneficial for brain activity. Therefore, the aim of the present systematic review was to further analyse the influence of the type of exercise performed and its volume intensity on brain oxidative stress markers.

Methods

Search strategy

A systematic review of the literature was conducted up to November 2012 across the following electronic databases: PUBMED, SCOPUS, SPORTS DISCUS, Web of Science, and The Cochrane Library. In addition, manual searching of the reference lists was carried out and results were combined in Endnote (EndNote X3 for Mac OS X, Dakota State University, Thomson Reuters). The date of the first published article related to brain oxidative stress and exercise was chosen as the initial date of the search.

The search strategy used in the mentioned electronic databases was established as: (swim* OR exercise OR training) AND ("oxidative stress" AND brain) for each database.

Inclusion and exclusion criteria

Studies proposed to be included in the review were checked for the following criteria: (a) the study was a full report published in a peer-reviewed journal; (b) only studies developed in healthy humans or rodents were included in the review; (c) one or more exercise programs were carried out; (d) key words combination referred to exercise, oxidative stress, or brain was included as a deeper and exhaustive search process.

Articles were included only if they met all of these four criteria and therefore articles were excluded if (a) they were published after November 2012; (b) full text of the articles was not found; (c) studies were published as an abstract; (d) exercise was not performed; (e) articles were not written in English, Spanish, or Portuguese; and (f) the studies were not performed in healthy "humans" or "rodents." Finally, (g) studies that used drugs administration before or after exercise were also excluded.

Identification of eligible studies

Eligible studies were longitudinal and cross-sectional observational studies developed in healthy humans or rodents which analysed the association between exercise and oxidative stress on brain and which did not administer any drug.

The abstracts of all articles identified through the search were read by two independent researchers (D. C. M. and V. A. A.) who selected the potentially eligible articles. In the next step, the two independent researchers (D. C. M. and V. A. A.) carefully read and evaluated these articles. A consensus meeting was arranged to sort out differences between D. C. M. and V. A. A. and finally decide if the potentially eligible articles were included or not. The reference list of every selected article was carefully checked to identify other potentially eligible studies.

Methodological quality assessment

The final sample of studies for review was subsequently analysed by a methodological quality assessment (MQA), according to a modified version of the Downs and Black Quality Index (Downs & Black, 1998) with the Ainge et al. (2011) modification for animal models (Table 1). This modified version consists of a total of 10 questions; 7 of them assess the quality of reporting (including animal-specific questions), 2 of them assess the internal validity (one each on bias and confounding), and 1 question assesses the power of each study. MQA was conducted separately by two researchers (D. C. M and V. A. A.). For each study, a "yes" or "no" was recorded for each question as either 1 or 0, respectively. Responses were summed to give a total out of 10, which was then expressed as a percentage. Finally, to identify general strengths and weaknesses across the group of studies, responses for each question were summed to give a total out of five questions. For all studies, a total quality score was calculated by counting up the number of positive items (a total score between 0 and 10), which was then expressed as a percentage. Studies were defined as high quality if they had a total score of 7 or higher. A total score of 5 and 6 was defined as low quality, and a score of less than 4 was defined as very low quality (Ruiz et al., 2009) (Table 2). Two reviewers (D. C. M. and V. A. A.) separately evaluated the quality of the studies. A consensus meeting was arranged to sort out differences between both reviewers.

Levels of evidence

Three levels of evidence were constructed: (a) strong evidence: consistent findings in three or more high-quality studies; (b) moderate evidence: consistent findings in two high-quality studies; and (c) limited or conflicting evidence: consistent findings in multiple

Table 1. Methodological quality assessment questions.

Modified from Ainge et al. and Downs and Black Quality Index	
Reporting	
General	
	1 Were the hypotheses/aims/objectives of the study clearly described within the introduction?
Animal characteristics	
	2 Was animal species/strain identified?
	3 Was the animal age at commencement of the study or at conception specified?
	4 Have the animal weights at commencement or at conception of study been specified?
	5 Have the housing details been specified?
Design and outcomes	
	6 Were the interventions of interest clearly described?
	7 Have all important adverse events that may be consequence of the intervention been reported?
Internal validity – bias	
Bias	
	8 Was an attempt made to blind those measuring the main outcomes of the intervention?
Confounding	
	9 Were losses of animals explained?
Power	
	10 Was the paper of sufficient power to detect a clinical important effect where the probability value for a difference being due to chance is less than 5%?

Methodological quality assessment questions modified from Ainge et al. (2011) and Downs and Black (1998).

Table 2. Methodological quality assessment

Author	Questions										
	1	2	3	4	5	6	7	8	9	10	Total (%)
Navarro et al.	1	1	1	0	1	1	0	0	0	1	60
Ohkuwa et al.	1	1	1	1	1	0	0	0	0	0	50
Falone et al.	1	1	1	1	1	1	0	0	0	1	70
Vollert et al.	1	1	1	1	1	1	0	0	0	1	70
Itoh et al.	1	1	1	1	1	1	0	0	0	0	60
Somani et al.	1	1	0	1	0	1	0	0	0	1	50
Liu et al.	1	1	1	0	1	1	0	0	0	0	50
Radak et al. (2001b)	1	1	1	0	0	0	0	0	0	1	40
Radak et al. (2006)	1	1	1	0	1	1	0	0	0	1	60
Qiao et al.	1	1	1	0	1	1	0	0	0	1	60
Aguiar et al. (2010)	1	1	1	1	1	1	0	0	0	1	70
Aguiar et al. (2008b)	1	1	1	1	1	1	1	0	0	1	80
Tsakiris et al.	0	1	0	1	1	1	0	0	0	1	50
Aydin et al.	1	1	1	0	1	1	0	0	0	1	60
Cechetti et al.	1	1	1	0	0	1	0	0	0	0	40
Aksu et al.	1	1	1	1	0	0	0	0	0	1	50
De Araujo et al.	1	1	1	1	1	0	0	0	0	1	60
Acikgoz et al.	1	1	1	1	0	1	0	0	0	0	50
Ogonovszky et al.	1	1	1	0	0	1	0	0	0	1	50
	Reporting							Internal validity (bias)	Internal validity (confounding)	Power	Average
Total/19	18	19	17	11	13	15	1	0	0	14	56.8

low-quality studies, inconsistent results found in multiple high-quality studies, or results based on one single study (Ruiz et al., 2009).

Data extraction

For all studies that met the eligibility criteria, all relevant data were extracted: characteristics of the sample, random or nonrandomized experimental designs, exercise protocols performed, chosen enzymes and its measurement methodology, methodology employed during the animals sacrifice and samples saving, brain protein concentration and oxidation estimation methodology, brain area selected, and statistical analysis carried out.

Results

Search results

Initial electronic searching across the above-mentioned five Internet databases led us to 1553 articles. The removal of 502 duplicates, 378 by Endnote program and 124 handled, resulted in 1051 individual articles to be subjected to inclusion and exclusion criteria. After examination of inclusion and exclusion criteria, 107 (removal of 944) articles were selected for further reading. A total of 88 articles did not meet the inclusion criteria after the methods examination. Finally, 19 manuscripts met the inclusion criteria and were included in the present review (studies flow showed in Fig. 1).

Methodological quality assessment

The modified MQA carried out in the 19 selected manuscripts is provided in Table 2. The total score of the Quality Index for each paper is shown in the last right column and expressed as percentage. Manuscripts ranged between 40% (Radak et al., 2001b; Cechetti et al., 2008) and 80% (Aguiar et al., 2008b) with an average quality index of 56.8%. Total rating for reporting and internal validity were considered, being the overall quality of reporting of the manuscripts 46% higher than the internal validity (Table 3). We defined 4 studies as high quality (score $\geq$7), 13 as low quality (5$\leq$ score $\leq$6), and 2 as very low quality (score $\leq$4).

Interventions and species/strain details (19/19), as well as objectives (18/19) and the age at start of the studies (17/19), were specified in the majority of the manuscripts. Furthermore, 11 of 19 studies referred to the weight of the animals and 1 of 19 studies reported important adverse events. Finally, no studies described a blinding intervention or referred some experimental death during their intervention in their Methods section.

Levels of evidence

Table 4 shows the data extraction of the studies reporting the influence of exercise on brain oxidative stress in rodents. No conclusive moderate evidence was obtained in the selected sample, being only four of them of high quality (Aguiar Jr et al., 2008b; Aguiar et al., 2010; Vollert et al., 2011; Falone et al., 2012). Among these four studies, brain oxidative stress was decreased in two of them (Vollert et al., 2011; Falone et al., 2012), and increased in the remaining two (Aguiar Jr et al., 2008b; Aguiar et al., 2010).

Sample of the selected studies

According to the inclusion and exclusion criteria, after the whole process no human study was obtained.

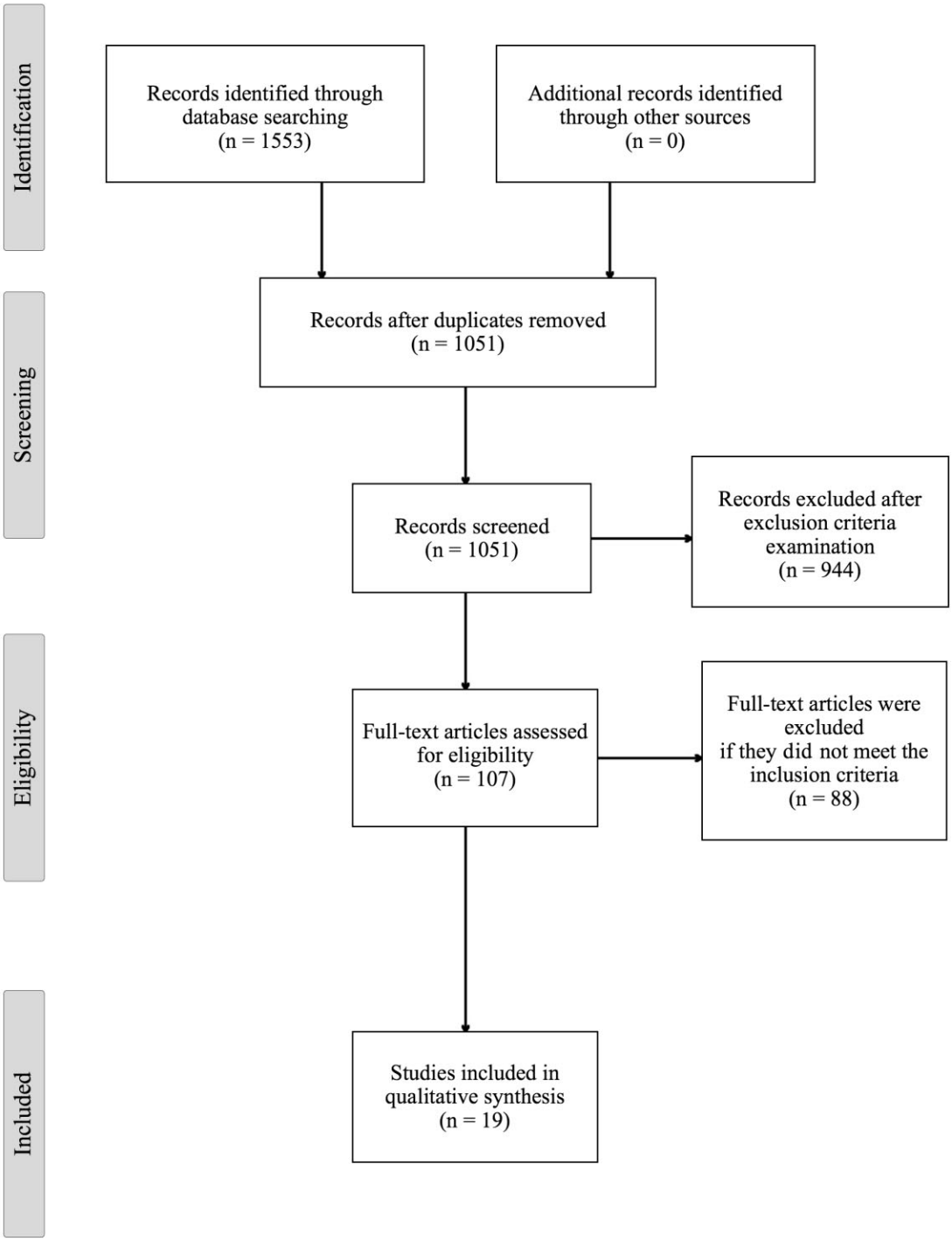

Fig. 1. Process from initial search to final inclusion of the manuscripts.

Therefore, only studies developed in rodents were analysed. Rodent characteristics for each study were registered. Fourteen of 19 manuscripts were carried out with two different strains of rats: Wistar (10/19) and Sprague–Dawley (4/19). The other five remaining studies were developed with four different strains of mice: CD1 (2/19), CF1 (1/19), Swiss (1/19), and Kunming albino (1/19). Any of the studies specified why each strain was chosen. Initial age of the animals was reported in 17 of 19 studies and ranged from 4 to 80 weeks old in rats and from 5 to 78 weeks old in mice. Weight of the rodents was registered in 11 of 19 studies, with a range at the start of the experience from 145 to 380 g in rats, and from 30 to 50 g in mice. Eighteen of 19 studies recorded the sex of the rodents. Male was the main gender chosen for rats (11/19) as well as for mice (4/19). The three remaining studies were developed in female animals (rats 2/19 and mouse 1/19) and one study was carried out in both sexes in mice.

Table 3. Methodological quality assessment results

Author	Reporting x/7	Total%*	Internal x/3	Validity %*	Total x/10	Rating %*
Navarro et al.	5	71.4	1	33.3	6	60
Ohkuwa et al.	5	71.4	0	0	5	50
Falone et al.	6	85.7	1	33.3	7	70
Vollert et al.	6	85.7	1	33.3	7	70
Itoh et al.	6	85.7	0	0	6	60
Somani et al.	4	57.1	1	33.3	5	50
Liu et al.	5	71.4	0	0	5	50
Radak et al. (2001b)	3	42.9	1	33.3	4	40
Radak et al. (2006)	5	71.4	1	33.3	6	60
Qiao et al.	5	71.4	1	33.3	6	60
Aguiar et al. (2010)	6	85.7	1	33.3	7	70
Aguiar et al. (2008b)	7	100	1	33.3	8	80
Tsakiris et al.	4	57.1	1	33.3	5	50
Aydin et al.	5	71.4	1	33.3	6	60
Cechetti et al.	4	57.1	0	0	4	40
Aksu et al.	4	57.1	1	33.3	5	50
De Araujo et al.	5	71.4	1	33.3	6	60
Acikgoz et al.	5	71.4	0	0	5	50
Ogonovszky et al.	4	57.1	1	33.3	5	50
Average	4.9	70.7	0.7	24.6	5.7	56.8

MQA modified Quality Index results for reporting, internal validity, and overall score for all articles reviewed. Results expressed as total out of 7, 3, and 10, respectively, and as a percentage.

*Percentage that meets the criteria.

Oxidative stress markers

Eleven markers related to oxidative stress were analysed among the selected studies: lipid peroxidation (LP) (13/19 studies), glutathione (GSH) (7/19), glutathione peroxidase (GPx or GSH-Px) (6/19), superoxide dismutase (SOD) (6/19), oxidized glutathione (GSSG) (5/19), glutathione reductase (3/19), catalase (CAT) (3/19), and total antioxidant status (1/19). Additionally, protein tissue (5/19), protein oxidation (3/19), and protein brain-derived neurotrophic factor (BDNF) (5/19) content were also measured. Finally, total brain (8/19), hippocampus (7/19), cerebral cortex (6/19), corpus striatum (5/19), prefrontal cortex (3/19), cerebellum (2/19), brainstem (1/19), diencephalon (1/19), and amygdala (1/19) were the brain areas selected to be measured in the reported studies (Table 3).

Exercise training programs

Running in a treadmill (13/19) and swimming (6/19) at different intensities, involving continuous or intervallic activities and with or without loads or different slopes, were the most common exercise programs reported in most of the studies. According to the effects of the different exercise interventions performed on brain oxidative stress, we have found the following results:

Exercise protocol may decrease oxidative stress

Ten of the 19 studies improved brain antioxidant capacity (Somani et al., 1995; Ohkuwa et al., 1997; Itoh et al., 1998; Liu et al., 2000; Radak et al., 2001b, 2006; Navarro et al., 2004; Qiao et al., 2006; Vollert et al., 2011; Falone et al., 2012). Navarro et al. (2004) carried out a moderate exercise treadmill (6, 9, and 12 m/min for 5 min each, every day) from 28 to 78 weeks of age extended in mice. The authors concluded that moderate exercise started at young age increases life span, decreases oxidative stress, and prevents the decline of cytochrome oxidase activity and behavioral performance at middle-aged but not at old-aged mice. This concurs with the study by Ohkuwa et al. (1997) who performed a protocol where rats were divided into four groups: two sedentary with or without voluntary running (physically active) and two exercise groups (running on a treadmill) with or without voluntary running. Groups were trained 2 days/week, at a speed of 10 m/min for the 3 first weeks and 3 days/week, at a speed of 15 m/min for the last 2 weeks. They observed that physically active and exercise group enhances the endogenous ability of the body to defend it against oxidative stress. GSH, GSSG, and ratio GSH/total GSH (ratio) levels in brain were higher in old than young rats. In the study performed by Falone et al. (2012), four mice groups with or without exercise for 2 or 4 months each group, respectively, were carried out. Exercised groups started the treadmill training program (running at 13 m/min for 20 min, 5 days/week) and the final running workload was reached by incrementing 1 min/day, starting from 10 min/day. The authors suggested that lately initiated exercise regimen strongly reduced molecular damage profiles and increased their antioxidant enzymatic capacity. Vollert et al. (2011) developed a moderate treadmill exercise protocol in rats for 4 weeks: 30 min/day at a speed of 10 m/min for 2 weeks (2×15 min sessions), 45 min at a speed of 15 m/

Table 4. Oxidative stress outcomes studied

Author/study quality score	Sp	R/NR	Ex	GSH	GSSG	GPx	GR	SOD	CAT	TAS	LP	BDNF	PT	PO	PB
Navarro et al. Quality score 6	CD1 mice	NR	Aer	–	–	–	–	Ex ↑Mn and Cu-Zn	Ex ↓old	–	Ex ↓TBARS Middle age	–	–	–	Total brain
Ohkuwa et al. Quality score 5	WS	NR	Aer	PA ↑old Ex ↑old	Ex ↑young (PA and Sed) Ex (↑old)	–	–	–	–	–	–	–	–	–	Total brain
Falone et al. Quality score 7	CD1 mice	R	Aer	Ex (↓ Ratio tGSH/GSSG in E2 vs S2 and E4 vs S4)	Ex↓ Ratio tGSH/GSSG in E2 vs S2 and E4 vs S4	GPx (Ex ↓E2 vs S2 Ex ↑S4 vs S2)	Ex (↑ E2 vs S2) Ex (NS E4 vs S4)	Ex ↓ E2 vs S2 Ex ↑ E4 vs S4	Ex ↓ E2 vs S2 Ex ↑ E4 vs S4	–	Ex (↑TBARS in E2 vs S2)(similar levels in S4 vs E4)	Ex ↓ E2 vs S2 Ex ↑ E4 vs S4	–	–	CC
Vollert et al. Quality score 7	WS	R	Aer	–	–	–	–	–	–	–	SLD ↑ MDA in H, CC, and AM compared C and Ex)	–	–	–	H, CC, and AM
Itoh et al. Quality score 6	WS	NR	Aer	Ex NS	Ex(↑type I) Sed>type I and type II	–	–	–	–	–	–	–	–	–	Total brain
Somani et al. Quality score 5	SD	R	An	Ex NS (CC and CS)	Ex (↓CC and BS)	GSH-Px (Ex ↓CS)	–	Ex (↑CS and BS)	–	–	–	–	–	–	CC, CS, BS, and H
Liu et al. Quality score 5	SD	NR	Aer	Ex NS	Ex NS	–	–	–	–	–	↓MDA	–	–	–	Total brain
Radak et al. (2001b) Quality score 4	WS	R	Aer	–	–	–	–	–	–	–	NS Ex	–	–	–	Total brain
Radak et al. (2006) Quality score 6	WS	R	Aer	–	–	–	–	–	–	–	–	Ex↑ DT↓	–	–	EPR- > CB BDNF- > H
Qiao et al. Quality score 6	Albino mice	R	An	–	–	–	–	Ex Ns	–	↑ Ex	Ex NS	–	–	–	DC
Aguiar et al. (2010) Quality score 7	Swiss mice	R	An	↓H (NS in PC and CS)	–	GPx (Ex ↑H and PC NS in CS)	Ex NS	–	–	–	–	–	–	–	H, CS, and PC
Aguiar et al. (2008b) Quality score 8	CF1 mice	R	An	–	–	–	–	–	–	–	Both Ex ↑TBARS	Both Ex↓	–	–	CC
Tsakiris et al. Quality score 5	WS	NR	Aer	–	–	–	–	–	–	Ex ↓after 2 and 5 h	–	–	Ex ↓	–	Total brain
Aydin et al. Quality score 6	SD	NR	An	End and Exh Ex (↓DR vs AL) (↑Intensity Ex ↓DR and AL)	–	GSH-Px End and Exh Ex (↓DR vs AL and sedentary) ↑Intensity Ex ↓DR)	Exh Ex (↓DR and AL)	–	–	–	↑Intensity Ex ↑TBARS in DR and AL	–	–	↑Intensity Ex ↑PO in DR and AL	CC
Cechetti et al. Quality score 4	WS	R	Aer	–	–	–	–	–	–	–	Ex NS	Ex NS	–	–	H, CC, CS, and CB
Aksu et al. Quality score 5	SD	NR	Aer	–	–	GPx (Ex NS)	–	Ex NS	–	–	Ex NS TBARS	–	–	–	H, PC, and CS
De Araujo et al. Quality score 6	WS	NR	Aer/An	–	–	–	–	–	Ex NS	–	Ex NS TBARS	–	–	–	Total brain
Acikgoz et al. Quality score 5	WS	NR	An	–	–	GPx (Ex NS)	–	Ex NS	–	–	Ex NS	–	–	–	H, PC, and CS
Ogonovszky et al. Quality score 5	WS	R	Aer/An	–	–	–	–	–	–	–	Ex NS	OT ↑	–	–	Total brain

Sp, species; R/NR, randomized/no randomized; Ex, exercise; GSH, glutathione; GSSG, oxidized glutathione; GSH-Px, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; CAT, catalase; TAS, total antioxidant status; LP, lipid peroxidation; BDNF, brain-derived neurotrophic factor; PT, protein tissue content; PO, protein oxidation content; PB, part of the brain measured; SD, Sprague–Dawley rat; WS, Wistar rat; An, anaerobic exercise; Aer, aerobic exercise; End, endurance exercise; Exh, exhaustive exercise; PA, physical active; Sed, sedentary; SLD, sleep deprivation; CC, cerebral cortex; CS, corpus striatum; BS, brain stem; H, hippocampus; DC, diencephalon; CB, cerebellum; PC; prefrontal cortex; AM, amygdala; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substance; OT, overtrained; DT, detrained; DR, diet restriction; AL; *ad libitum*; NS, not significant.

min for 1 week (3 × 15 min sessions), and finally 60 min at 15 m/min (4 × 15 min sessions), and analysed the potentially protective effect of exercise before acute sleep deprivation. The authors reported that acute sleep deprivation increases oxidative stress in the cortex, hippocampus, and amygdala while prior treadmill exercise prevents against this increase. Similarly, Itoh et al. (1998) divided rats into three groups: sedentary, type I training (constant speed of 20 m/min for 15 min and 22.5 m/min for 5 min for the first week; 20 m/min for 20 min and 22.5 m/min for 5 min for the second week; 22.5 m/min for 20 min and 22.5 m/min for 5 min for the third week), and type II training (running at 20 m/min for 30, 45, and 60 min for the first, second, and third weeks, respectively). They observed that regular moderate endurance exercise increased antioxidant capacity in rats at different treadmill training protocols.

Other protocols have been with slope or increasing the intensity abruptly. Somani et al. (1995) performed an incremental exercise program where rats ran 5 days/week for 7.5 weeks. In the first 2 weeks, animals ran at a speed of 8, 15, and 19 m/min for 5 min each speed (i.e., 15 min) and for 10 min each speed (i.e., 30 min) the second week. In the third and fourth weeks, speed increased to 19, 27, and 30 m/min for 10 min each speed. Same speed and long time were carried out for the last 3.5 weeks. The angle of inclination was increased gradually up to 10°. The authors observed an increase in SOD enzymatic activity after exercise in different brain areas.

In the study by Liu et al. (2000), the animals in the chronic exercise groups were habituated to treadmill exercise over a 2-week period, where the duration and speed of exercise progressively increased to 120 min at 27 m/min. For 2 weeks thereafter, animals were exercised at this level for 8 weeks, 5 days/week. Animals in the acute exercise groups were also conditioned to the treadmill over a 2-week period but only for 10 min at 13 m/min for 3 days/week. Immediately before death, animals were made to run in the treadmill at 27 m/min until exhaustion. The authors observed an increase on brain antioxidant levels with chronic but not with acute exercise.

Other studies employed swimming protocols, as the one performed by Radak et al. (2001b) in which young and middle-aged rats were trained during 60 min/day, 5 days/week for 6 weeks, and 90 min/day, 5 days/week, the 3 remaining weeks. The authors observed that swimming improves some cognitive functions, with the parallel attenuation of the accumulation of oxidative-damaged proteins. The same authors (Radak et al., 2006) distributed rats into three groups: control, exercise, and detrained groups. Exercise and detrained rats swam for 8 weeks, 60 min/day, 5 days/week for 4 weeks. Then, for the remaining 4 weeks, exercise was increased to 120 min/day for 5 days/week. After 8 weeks of training, the detrained group was kept as the control group for an additional 8 weeks. The authors concluded that exercise training is likely to benefit the effect in the production of reactive oxygen species (ROS) and the related oxidative damage. Furthermore, swimming but in mice, Qiao et al. (2006) distributed mice into three groups: control, and anaerobic exercise with short or long rest interval (10 or 40 s, respectively) groups. These exercise groups were each subdivided into four subgroups: 2, 4, 6 days and behavioral observation group. Mice swam with a load tied to the tails equal to the 10% of their body mass the first and second days, a 13% the third and fourth days, and a 15% of the fifth and sixth days. Daily swim lasted 8 × 10 s. The authors found that intermittent anaerobic exercise increases brain antioxidant capacity.

Exercise may increase oxidative stress

In contrast, 6 of 19 studies found increments of oxidative stress markers after an exercise protocol (Somani et al., 1995; Liu et al., 2000; Tsakiris et al., 2006; Aguiar Jr et al., 2008b; Aydin et al., 2009; Aguiar et al., 2010). Aguiar et al. (2010) divided mice in sedentary or high-intensity exercise groups. The researchers adapted a high-intensity exercise protocol from a high-intensity sprint interval training descriptions (Troup et al., 1986; Kubukeli et al., 2002). Thus, 60-min high-intensity sprint interval training was performed with two 20-min bouts of exercise separated by two 10-min periods of rest. In this protocol, they removed the resting time to avoid recovery and to reach high intensities of exercise. When the animals reached the stipulated maximum volume of exercise (60 min), they lowered this volume in the following week to increase the speed running. After this protocol, the authors described an increase in the vulnerability of the striatum to high-intensity exercise. The same authors also (Aguiar Jr et al., 2008b) performed an incremental running program in mice during 8 weeks (first 4 weeks 13.5 m/min and last 4 weeks 16.5 m/min of speed), 5 days/week for 40 days. The intermittent exercise group performed the exercise three times a day for 15 min and the continuous group exercised once for 45 min. The authors reported that intense exercise promoted brain mitochondrial dysfunction as well as an increase in the frontal cortex thiobarbituric acid-reactive substance levels in exercised mice. In agreement to the above-mentioned study, Somani et al. (1995) observed that different brain areas contained different activities of antioxidant enzymes, as well as GPx and GSSG levels, which were preferentially altered as a result of exercise training to cope with oxidative stress.

In the study performed by Tsakiris et al. (2006), short (2 h) as well as prolonged (5 h) forced swimming also induced oxidative stress in rats. Moreover, a Na^+, K^+-ATPase, and Mg^{2+}-ATPase activation was observed under the above-mentioned experimental conditions. Similarly, Aydin et al. (2009) divided rats in dietary restriction group or *ad libitum* food intake group, and each group was further subdivided into three groups:

sedentary, endurance exercise (5 days/week for 8 weeks), and maximal exercise (exhaustive swimming exercise) groups. At the end of the eighth week, rats in the exhausted exercise group were forced to swim until exhaustion. The authors concluded that long-term dietary restriction may protect against endurance and exhaustive swimming exercise-induced oxidative stress, which were used as an oxidant stressor. Finally, in the above-mentioned study by Liu et al. (2000), the authors observed a decrease in brain antioxidant levels with acute exercise.

Exercise does not affect oxidative stress

Five of the 19 studies did not observe changes in brain oxidative stress markers (Ogonovszky et al., 2005; Acikgoz et al., 2006; Cechetti et al., 2008; Aksu et al., 2009; de Araujo et al., 2009). Studies as the one performed by Cechetti et al. (2008) found that a daily moderate intensity exercise in rats, 2 weeks for 20 min/day of running, did not affect any oxidative stress parameter in hippocampus, suggesting that daily moderate exercise does not cause significant oxidative stress nor induce adaptations of the cellular antioxidant system. Treadmill training also did neither change BDNF content in the brain areas studied. Aksu et al. (2009) performed 10 trial groups with 8 animals in each. Acute exercise groups composed of groups that ran on a treadmill at a speed of 10 m/min (A1), 15 m/min (A2), and 20 m/min (A3) for 1 h and an exhaustive exercise group (E). Chronic exercise groups composed of rats that ran on a treadmill at a speed of 10 m/min (R1), 15 m/min (R2), and 20 m/min (R3), 1 h/day, 5 days/week, for 8 weeks. There were also three control groups: a group of non-exercising rats (C), a handled group of rats that were put on the treadmill without doing exercise for 1 h, 5 days/week, for 8 weeks (CR), and a handled group of rats that were put on the treadmill without doing exercise for 1 h (CA). In acute exhaustive exercise group (E), the rats were forced to run at a speed of 25 m/min at a slope of 5° until exhaustion. This study also observed that acute as well as chronic exercise protocols do not alter oxidative stress in prefrontal cortex, striatum, and hippocampus.

In the study by De Araujo et al. (2009), rats were divided into three experimental groups: sedentary, trained at the metabolic transition intensity (speed equivalent to the aerobic/anaerobic threshold, 40 min/day, 5 days/week, for 8 weeks), and trained (speed 25% above the aerobic/anaerobic threshold, 40 min/day, 5 days/week, for 8 weeks). They did not observe alterations on brain CAT enzyme activity by this protocol. Acikgoz et al. (2006) found that an acute exhaustive protocol in rats running at 25 m/min with a slope of 5° until exhaustion did not cause LP in the hippocampus, prefrontal cortex, and striatum during the post-exercise period. Finally, Ogonovszky et al. (2005) distributed 28 Wistar rats in control, moderately trained (swimming 60 min/day, 5 days/week, for 8 weeks), strenuously trained (swimming increased by 30 min/week until it reached 4.5 h for the last week), and overtrained group (swimming 60 min/day, 5 days/week, for 6 weeks and then the duration was abruptly increased to 4.5 h for the remaining 2 weeks). Under their experimental conditions, overtraining did neither induce brain oxidative stress.

Discussion

The purpose of this systematic review was to study the effects of exercise on brain oxidative stress. Aerobic moderate exercise appears to promote a protective antioxidant function on brain. However, studies referred to aerobic exhausted exercise, anaerobic exercise, or the combination of both types of training report inconclusive or conflicting findings. The high heterogeneity observed among the exercise protocols developed in the studies makes it difficult to draw clear conclusions regarding exercise volume and intensity.

As we mentioned above, running in a treadmill and swimming were the more common activities carried out. We aimed to analyse, independently on the type of exercise, the effects of exercise on the brain antioxidant capacity. Moderate aerobic training or simply voluntary exercise (running on a wheel) ameliorates antioxidant capacity (Ohkuwa et al., 1997; Itoh et al., 1998; Radak et al., 2001b, 2006; Navarro et al., 2004; Vollert et al., 2011; Falone et al., 2012) as well as regular moderate exercise improves brain function (Radak et al., 2006), memory (Radak et al., 2001a, b), proteasome activation, and up-regulation of the antioxidant system (Radak et al., 2000b). Furthermore, daily moderate exercise has been shown to reduce damage of hippocampal slices from Wistar rats exposed to *in vitro* ischemia (Scopel et al., 2006; Cechetti et al., 2007). Anaerobic exercise in a progressive exercise program can also improve different activities of antioxidant enzymes in brain (Somani et al., 1995). Similarly, anaerobic exercise with 10 s (short) or 40 s (long) rest intervals increased the antioxidant capacity from different tissues (Qiao et al., 2006) at the same time that running on a treadmill until exhaustion did not induce LP in the hippocampus (Acikgoz et al., 2006). Surprisingly, some other studies in which rats were overtrained in long term of strenuous exercise or when the duration increased abruptly did not induce brain oxidative stress (Fry et al., 1991; Petibois et al., 2003; Ogonovszky et al., 2005), and similarly acute and chronic exercise neither promoted oxidant stress in prefrontal cortex, striatum, and hippocampus (Aksu et al., 2009).

In contrast, although some studies (Somani et al., 1995; Ogonovszky et al., 2005; Qiao et al., 2006) have found antioxidant properties after aerobic extenuation or anaerobic programs, the body of the revised literature suggests that anaerobic high-intensity and strenuous exercises, independently of the capacity performed, can

increase, in general, oxidative stress (Tsakiris et al., 2006; Aguiar Jr et al., 2008b; Aydin et al., 2009; Aguiar et al., 2010). Consequently, we hypothesize that regular aerobic moderate training or physical active programs are the most appropriated exercises to positively enhance brain antioxidant response.

SOD, GSH, GSSG, GPx enzymes, and LP were the oxidative stress markers more frequently analysed along the studies. When analysing these outcomes, aerobic exercise promoted a positive effect (increase or maintain the same level) on SOD levels in 100% of the cases, whereas GSH, GSSG, and GPx showed a more inconclusive response, with a slightly trend to a positive effect of aerobic exercise. Finally, aerobic exercise improved LP in 90% (Liu et al., 2000; Radak et al., 2001b; Navarro et al., 2004; Ogonovszky et al., 2005; Cechetti et al., 2008; Aksu et al., 2009; de Araujo et al., 2009; Vollert et al., 2011; Falone et al., 2012) of the studies while it was decreased in 50% (Acikgoz et al., 2006; Qiao et al., 2006) of the studies that performed anaerobic high-intensity exercise protocols.

Oxidative stress elicits different responses depending on the organ tissue type and its endogenous antioxidant levels with an acute and chronic exercise. In the study performed by Liu et al. (2000), brain was positively responsive to chronic exercise and its response was different compared with other organs analysed.

Brain was the tissue selected in the present review due to the little information regarding whether exercise above certain intensity or duration could be harmful in the brain function (Ogonovszky et al., 2005). Despite that there is not a consensus about which parts of the brain should be analysed and their reasons, three studies described why they selected a specific brain area to measure; cerebral cortex, brain stem, corpus striatum, and hippocampus are the regions involved in motor control and cognitive functions by exercise and therefore, for such authors, these must be the selected areas to study when analysing the exercise effects on brain (Somani et al., 1995). Moreover, hippocampus is also recommended to be selected because it contains high concentrations of glucocorticoid receptors (Acikgoz et al., 2006). For these authors, prefrontal cortex and corpus striatum should additionally be measured because they have high dopamine content (Acikgoz et al., 2006). Theoretically, exhaustive exercise may cause oxidative stress in the brain. First, exercise enhances brain dopamine synthesis (Sutoo & Akiyama, 2003). Dopamine may form ROS through either dopamine metabolism by monoamine oxidase or autoxidation (Halliwell & Gutteridge, 1999). Second, exercise leads to increased serum glucocorticoid levels. Corticosterone increases the toxicity of oxygen radical generators (McIntosh & Sapolsky, 1996), and may increase the basal levels of ROS (McIntosh & Sapolsky, 1996), altering antioxidant enzyme activities in the brain (McIntosh et al., 1998).

Regarding the MQA performed in the studies from the present review, the total overall quality of reporting was higher (71%) than the internal validity (25%), with an average quality of 57%. These percentages provide a poor internal validity of the manuscripts, and thus future studies should report adverse events or experimental deaths and should employ blinding interventions with the purpose of increasing the internal validity, and therefore to improve the quality of the studies. However, even taking the above-mentioned reasons in consideration, the main quality of the studies analysed provided an acceptable level to consolidate the results of this systematic review.

Unfortunately, the exhaustive process carried out in the present systematic review does not provide a consensus about the best specific exercise program protocol to protect brain against oxidative stress, and neither about which part of the brain should be specifically analysed.

Overall, the scope of this systematic review was to overview the literature addressing the influence of exercise on brain oxidative stress. To our knowledge, no study has been deeply investigated this relationship. Because most of the studies in which brain oxidative stress has been studied after a parallel intervention (e.g., drugs administration), it is interesting to analyse the effects of different types of exercise on brain oxidative stress markers by itself, studied without any alteration.

Limitations and strengths

The present study has several limitations that need to be mentioned. First, the heterogeneity among the exercise protocols developed among the studies is huge. The exercise protocols make them difficult to draw clear conclusions regarding exercise volume and intensity. Second, in the selected manuscripts, the authors have not described blinding interventions and losses of animals, which would have helped improve the methodological quality of the studies. On the other hand, this is the first systematic review addressing the influence of exercise on brain oxidative stress with no alteration (e.g., drugs). Furthermore, a rigorous MQA, including levels of evidence, was carried out through all the selected manuscripts.

Perspectives

The wide range of exercise protocols at different intensities and volumes does not allow us to provide reliable conclusions. This lack of homogeneity in the protocols could be due to the difficulty to establish the intensity of the effort when using animal models.

Future investigations should be exhaustively controlled and be focused on brain oxidative stress markers regarding the different specific regions of the brain and a wide range of conditions as intensity and type of exercise, and drink or food intake, which could all of them

modify the findings. Moreover, establishing standardized exercise protocols in order to specifically study aerobic or anaerobic metabolism will help to improve our knowledge in this topic. In addition, it would be of interest to test the effect of the spontaneous physical activity (e.g., through running wheels) on brain oxidative stress in future studies.

Despite that literature tends to globalize exercise like a way to improve brain antioxidant capacity, studies referred to aerobic exhausted exercise, anaerobic exercise, or the combination of both types of training still report confusing findings. Regular moderate aerobic exercise appears to be highly contrasted to protect against brain oxidative stress. Therefore, among all the types of training programs analysed in the present review, moderate aerobic exercise is the most contracted appropriate activity to promote a protective antioxidant capacity on brain.

At research level, this study is interesting for the scientific community in order to improve the design and standardization of exercise protocols in their experiments. Moreover, this review may help sports practitioners, personal trainers, or health providers to select moderate aerobic exercise in order to reduce brain oxidative stress (especially on weaker populations like Alzheimer, dementia, or other cognitive diseases).

Key words: enzymatic activity, oxidative stress, physical extenuation, rats, brain, anaerobic exercise, aerobic exercise, exercise protocol.

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

Daniel Camiletti-Moirón, Virginia A. Aparicio, Elena Nebot, Gerardo Medina, Rosario Martínez, Garyfallia Kapravelou, Ana Andrade, Jesús M. Porres, María López-Jurado, Pilar Aranda Ramírez.

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Original/*Deporte y ejercicio*

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 know regarding to the high-protein diets and high intensity exercise on oxidative stress production. The aim of this study was to analyse the effects of 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), cooper/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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UNA DIETA ALTA EN PROTEÍNA PRODUCE ESTRÉS OXIDATIVO EN EL CEREBRO DE RATAS: ACCIÓN PROTECTORA DEL EJERCICIO DE ALTA INTENSIDAD SOBRE LA PEROXIDACIÓN LIPÍDICA

Resumen

Introducción: **Es conocido que la proteína de soja así como la práctica de ejercicio físico aeróbico pueden incrementar la capacidad antioxidante y con ello reducir la sobreproducción de radicales libres en el cerebro. Sin embargo, existe desconocimiento sobre el efecto del consumo de dietas hiperproteicas y el entrenamiento de alta intensidad (EAI) sobre dicho estrés oxidativo. El objetivo del presente estudio fue analizar la influencia del consumo de una dieta hiperproteica y de EAI sobre marcadores de estrés oxidativo en cerebro.**

Métodos: **Cuarenta ratas Wistar macho adultas fueron aleatoriamente distribuidas en 4 grupos experimentales (n=10): dieta normoproteica o hiperproteica, con o sin EAI durante un periodo experimental de 12 semanas. Se determinaron los principales marcadores de daño oxidativo en cerebro como sustancias reactivas del ácido tiobarbitúrico (TBARs) y el contenido de grupos carbonilos (PCC). Además, se midieron las actividades enzimáticas superóxido dismutasa del manganeso (Mn-SOD), de cobre/zinc (CuZn-SOD) y catalasa (CAT), así como el nivel de proteína del factor nuclear eritroide-2 (Nrf2).**

Resultados: **Los niveles de TBARs, PCC, tSOD, Mn-SOD, CuZn-SOD y CAT fueron significativamente mayores en los grupos hiperproteicos en comparación con los normoproteicos (todas, p<0,05). La expresión de la proteína Nrf2 fue mayor en los grupos hiperproteicos y con EAI en comparación con los grupos normorpoteicos y sedentarios, respectivamente (ambos, p<0,01). Se observó una interacción en la disminución de los niveles de TBARs, tSOD y CuZn-SOD producida por el EAI en el grupo hiperproteico que no fue reflejada en el grupo normoproteico (p=0,05).**

Conclusions: **The high-protein diets consumption produce 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.*

Conclusión: **El consumo de una dieta hiperproteica 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 hiperproteica.**

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Palabras claves: *Superóxido Dismutasa. Catalasa. Sustancias Reactivas al Ácido Tiobarbitúrico. Factor 2 Relacionado con NF-E2. Proteínas de Soja. Hipertrofia.*

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.

Introduction

Reactive oxygen species (ROS) are by-products of aerobic cellular metabolism that can induce oxidative stress[1,2]. The major antioxidant enzymes in the rat brain are superoxide dismutase (SOD) and catalase (CAT)[3]. 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[1,2]. In addition, the oxidative damage repair systems are important in order to minimize the dangerous effects of high production of ROS[4,5]. 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[6-8].

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 aldehydes or dienals (e.g., 4-hydroxynonenal), which in turn may cause neuronal apoptosis[9]. 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[10-12].

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[13]. 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[14,15]. 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[16,17]. 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[18,19]. Recent studies have observed that chronic exercise activates the Nuclear factor erythroid 2 related factor 2 (Nrf2) in human skeletal muscle and rat kidney,[20,21] 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[22,23]. 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.

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±2ºC). 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 -80ºC 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 rats[25] 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 rats[27-29]. 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.

Table I
Formulation of the experimental diets

Nutritional Composition (g/100g DM)	*Protein diet*	
	Normal protein	*High protein*
Soy protein supplement	13.1	57.4
Mineral mix (AIN-93M-MX)	3.5	3.5
Vitamin mix (AIN-93-VX)	1	1
Fat (olive oil)	4	4
Choline chloride	0.25	0.25
Cellulose	5	5
Starch	62.4	28.6
Methionine	0.5	-
Sucrose	10	-

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 on gaining muscle mass and strength who usually combine high-protein diets with HIE[29] (see Table II). Therefore, our training protocol follows the established principles for human strength training, involving weights, repetitions, and sets to maximize muscle gain[30].

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 al[29]. The entire training process was designed and controlled by sport scientists in collaboration with experienced researches trained to work with rats.

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

Table II
Details of the high-intensity exercise program

Week	*Work time (min)*	*Sets*	*Time between sets (min)*	*Weight (% 1 RM)*
1	2	10	1	55
2	2	10	1	60
3	2	10	1	65
4	2	10	1.5	70
5	2	10	1.5	70
6	2.5	10	1.5	75
7	2.5	12	1.5	75
8	2	12	2	80
9	2.5	12	2	80
10	1.5	12	2	85
11	2	12	2.5	85
12	1	12	2.5	85

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[31]. The results were expressed as nmol of Malonildialdehide 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[32]. 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.[33] 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 tSOD 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 Aebi[34] monitoring the disappearance of H_2O_2 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 Lowry[35].

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 (lgepal), 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 Aproptinin. 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 -80ºC until further use. The protein concentration was measured by the method of Lowry *et* al.[35]. 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 4ºC. 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 between 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 III
Effects of the dietary protein amount and high-intensity exercise on final body weight, carcass weight, food intake and brain weight

	Normal protein		*High protein*		*SEM*	*p values*		
	Sedentary	*Exercise*	*Sedentary*	*Exercise*		*Protein amount*	*Exercise*	*Protein amount*Exercise*
Food intake (g/day)	20.317c	15.622a,b	16.948b	14.806a	0.177	<0.001	<0.001	0.001
Final body weight (g)	351.216a	313.042a	317.594a	327.174a	5.085	0.344	0.168	0.025
Carcass weight (g)	172.120a	163.210a	178.870a	169.229a	2.897	0.278	0.118	0.950
Brain (g)	1.925a	1.881a	1.894a	1.917a	0.015	0.927	0.737	0.274
Brain (g/100g body weight)	0.552a	0.605a	0.603a	0.588a	0.009	0.357	0.297	0.075
Brain (g/100g carcass weight)	1.128a	1.160a	1.064a	1.142a	0.016	0.212	0.094	0.484

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).

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

	Normal protein		*High protein*		*SEM*	*p values*		
	Sedentary	*Exercise*	*Sedentary*	*Exercise*		*Protein amount*	*Exercise*	*Protein amount*Exercise*
TBARs (nmol MDA/mg protein)	19.684a	23.553a,b	27.768b	22.875a,b	0.880	0.042	0.773	0.018
PCC (nmol/mg protein)	3.496a	2.440a	4.878a	4.948a	0.332	0.006	0.463	0.403
tSOD (U/mg protein)	137.474a	154.578a,b	192.837c	173.469b,c	3.551	<0.001	0.874	0.015
Mn-SOD (U/mg protein)	63.990a	71.828a,b	79.299b	80.359b	1.912	0.004	0.252	0.381
CuZn-SOD (U/mg protein)	73.484a	82.750a	113.538b	93.110a	2.578	<0.001	0.286	0.007
CAT (μmolH2O2/min/mg protein)	2.852a	4.274b	3.526a,b	5.640c	0.126	<0.001	<0.001	0.178

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, cooper 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).

Food consumption, body weight and body composition

Food intake is markedly affected by diet composition[36,37] and physical activity[38,39]. Few studies in animals as well as in humans have illustrated that high-protein diets[36,37] 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[40]. These assertions are in agreement with our findings that the high-protein and the HIE groups displayed a reduced food intake when com-

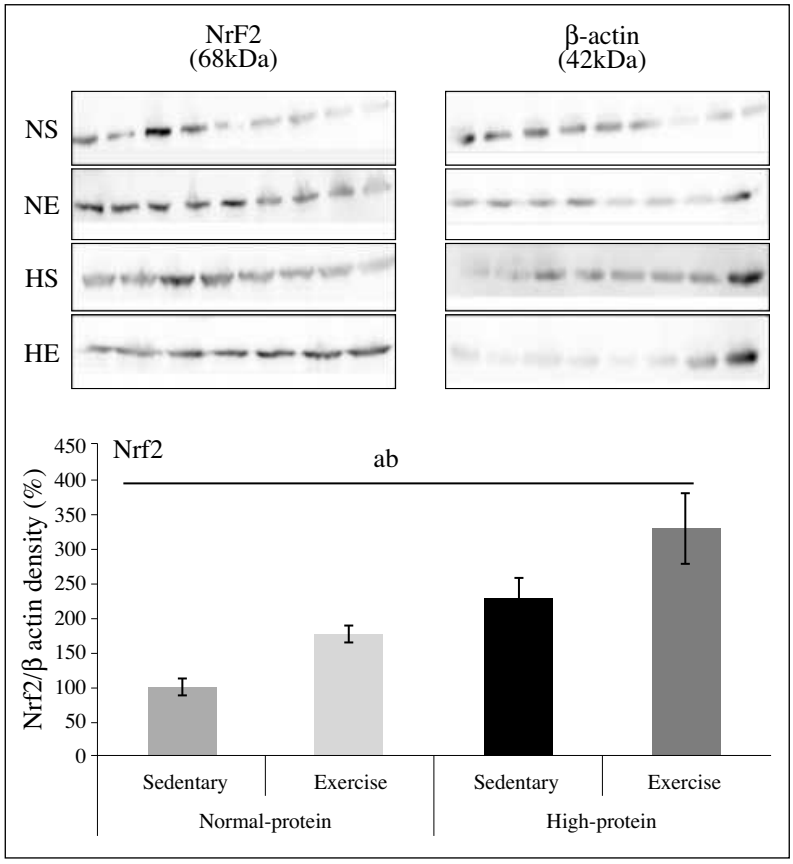

Fig. 1.—Effects of the high-protein diet and high-intensity exercise on brain Nrf2 protein levels, n=8.
The representative western blots show the Nrf2 bands (left lines) and the β-actin bands used as a loading control (right lines). Annotation indicates significant effect of a = exercise, b = anabolic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

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[14,15], 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[41], 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[42] 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.[43], 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[44]. 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[22,45]. 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[23,46].

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[47]. In addition, Margonis et al.[47] 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[48] 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[48].

In spite of the controversial findings regarding the HIE, acute exercise promotes free radicals and ROS generation, which may lead to lipid peroxidation[49]. 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[49], 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[50]. 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.

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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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1.2. KIDNEY

High-intensity exercise may compromise renal morphology in rats

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High-Intensity Exercise May Compromise Renal Morphology in Rats

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Key words
- strength training
- plasma
- urine
- kidney
- renal morphology
- rat

Abstract

We investigated the renal effects of a high-intensity exercise (HIE) program based on strength training. 20 Wistar rats were randomly assigned to 2 experimental groups performing HIE or control over 12 weeks. Urinary volume, pH, citrate and calcium, and plasma urea, total proteins, creatinine, albumin, lactate dehydrogenase, creatine kinase (CK), calcium, magnesium, corticosterone and testosterone were measured. We also studied renal morphology with the Fibrosis HR® software. Plasma urea and CK concentrations were higher in the HIE compared to the control group ($p<0.05$), whereas plasma creatinine was lower ($p<0.01$). Plasma corticosterone was higher ($p<0.05$) and testosterone lower ($p<0.01$) in the HIE group. Except for the higher urinary volume found in the HIE group ($p<0.05$), no differences between groups were observed in the rest of urinary parameters analyzed. Renal interstitial connective tissue was ~30% higher in the HIE group ($p<0.05$). Glomerular tufts and mesangial areas were also higher in the HIE group (all, $p<0.05$). No differences between groups were observed in the glomerular area. Overall, HIE promoted a worse morphological renal profile that might be associated with a higher risk for incidence of kidney disease in the long-term. The stress induced by the type of exercise performed could be on the basis of this worse morphological renal status.

Introduction

The effect of exercise, and more specifically its type, dose and intensity, on renal status is rather unknown. This is especially relevant nowadays due to the fact that chronic kidney disease (CKD) is a silent illness that is becoming an emergent public health burden [20]. On the one hand, exercise might reduce kidney inflammation, improve glomerular filtration rate and increase plasma albumin concentrations [32, 36–39]. Because it is well established that inactivity contributes to CKD [42], these patients could benefit from resistance training interventions [23]. Resistance training may increase nitrogen (N) retention and protein synthesis, ameliorate loss of muscle mass and its function and consequently alleviate proteinuria and thereby kidney disease in this segment of the population (elderly, CKD, or subjects exposed to weightlessness) [10, 14, 23]. On the other hand, high-intensity or strenuous exercise can result in muscle damage evidenced by increased blood levels of muscle proteins such as creatine kinase (CK), lactate dehydrogenase (LDH) and myoglobin [41, 45]. Renal function can be impaired when plasma concentration of these proteins increases [19]. In addition, intense resistance training, such as strength training, could promote hypoxia, glucose depletion or oxidative stress, which may lead to endoplasmic reticulum stress, inducing glomerular and tubular damage in patients with acute and CKD [13, 22]. Furthermore, in the last decade, strength training has become one of the most popular physical activities in developed countries and steadily increasing numbers of gyms have been opened [12].

To date, only 2 studies have examined kidney morphology after an exercise intervention in depth, and they were performed in rats subjected to weightlessness [14] or suffering from hypertension [2]. Moreover, the effect of exercise, more specifically high-intensity exercise (HIE) on renal morphology under normal conditions or in healthy individuals remains unclear. Therefore, the present study sought to examine the effects of a HIE protocol based on strength training on plasma, urinary and morphological renal markers in rats.

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Materials and Methods

Animals and experimental design

A total of 20 albino male Wistar rats were assigned to 2 groups (n = 10), performing HIE or sedentary. The animals, aged 8 weeks and having an initial body weight of 150 ± 8 g, were housed in individual stainless steel metabolism cages designed for the separate collection of urine. The cages were located in a well-ventilated thermostatically controlled room (21 ± 2 °C), with relative humidity ranging from 40 to 60 %. A 12:12 reverse light-dark (08:00–20:00 h) cycle was implemented to allow exercise training during the dark period. Throughout the experimental period all rats had free access to distilled water and the animals consumed the diet ad libitum. Experimental diets were formulated to meet the nutrient requirements of rats [1] based on the AIN-93M formulation described by Reeves et al., but included modifications in the protein source and content and the oil source [40]. A protein content of 10 % was chosen according to the American Institute of Nutrition (AIN-93M) [40]. Commercial soy protein isolate was used as the source of protein since this protein is widely available and used by athletes.

One week prior to the experimental period, the animals were allowed to adapt to the experimental conditions. The rats' body weights were measured weekly and at the same time of day, and the amount of food consumed by each rat was registered daily (Ohaus® Adventurer™*Pro.* Capacity to 3 100 g, readability of 0.01 g. New Jersey, USA).

During week 11, a 12-h urine sample from each animal was collected for biochemical analysis. The urine volumes were recorded and samples were transferred into graduated centrifuge tubes for pH, calcium and citrate analysis. At the end of the experimental period, the animals were anaesthetized with ketamine-xylazine and euthanized by cannulation of the abdominal aorta. Blood was collected (with heparin as anticoagulant) and centrifuged at 3 000 rpm for 15 min to separate the plasma, which was subsequently removed and frozen in liquid nitrogen (N) and stored at −80 °C. The carcass weights were recorded. Carcass weight is the weight of the slaughtered animal's cold body after being skinned, bled and eviscerated and following removal the head, the tail and the feet. The left kidneys were extracted, weighed and immediately stored in formalin for subsequent histological analyses.

All experiments were undertaken in accordance with the Ethical Standards in Sport and Exercise Science Research [18] as well as the Directional Guides Related to Animal Housing and Care (European Community Council, 1986) [15] and followed the Canadian Council on Animal Care (CCAC) guidelines. All procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada.

Resistance training

The experimental group was trained following a strength training protocol in a motorized treadmill (Panlab Treadmills for 5 rats, LE 8710R) with weights in a bag 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 through regular exercise at gyms. Therefore, our training protocol follows the established principles for human strength training, involving weights, repetitions and sets to maximize muscle gains [12]. Thus, the aim of the present study was not to protect renal health through exercise (i. e., aerobic exercise).

Table 1 Details of the resistance-training program.

Week	Work time (min)	Sets	Time between sets (min)	Weight (% 1 RM)
1	2	10	1	55
2	2	10	1	60
3	2	10	1	65
4	2	10	1.5	70
5	2	10	1.5	70
6	2.5	10	1.5	75
7	2.5	12	1.5	75
8	2	12	2	80
9	2.5	12	2	80
10	1.5	12	2	85
11	2	12	2.5	85
12	1	12	2.5	85

RM: repetition maximum

The training group exercised on alternate days (3–4 sessions/week). The animals ran 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, first 3 days without weight and the last 4 days with 20 % of their body weight. The training protocol used in the present study has been previously developed and deeply described by Aparicio et al. [6]. The entire training process was designed and controlled by sport scientists in collaboration with experienced researches used to working with rats. The number of sessions performed each week, the number of sets per session and the time spent in each set as well as the load carried by the animals is shown in **Table 1**. From the first week of the experimental period until the completion of the study, the training weights (loads) were progressively increased and individually adjusted once per week to the percentage of one repetition maximum (1 RM), defined as the maximum load that the rat could carry in the bag. The 1 RM test was conducted as follows. The rat was first placed on a flat, horizontal and non-slippery surface with a specific loaded bag that was tied to its tail. The rat was then acoustically stimulated and immediately reacted by moving forward. This procedure was repeated several times, with the load being increased every time, until the load was so heavy that the rat could not move forward even when actively stimulated. The load achieved at this point was considered the 1 RM and was measured weekly in all animals to adapt the %1 RM load during the training period.

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

Chemical analyses

The total N content of the protein supplement and quadriceps was determined according to Kjeldahl's method. Crude protein amounts were calculated as N × 6.25. The urine calcium content was determined by atomic absorption spectrophotometry using a PerkinElmer Analyst 300 spectrophotometer (PerkinElmer, Wellesley, MA, USA), and the results were validated using standard reference materials CRM-189, CRM-383, and CRM-709.

The urinary pH was analyzed using a bench pH-meter (Crison, Barcelona, Spain), and urinary citrate level was analyzed using a commercial kit (Spinreact, S. A. Gerona, Spain). The plasma urea, total proteins, albumin, LDH, CK, calcium and magnesium concentrations were measured using an autoanalyzer (Hitachi-Roche p800, F. Hoffmann-La Roche Ltd. Switzerland).

Plasma corticosterone concentrations were measured by radioimmunoassay using a commercially available Corticosterone Rat/Mouse (DRG International Inc, USA) I-125 Kit without modification. All samples were assayed in duplicate and in the same assay. The intra assay coefficient of variation was 4.4%, and the sensitivity was 7.7 ng/ml. Plasma testosterone concentrations were measured by radioimmunoassay using a commercially available TESTO-CTK I-125 Kit (Dia Sorin, Italy) without modification. All samples were assayed in duplicate and in the same assay. The intra assay coefficient of variation was 5.1% and the sensitivity was 0.02 ng/ml. Corticosterone/testosterone as well as testosterone/corticosterone indexes as expressed corticosterone and testosterone in nmol/L were also calculated.

Histological analysis

The left-kidney samples were fixed in 4% buffered formalin and embedded in paraffin. Subsequently, 4-micrometer-thick sections were obtained and stained with 1% Picro-sirius red F3BA (Gurr, BDH Chemicals Ltd, Poole, United Kingdom) [43]. This technique facilitates the visualization of connective fibers as deep red stains on a pale yellow background [43]. The sections were assessed by optical microscopy. 40 images per sample were captured: 20 of the glomerulus to determine the morphometry and the intraglomerular connective tissue and 20 of the tubulointerstitial area to measure the interstitial connective tissue. All images were acquired using the 20× lens and analyzed with the Fibrosis HR® software [31]. This image analysis application allowed us to automatically quantify morphometric parameters by using various image-processing algorithms [31].

We estimated the following 8 morphological variables that we describe for the better understanding of the present results: a) Percentage of interstitial connective tissue in relation to the image area, excluding the glomerular area (the connective tissue that is in the gap over the Bowman's capsule). b) The area of interstitial connective tissue (including Bowman's capsule).

The Fibrosis HR® software divides glomerular tufts into 2 categories: "glomerular tuft I" and "glomerular tuft II". The variable "glomerular tuft I" corresponds to the renal corpuscle excluding the Bowman's capsule. The variable "glomerular tuft II" corresponds to the renal corpuscle excluding the Bowman's capsule and considering the area of the capillary lumens and urinary spaces in the glomerulus. c) Glomerular tuft I area. d) Glomerular tuft II area. e) Glomerular tuft I percentage (percentage of glomerular tuft I related to the glomerular area). f) Glomerular tuft II percentage (percentage of glomerular tuft II related to the glomerular area). g) Mesangial area. h) Glomerular area.

Statistical analysis

Results are presented as mean and standard deviation, unless otherwise indicated. Analysis of variance (ANOVA) was used to compare the HIE and the sedentary group with final body weight, food intake, muscle, urinary, plasma and renal morphology parameters as dependent variables. All comparisons were conducted with the Statistical Package for Social Sciences (SPSS, version 19.0 for Windows; SPSS Inc., Chicago, IL), and the level of statistical significance was set at $p < 0.05$. Additionally, standardized effect size statistics were estimated in all the comparisons through the *Cohen's d* test.

Results

The effects of HIE on the final body weight, food intake, muscle, plasma and urinary parameters are shown in **Table 2**.

	High-intensity exercise	Sedentary	p	Effect size†
final body weight (g)	313.0 (31.4)	350.4 (27.9)	0.009	1.25
carcass weight (g)	163.2 (17.6)	170.9 (18.2)	0.337	−0.43
food intake (g/day)	16.5 (2.70)	15.7 (1.76)	0.060	0.35
quadriceps N content (g/100 g DM)	15.1 (1.7)	12.9 (1.3)	<0.001	1.45
Plasma				
urea (mg/dl)	30.8 (4.60)	25.0 (5.12)	0.015	1.19
total proteins (g/dl)	5.49 (0.19)	5.61 (0.87)	0.173	−0.03
creatinine (mg/dl)	0.48 (0.06)	0.67 (0.13)	0.001	−0.30
albumin (mg/dl)	3.18 (0.47)	2.73 (0.83)	0.145	0.68
lactate dehydrogenase (u/L)	1147 (1625)	677 (217)	0.430	0.41
creatine kinase (u/L)	1957 (660)	1370 (563)	0.027	0.95
calcium (mg/dl)	27.6 (4.6)	32.4 (8.8)	0.154	−0.68
magnesium (mg/dl)	2.17 (0.50)	3.41 (1.81)	0.069	−0.93
corticosterone (nmol/L)	28.1 (4.56)	23.3 (6.15)	0.004	0.88
testosterone (nmol/L)	2.95 (2.53)	4.72 (3.38)	0.045	−0.59
corticosterone/testosterone	8.12 (6.12)	4.39 (3.86)	0.020	0.72
testosterone/corticosterone	0.20 (0.14)	0.43 (0.39)	0.002	−0.78
urine				
urinary calcium (g/L)	2.29 (0.86)	2.27 (0.86)	0.914	0.02
urinary calcium (mg/day)	0.96 (0.60)	0.51 (0.27)	0.064	0.96
urinary citrate (g/L)	2.81 (1.21)	2.88 (1.83)	0.917	−0.04
urinary pH	6.84 (0.45)	7.25 (0.54)	0.084	−0.82
urinary volume (ml/h)	0.36 (0.13)	0.23 (0.10)	0.025	1.12

Values expressed as mean (standard deviation). N: Nitrogen. DM: dry matter. † Effects size statistics are expressed as *Cohen's* d. This statistical test was included to show the relative magnitude of the difference in scores between a treatment (exercise) and a non-treatment (control) group

Table 2 Effects of high-intensity strength exercise on plasma and urinary parameters.

Final body weight, food intake and muscle N content

Final body weight was 11% lower in the HIE compared to the sedentary group, whereas food intake was 7% higher (both, $p<0.01$). Quadriceps N content was higher in the HIE group than in the sedentary group ($p<0.05$).

Plasma and urinary parameters

Plasma urea and CK concentrations were higher in the HIE group than in the sedentary group (both, $p<0.05$). Plasma creatinine concentrations were lower in the HIE group than in the sedentary group ($p<0.01$). No differences between groups were observed on plasma albumin, total proteins, calcium, magnesium or LDH concentrations.

Plasma corticosterone was 20% higher ($p<0.01$) at the same time that plasma testosterone was 60% lower in the HIE compared to the sedentary group ($p<0.01$). Plasma corticosterone/testosterone ratio was 46% higher in the HIE compared to the sedentary group, whereas testosterone/corticosterone ratio was 115% lower (both, $p<0.01$).

Except for the higher urinary volume found in the HIE compared to the sedentary group ($p<0.05$), no differences between groups were observed in the rest of the urinary parameters analysed (urinary pH, calcium and citrate).

The effects of HIE on kidney weight and morphology are shown in **Table 3**.

Kidney weight and morphology

No differences between groups were observed on kidney wet mass weight, as expressed in absolute value as well as in relation to the animal final body weight (both, $p>0.05$).

Kidney interstitial connective tissue was 30% higher in the HIE compared to the sedentary group ($p<0.05$). Percentages of glomerular tuft areas (referred to glomerular areas) as well as glomerular tufts and mesangial areas were higher in the HIE compared to the sedentary group (all, $p<0.05$). No differences between groups were observed in the glomerular area.

Discussion

The findings of the present study show that rats involved in a HIE protocol displayed a worse renal morphological profile when compared to the sedentary group, which might present a higher risk for incidence of kidney disease in the long-term. The stress induced by the type of exercise performed under our experimental design may be related to this inferior morphological renal status.

In the general healthy population, exercise appears to improve global renal status [32, 36–38]. Exercise could improve microalbuminuria [32, 38, 39], and we have observed higher albumin concentrations in our trained group, which could mean a reduction of the microalbuminuria. Exercise could also decrease renal inflammation [38]. We have observed a lower, but not significant, kidney weight in our trained animals, which could mean a lower renal inflammation or a lower renal hypertrophy. It should be noted that in a similar previous study performed by our group with the same exercise protocol, our trained animals exhibited a significantly lower kidney weight [5], which could be explained by the groups size or the level of protein employed.

Hypertension is also an important risk factor for CKD, and regular exercise can efficiency help to decrease blood pressure [24, 44]. In the study by Agarwal et al. [2], spontaneously hypertensive rats performed 16 weeks of moderate-intensity exercise on a treadmill (5 days per week; 60 min per day at 20 m/min, which corresponds approximately to 60% of maximal aerobic velocity), and this exercise protocol preserved renal hemodynamic and structure. Furthermore, exercise-induced effects, at least in part, were found to be pressure-independent [2].

Patients with chronic renal failure usually present the syndrome of "protein-energy malnutrition", which is a relevant factor for morbidity and mortality in this population and requires early detection and vigorous treatment [4]. These patients could benefit from resistance training interventions [23]. Indeed, Ding et al. [14] explored the effects of long-term weightlessness on the renal tissue and investigated the simulated microgravity on the renal morphological damage and related molecular mechanisms in rats. Resistance training (4 sets, 12 repetitions for each set at 65–75% of 1RM, 5 times per week for 8 weeks) reduced kidney cell apoptosis and expression of HSP70 protein and attenuated the kidney impairment imposed by weightlessness [14]. Quadriceps N (protein) content was higher in trained animals, which might confirm the effectiveness of the strength training protocol performed in the present study on increasing muscle mass.

The maintenance of urinary acid/base homeostasis is also important in order to preserve renal health [4]. A decrease in urinary pH, hypocitraturia and hypercalciuria are risk factors for kidney stone formation [3, 34]. In our study, no noticeable differences in these urinary parameters were observed, and consequently both groups presented similar risk of nephrolithiasis. However, the ~37% higher urinary volume together with the

Table 3 Effects of high-intensity strength exercise on kidney morphology.

	High-intensity exercise	Sedentary	p	Effect size†
kidney (g) (mean right and left)	0.88 (0.12)	0.92 (0.10)	0.437	0.48
kidney (g/100 g body weight)	0.28 (0.02)	0.26 (0.03)	0.202	0.28
kidney interstitial connective tissue (%)	3.97 (0.95)	2.71 (1.24)	0.019	1.14
kidney interstitial connective tissue (μm²)	5277.6 (1304)	3656.9 (1634)	0.023	1.10
glomerular tuft I (%)	22.13 (5.23)	16.74 (6.50)	0.065	0.91
glomerular tuft I area (μm²)	9180.6 (2386)	6616.3 (2652)	0.036	1.02
glomerular tuft II (%)	52.46 (11.9)	37.08 (16.38)	0.028	1.07
glomerular tuft II area (μm²)	21448 (5200)	14573 (5870)	0.013	1.23
mesangium area (μm²)	5673.1 (1415)	4172.2 (1510)	0.034	1.03
glomerular area (μm²)	41328 (4108)	40405 (3350)	0.406	0.24

Values expressed as mean (standard deviation). The variable "glomerular tuft I" corresponds to the renal corpuscle excluding the Bowman's capsule. The variable "glomerular tuft II" corresponds to the renal corpuscle excluding the Bowman's capsule and factoring in the area of the capillary lumens and urinary spaces in the glomerulus. † Effects size statistics are expressed as *Cohen's* d. This statistical test was included to show the relative magnitude of the difference in scores between a treatment (exercise) and a non-treatment (control) group

~20% higher levels of plasma urea found in the HIE group could mean a higher renal filtration (i.e., hyperfiltration) in the trained group [33]. Moreover, most of the morphological renal variables studied exhibited a worse profile, with higher kidney interstitial connective tissue, glomerular tufts and mesangium areas in the HIE group. Different hypotheses could explain these findings:

1) During heavy physical exercise (such as that performed in our strength training protocol), 2 phenomena occur: the decrease of the glomerular filtration rate and the release into the blood of some molecules from muscles such as CK, LDH and metmyoglobin [11,29,41,45]. Renal filtration of metmyoglobin released from damaged muscle and filtered at the glomerulus is known to cause acute renal injury in exercise rhabdomyolysis [29,30,35]. A 10-fold increase of CK is common in athletes after exercise [8,11]. In humans, serum CK 5 times higher than normal usually confirms rhabdomyolysis [29]. We have observed higher levels of CK in our HIE group, but in a lower magnitude. Therefore, the higher levels of CK may indirectly suggest that metmyoglobin has been liberated. Also noteworthy, yet without statistical significance, is the 3 times higher level of LDH observed in the HIE group. In fact, in the study by Colombini et al. [11] CK activity from 9 professional cyclists during the Giro d'Italia 3-week stage race increased during the second part of the race, and LDH activity progressively increased during the entire course of the race. There was a negative correlation between CK activity and the delta prerace-day 12 of glomerular filtration rate. The authors concluded that the effect of prolonged strenuous muscular effort on biochemical laboratory parameters in professional road cyclists was confirmed. In agreement with our results, the authors also observed that creatinine is unaffected by response to physical stress-induced muscular damage [11].

2) Cortisol is a glucocorticoid released from the adrenal cortex in response to stress, which is believed to play an important role in the remodelling of tissue [28] in response to intense exercise such as ours [16,25,26]. Indeed, resistance HIE protocols such as a 10-station heavy-resistance exercise protocol with 3 sets of 10 RM and very short rest periods between sets, or a sprint intervals protocol [25,26] that stimulate the greatest lactate response are correlated with high plasma cortisol levels. Moreover, protocols that result in the greatest concentrations of circulating CK 24-h post-exercise, also result in the greatest rises in circulating cortisol [27]. Moreover, high plasma corticosterone levels have been reported in rats after a moderate aerobic treadmill exercise protocol (60 min/d, 5 d/wk at 42 cm/s and 0% grade) [17]. We have confirmed these findings and observed higher levels of plasma corticosterone in our HIE groups. Sustained delivery of supraphysiological levels of corticosterone play a role in modifying kidney structure and function [9].

Our trained group also presented ~60% less testosterone than the sedentary group. Gonadal dysfunction is a frequent finding in men with CKD and with end-stage renal disease. Testosterone deficiency is present in 26–66% of men with different degrees of renal failure [21]. Experimental and clinical evidence suggests that testosterone may have important clinical implications with regard to kidney disease progression [21].

3) Finally, disturbances promoted by intense resistance training, such as hypoxia, glucose depletion or oxidative stress, may lead to endoplasmic reticulum dysfunction, which can induce endoplasmic reticulum stress. Accumulating evidence indicates that endoplasmic reticulum stress contributes to glomerular and tubular damage [13,22].

The differences found between the HIE and the sedentary group in corticosterone-testosterone, as well as in testosterone-corticosterone ratios, may indicate a possible overtraining status in the HIE group [7]. This overload may also influence the 3 hypotheses stated above.

The present study has some limitations that must be discussed. First, the physiological responses observed in rodents must be confirmed in humans. In other words, the responses found after 3 months of training using our experimental exercise protocol in rodents cannot be directly extrapolated to the potential effects over decades in human subjects. Moreover, although we have tried to mimic the training methodology performed by humans, this protocol does not exactly reflect human strength training. Second, measuring additional markers of renal function such as the glomerular filtration rate would have been of interest in the interpretation of the present study results. On the other hand, this is the first study analyzing the effects of high-intensity strength training on renal morphology in healthy animals under normal experimental conditions.

Conclusions

Overall, under our experimental design, rats involved in a HIE protocol displayed a worse renal morphological profile when compared to the sedentary group, which might be associated with a higher risk for incidence of kidney disease in the long term. The stress induced by the type of exercise performed in the present study (strength training) could be related to this worse morphological renal status.

Some conclusions regarding exercise intensity and type could be extracted from this study. Renal benefits of exercise could depend on the duration and intensity thereof, and on individual physiological conditions [36]. It is therefore necessary to further examine the renal effects at different doses, intensities and types of exercise. This is also important for being able to prescribe the appropriate exercise program for better renal health for any given individual/patient. More studies are needed to develop evidence-based exercise training guidelines.

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Effects of high-protein diets and high-intensity exercise on kidney oxidative stress in rats

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

The effects of high-protein diet consumption and HIE on final body weight, carcass weight, kidney weight and food intake are shown in **Table 3.** High-protein and HIE groups exhibited lower food intake when compared to the normal-protein and untrained groups (both, $p<0.001$). Kidney wet mass expressed in absolute value, or referred to the final body weight or the carcass weight was significantly higher in the high-protein when compared to the normal-protein groups (all, $p<0.001$).

Significant protein amount*HIE interactions were observed for daily food intake and final body weight derived from a higher HIE-induced decrease in the normal-protein groups when compared to the high-protein animals ($p=0.001$ and $p=0.025$, respectively). In addition, a significant protein amount*HIE interaction was also found for kidney wet mass referred to the final body weight derived from a higher HIE-induced decrease in this parameter for the high-protein groups that was not observed in the normal-protein animals ($p=0.016$).

Oxidative stress markers

The effects of high-protein diet and HIE on kidney oxidative stress markers are shown in **Table 4.** The high-protein groups showed

significantly higher values of TBARs content when compared to the normal-protein groups ($p<0.001$). In contrast, high-protein groups displayed significantly lower levels of t-SOD, Mn-SOD, CuZn-SOD and GPx activity when compared to the normal-protein groups ($p<0.001$, $p<0.001$, $p=0.020$ and $p<0.001$, respectively).

Significant protein amount*HIE interactions were found for kidney PCC, t-SOD, and Mn-SOD activity derived from a greater HIE-induced increase in the high-protein groups that was not observed in the normal-protein groups ($p<0.001$, $p=0.05$ and $p<0.001$, respectively). A significant protein amount*HIE interaction was also observed for GPx derived from a higher HIE-induced increase in the normal-protein groups that was not observed in the high-protein animals ($p=0.002$).

Plasma and renal morphology parameters

The effects of high-protein diet and HIE on plasma and kidney morphology parameters are shown in **Table 5.** Plasma urea was significantly higher ($p=0.024$), and total protein and creatinine levels were lower ($p=0.002$ and $p<0.001$, respectively) in the high-protein animals when compared to the normal-protein groups, respectively. A significant protein amount*HIE interaction was found for plasma

creatinine content derived from a higher HIE-induced decrease in this parameter for the normal-protein groups when compared to the high-protein groups (p=0.003).

The high-protein groups exhibited significantly higher glomerular tuft I area, mesangium area and glomerular area when compared to the normal-protein groups (p=0.018, p=0.018 and p=0.002, respectively). A significant protein amount*HIE interactions was found for glomerular tuft II area derived from a higher HIE-induced increase in the normal-protein groups that was not observed in the high-protein groups (p=0.030). A significant protein amount*HIE interactions was also observed for mesangium percentage that resulted from a higher HIE-induced increase in the high-protein groups that was not observed in the normal-protein groups (p=0.006).

Table 3. Effects of the dietary protein amount and high-intensity exercise on final body weight, carcass weight, food intake and kidney weight.

	Normal protein		High protein			*p* values		
	Untrained	HIE	Untrained	HIE	SEM	Protein amount	HIE	Protein amount*HIE
Food intake (g/day)	20.32c	15.62a,b	16.95b	14.81a	0.177	<0.001	<0.001	0.001
Final body weight (g)	351.22a	313.04a	317.59a	327.17a	5.085	0.344	0.168	0.025
Carcass weight (g)	172.12a	163.21a	178.87a	169.23a	2.897	0.278	0.118	0.950
Kidney (g)	0.92a,b	0.87a	1.17c	1.06b,c	0.021	<0.001	0.068	0.513
Kidney (g/100g body weight)	0.26a	0.28a,b	0.37c	0.32b	0.006	<0.001	0.168	0.016
Kidney (g/100g carcass weight)	0.54a	0.53a	0.65b	0.63b	0.008	<0.001	0.333	0.437

SEM, standard error of the mean; HIE, high-intensity exercise.

Values expressed as mean of 10 rats. The same letter in the same row indicates no significant difference between groups ($p>0.05$).

Table 4. Effects of the dietary protein amount and high-intensity exercise on kidney oxidative stress markers.

	Normal protein		High protein			*p* values		
	Untrained	HIE	Untrained	HIE	SEM	Protein amount	HIE	Protein amount*HIE
TBARs (nmol MDA/mg protein)	12.8a	18.6a	55.3b	55.6b	2.382	<0.001	0.527	0.564
PCC (nmol/mg protein)	5.1c	2.1a	3.4b	4.8c	0.127	0.030	0.004	<0.001
t-SOD (U/mg protein)	247.3b	215.5a	197.3a	198.6a	4.077	<0.001	0.068	0.050
Mn-SOD (U/mg protein)	136.9b	109.2a	100.8a	107.7a	2.255	<0.001	0.027	<0.001
CuZn SOD (U/mg protein)	110.4a	106.3a	96.6a	90.8a	2.999	0.020	0.416	0.896
CAT (µmolH2O2/min/mg protein)	125.3a	123.6a	105.8a	125.6a	4.385	0.323	0.307	0.229
GPx (nmolNADPH/min/mg protein)	28.6b	44.9c	16.0a	19.4a	0.936	<0.001	<0.001	0.002

SEM, standard error of the mean; HIE, high-intensity exercise; TBARs, thiobarbituric acid-reactive substances; PCC, protein carbonyl content; t-SOD, total superoxide dismutase; Mn-SOD, manganese superoxide dismutase; CuZn-SOD, cooper and zinc superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

Values expressed as mean of 10 rats. The same letter in the same row indicates no significant difference between groups ($p>0.05$).

Table 5. Effects of the dietary protein amount and high-intensity exercise on plasma and kidney morphology.

	Normal protein		High protein			*p* values		
	Untrained	HIE	Untrained	HIE	SEM	Protein amount	HIE	Protein amount*HIE
Plasma								
Urea (mg/dl)	24.52a	30.82b	29.10a,b	33.10b	0.727	0.024	0.001	0.434
Total Protein (g/dl)	5.62b	5.49a,b	5.27a,b	5.13a	0.051	0.002	0.188	0.969
Creatinine (mg/dl)	0.66b	0.48a	0.43a	0.42a	0.013	<0.001	0.001	0.003
Albumin (mg/dl)	2.75a	3.18a	2.72a	2.83a	0.122	0.448	0.269	0.511
Morphology								
Interstitial connective tissue (%)	2.71a	3.97a	3.33a	3.65a	0.179	0.683	0.034	0.199
Interstitial connective tissue(µm²)	3657a	5278a	4245a	4764a	241.098	0.939	0.033	0.261
Glomerular tuft I (%)	16.74a	22.13a	21.76a	21.89a	0.963	0.222	0.160	0.181

Glomerular tuft I area (µm²)	6616a	9181a,b	10155b	9627a,b	403.582	0.018	0.215	0.063
Glomerular tuft II (%)	37.08a	52.46b	44.86a,b	47.75a,b	2.018	0.705	0.030	0.130
Glomerular tuft II area (µm²)	14573a	21448b	20891b	20701b	783.234	0.084	0.040	0.030
Mesangium (%)	64.74b	62.32a,b	60.74a	63.86a,b	0.475	0.204	0.714	0.006
Mesangium area (µm²)	4172a	5673a,b	6178b	6106b	245.713	0.018	0.155	0.118
Glomerular area (µm²)	40405a	41328a	46590b	44381a,b	682.935	0.002	0.641	0.259
Fibrosis-T (%)	2.09a	3.02b	1.99a	2.50a,b	0.118	0.195	0.004	0.394
Fibrosis-T area (µm²)	3660a	5279b	3472a	4377a,b	206.465	0.195	0.004	0.394

SEM, standard error of the mean; HIE, high-intensity exercise.

Values expressed as mean of 10 rats. The same letter in the same row indicates no significant difference between groups ($P>0.05$).

2. OXIDATIVE EFFECTS OF ANABOLIC ANDROGENIC STEROIDS AND HIGH-INTENSITY EXERCISE

2.1. BRAIN

High-intensity exercise modifies the effects of Stanozolol on brain oxidative stress in rats

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Supplementary files.

Title: High-intensity exercise modifies the effects of Stanozolol on brain oxidative stress in rats

ABSTRACT

We analysed the effects of high-intensity exercise (HIE) and Anabolic androgenic steroids (AAS) on brain redox status. Forty male Wistar rats were randomly distributed in 4 experimental groups (n=10) with or without HIE and with or without weekly Stanozolol administration. Thiobarbituric acid-reactive substances (TBARs) and protein carbonyl content (PCC) were assessed. Total superoxide dismutase (tSOD), manganese superoxide dismutase (Mn-SOD), cooper/zinc superoxide dismutase (CuZn-SOD) and catalase (CAT) activities were measured. Finally, protein expression level of glutathione peroxidase (GPx), NAD(P)H dehydrogenase, Quinone 1 (NQO1), NF-E2-Related Factor 2 (Nrf2), glial fibrillary acidic protein (GFAP), nuclear factor kappa β p65 (NF-κβ) and signal transducer and activator of transcription 3 were determined. Brain PCC concentrations were lower in the HIE groups compared to the untrained controls, whereas CAT activity was higher (both, $p<0.01$). Both HIE and AAS groups exhibited higher expression of GFAP and GPx, but lower NQO1 levels (all, $p<0.05$). There was

increased expression of NF-κβ in the AAS groups ($p<0.01$). In addition, there was increased expression of Nrf2 in the HIE groups ($p<0.001$). Several HIE*AAS interactions were found on TBARs content and GFAP expression, with HIE downregulating and upregulating AAS-mediated increase in TBARs and GFAP, respectively ($p<0.05$). Overall, HIE appeared to reduce the AAS-mediated negative effect on brain redox status.

Keywords: Anabolic agents; Superoxide dismutase; catalase; glial fibrillary acidic protein; nuclear factor kappa B; resistance training.

INTRODUCTION

Oxidative stress is a condition in which the delicate balance existing between free radicals production and their subsequent amelioration via the antioxidant defence system becomes skewed in favour of free radical expression [39]. Therefore, oxidative damage repair systems are important in order to minimize the dangerous effects of pro-oxidant reactive oxygen species (ROS) [20]. The brain readily suffers oxidative damage due to its higher metabolic rate, lipid content and lower levels of catalase (CAT) [37]. Consequently, brain oxidative stress has been implicated in several neurodegenerative disorders such as Parkinson's

disease, Alzheimer disease, multiple sclerosis, and amyotrophic lateral sclerosis [9,28].

It is well established that regular exercise plays an important preventive and therapeutic role on oxidative stress-associated brain diseases [42]. However, the benefits of high-intensity exercise (HIE) on brain function are under debate due to the potential overproduction of ROS that this type of exercise can induce [12]. This overproduction of ROS can alter the concentrations of different early biomarkers of oxidative stress such as plasma total antioxidant capacity (TAC) or erythrocyte reduced glutathione (GSH) and CAT activity, suggesting modifications in blood redox status [63].

Anabolic androgenic steroids (AAS) have both protein synthesizing (anabolic) and masculinizing (androgenic) effects on the body [56]. Although AAS may be prescribed for patients with pathological conditions (e.g. hypogonadism or sarcopenia) [11], they are widely used among professional athletes, competitive and recreational body builders or even non-athletic adolescents because AAS are some of the most powerful performance enhancing substances [29]. Severe effects such as adverse plasma and hepatic lipid profile can emerge with prolonged use or high doses of AAS [6]. Regarding brain function, AAS

may adversely affect neural activity in the hypothalamus and forebrain [45], by promoting neurodegenerative and apoptotic effects [61].

Some studies have already demonstrated the combined effects of HIE and AAS in different tissues. For example, concerning muscle mass, the combination of these interventions induced comparable hypertrophy in all major fibre types of soleus, tibialis anterior and gastrocnemius muscle [23,26]. In contrast, the beneficial effects provided by HIE on hippocampal cell proliferation and apoptotic signalling as well as the improved heart antioxidant capacity were impaired by Nandrolone [15,44]. On the other hand, Stanozolol treatment protected rat skeletal muscle mitochondria against oxidative damage of proteins and changes in membrane fatty acid composition induced by acute exercise [53]. Thus, the involvement of specific molecular mediators on the biological effects of HIE and/or AAS depend on numerous factors such as the training protocol designed, animal model investigated, age, sex, AAS dose, metabolism or treatment regimen [22,50].

Several oxidative stress brain markers and antioxidant enzymes have been used to evaluate brain damage. Astrocytes play a key role in brain physiology and diverse neurodegenerative diseases [55]. Glial fibrillary acidic protein (GFAP) is a specific astrocyte marker, which

increases as a sign of astrogliosis, associated with conditions of brain injury [24]. Glial activation, in response to injury stimuli, commonly involves changes in GFAP and antioxidant defence [51]. The NF-E2-Related Factor 2 (Nrf2) plays a central role in the regulation of phase 2 enzymes, such as glutathione peroxidase (GPx), glutathione s-transferase (GST) and NAD(P)H dehydrogenase, Quinone 1 (NQO1) [49,67]. Recent studies have observed that chronic exercise activates the Nrf2 in human skeletal muscle and rat kidney whereas acute exercise promotes myocardial Nrf2 function [27,54].

Signal transducer and activator of transcription 3 (STAT3) is activated by cytokines, growth factors, and receptor- or nonreceptor-tyrosine kinases [19,36]. A previous study has demonstrated that manganese superoxide dismutase (Mn-SOD), a primary cellular defence enzyme involved in protecting cells from oxidative stress [14], is a direct target of STAT3 in ischemia reperfusion-induced neuronal cell death. Hence, the loss of STAT3 activity reduces Mn-SOD expression after cerebral ischemia [33].

Given that hypertrophy resistance training is the main exercise modality practiced by AAS abusers [31] and the effect of androgens in combination with HIE on brain redox status has been scarcely

investigated, the purpose of the present study was to investigate the effects of an hypertrophy resistance training protocol (i.e. HIE) and AAS administration on brain redox status.

MATERIALS AND METHODS

Animals and experimental design

A total of 40 albino male Wistar rats were randomly distributed into 4 experimental groups derived of 2 interventions: HIE (untrained vs. HIE) (n=20) and AAS-administration (non-AAS vs. AAS) (n=20). Each specific intervention (i.e. untrained and non-AAS, untrained and AAS, HIE and non-AAS, HIE and AAS) was developed in groups of 10 rats and the experimental period lasted 12 weeks.

The animals (aged 8 weeks) with an initial body weight of 161±13 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 the animals. The rats were located in a well-ventilated thermostatically controlled room (21±2ºC). 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 N_2 and kept at -80ºC until further analyses.

All experiments were undertaken in accordance with the Ethical Standards in Sport and Exercise Science Research [30] as well as the Directional Guides Related to Animal Housing and Care (European Community Council, 1986) [25]. All procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada (2011-343).

High-intensity exercise

The animals were trained following a resistance training protocol on 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 [5]. 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 has been previously developed and deeply described by Aparicio et al [5]. The entire training process was designed and controlled by sport scientists in collaboration with experienced researchers trained to work with rats. The number of sessions performed each week, the number of sets per session, the time spent in each set, and the load carried by the animals is shown in **Table 1.**

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

Anabolic androgenic steroids administration

Following similar studies performed in rats, the animals received 10 mg/kg body weight of Stanozolol once a week by intramuscular injection in the gluteus (alternating the lateral side each week) for 12 weeks. This dose is comparable to the dose that has been reported as being frequently used by athletes (600 mg/week or approximately 8 mg/Kg/week) [16,17]. We used a commercially available Stanozolol solution of 50 mg/ml (Winstrol Depot, Desma Pharma group). The non-AAS administered group was injected with saline solution as placebo.

Chemical analyses

Brain homogenate preparation for oxidative damage markers and antioxidant activity

Brain samples (1 g) 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 for 30 sec followed by treatment with Sonoplus HD 2070 ultrasonic homogenizer (Bandelin) at 50% power for 10 sec. Homogenates were centrifuged at 19,921 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. [46]. The results were expressed as nmol of malondialdehyde per mg of protein (nmolMDA/mg).

Protein carbonyl content (PCC)

Total carbonyl content in brain was used as a biomarker of protein oxidation. The content was determined by spectrophotometry using a protein carbonyl colorimetric assay kit (Cayman, USA) according to Levine et al. [35]. Results were expressed as nmol of reactive carbonyl compounds/mg protein of tissue.

Antioxidant enzyme activity

Total superoxide dismutase (tSOD) activity was measured as described by Ukeda et al. [62] and adapted to a micro-plate reader. Manganese superoxide dismutase (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). Cooper/zinc superoxide dismutase (CuZn-SOD) activity was determined by subtracting the Mn-SOD activity from the tSOD 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 as described by Aebi [2] by monitoring the disappearance of H_2O_2 in the presence of brain homogenate at 240 nm and was expressed as μmol of H_2O_2

consumption per minute per milligram of protein. Protein concentration was determined by the method of Lowry [40].

Western blotting

Brain aliquots (1 g) were homogenized (1:10 w/v) in 20 mM Tris·HCl (pH 8.0) containing 0.1% octylphenoxypolyethoxyethanol (lgepal), 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 Aproptinin. Samples were homogenized with a Micra D-1 homogenizer (ART moderne labortechnik) at 18,000 rpm for 30 seconds followed by treatment with Sonoplus HD 2070 ultrasonic homogenizer (Bandelin) at 50% power for 10 seconds. Homogenates were centrifuged at 19,621 g and 4°C for 45 min (BECKMAN, Allegra 64R), supernatants were collected and stored at -80ºC until use. Protein concentration was quantified using the Bradford assay method (Bio-Rad, Hercules, CA). Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis under reducing conditions and then transferred to nitrocellulose membranes. Western blots were performed according to standard methods. Membranes were blocked in 5%

skimmed milk, and then incubated (overnight at 4ºC or room temperature for 3-4 h) with the antibody of interest, followed by incubation with a horseradish peroxidase-conjugated secondary antibody. The visualization of immunoreactive bands was performed using the ECL Plus Western blotting detection system (GE Healthcare, Pascataway, NJ). The primary antibodies were directed against NF-κβ p65 (Epitomics, Burlingame, CA; 1:1000); GFAP (Cell signalling Technology, Danvers, MA; 1:1000); GPx (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200); NQO1 (Abcam, Inc., Cambridge, MA; 1:2500); STAT3 (Cell Signalling Technology, Danvers, MA; 1:1000) and Nrf2 (Abcam, Inc., Cambridge, MA; 1:1500). The quantification was performed by volume densitometry using Image J software (NIH, Bethesda, MD) and normalization to ponceau reagent with the exception of Nrf2 that was normalized to β-actin.

Statistical analyses

Results are presented as mean and standard error of the mean (SEM), unless otherwise indicated. The effects of the HIE (untrained vs. HIE) and the AAS administration (non-AAS vs. AAS) on food intake, final body weight, brain weight, and oxidative stress markers, including their two-way interactions, were analysed by two-way factorial analysis of

variance (ANOVA), with HIE and AAS as fixed factors. Two-way interactions terms were introduced into the models to test interactions between both interventions (i.e. HIE*AAS). A significant *p* value indicates that there are differences in at least two of the groups. In addition, multiple comparisons between groups were made considering Bonferroni's adjustment in order to identify between which groups the differences were significant (e.g. untrained without AAS vs. exercise with AAS).

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, brain weight and food intake

The effects of HIE and AAS-administration on final body weight, brain weight and food intake are shown in **Table 2.** Food intake and final body weight were significantly decreased in the HIE when compared to the untrained groups ($p<0.001$ and $p=0.007$, respectively). A significant HIE*AAS interaction was found for daily food intake derived from a higher HIE-induced decrease in the non-AAS when compared to the

AAS-administered animals (p=0.007).

Oxidative stress markers

The effects of HIE and AAS-administration on brain oxidative stress markers are shown in **Table 3.** The HIE groups exhibited significantly lower brain PCC and increased brain CAT when compared to the untrained animals (p=0.002 and p<0.001, respectively). A significant HIE*AAS interaction was found for brain PCC derived from a higher HIE-induced decrease observed in the non-AAS when compared to the AAS-administered animals (p=0.009). Likewise, there was a significant HIE*AAS interaction on brain TBARs content, tSOD, Mn-SOD and CuZn-SOD activity caused by the HIE-induced decrease in the above mentioned parameters in the AAS-administered that was not observed in the non-AAS administered animals (p=0.019, p=0.002, p=0.003 and p=0.011, respectively).

Figure 1 shows the effects of the HIE and AAS on the expression of GFAP, GPx, NF-κβ, NQO1, STAT3 and Nrf2 proteins in rat brain. Both HIE and AAS groups exhibited higher expression of GFAP and GPx, but lower NQO1 levels when compared to the untrained and placebo animals (all, p<0.05). There was an increased expression of NF-κβ and NrF2 in the AAS and HIE groups when compared to the placebo

and untrained animals, respectively ($p<0.01$). In addition, there was a HIE*AAS interaction in GFAP expression, with HIE upregulating the AAS-mediated increment in protein expression ($p<0.05$).

DISCUSSION

The main findings of the present study were: 1) HIE decreased PCC concentrations and the expression of NQO1, increasing CAT activity and the expression of GFAP, GPx, and Nrf2 protein, 2) AAS administration increased the expression of GFAP, GPx, NF-κβ protein whereas it decreased NQO1 expression, and 3) Brain oxidative stress markers were differentially affected by the combination of HIE and AAS interventions.

Food consumption, body weight and body composition

In a similar way to what has been reported by other authors [10,64] and corroborated by our group [4], food intake as well as body weight [32,65] were markedly affected by HIE. Thus, the food intake and body weight diminution observed in the present study may be attributed to the HIE carried out and mediated through the increased production of cortisol induced by this type of protocol [7].

High-intensity exercise, anabolic androgenic steroids and brain oxidative stress

In view of the controversial findings regarding the effects of HIE on brain oxidative stress [12], our first concern was to elucidate whether the frequent type of exercise carried out by AAS users (i.e. HIE) might affect positively and/or negatively brain redox status. On the one hand, some authors suggest that intermittent anaerobic exercise and acute exhausted exercise increase brain antioxidant capacity and do not induce lipid peroxidation [1,48]. Concerning oxidative damage, despite the high levels of lipid peroxidation in brain tissue due to the high content of polyunsaturated fatty acids and the free radical metabolism [37], some studies have demonstrated that HIE does not alter lipid peroxidation in whole brain [39], hippocampus [1,66], cerebellum [66], prefrontal cortex [1] and striatum [1]. On the other hand, under HIE training, ROS production may be strongly and persistently increased, and the antioxidant response may not be effective to reset the system to the original levels of brain redox homeostasis [3]. In fact, HIE has been shown to induce oxidative stress and increase lipid peroxidation in mouse brain [18,52]. Taking into account these contradictory results in the literature our findings agree with some of the aforementioned studies in that no significant evidence was observed in brain TBARs levels after a HIE protocol. Thus, it appears that the intensity and length of training

protocol assayed in the present study was not sufficient to generate an imbalance between pro- and anti-oxidant forces in the brain.

Reactive oxygen species may also lead to the production of oxidative damage to proteins that is accompanied by an increase in the number of carbonyl residues. In this regard, Tsakiris et al. [59] reported that either short or prolonged forced swimming exercise increase ROS production, which could cause protein damage. The same trend was observed in the study by Aydin et al. [8], where brain PCC was significantly increased after swimming until exhaustion. In contrast, lower levels of PCC in the hippocampus of 12-months old rats were found after daily moderate intensity exercise for 15 weeks (18 m/min, on a 0% incline, for 30 min) (Marosi et al. [41]. In accordance to previous findings, our results suggest that HIE might be responsible for lower levels of brain PCC when compared to untrained groups, suggesting that the oxidants produced by HIE were not able of affecting brain proteins.

Some authors have previously described that HIE did not induce significant oxidative stress to alter brain antioxidant enzyme activities [1,66]. Our results agree with these findings with regard to Mn-SOD and CuZn-SOD activities, but not to CAT activity that increased significantly in response to the HIE protocol. Catalase is an enzyme that is highly

modulated by exercise and especially by endurance training, in which the formation of ROS by leakage of superoxide radicals in the electron transporter chain is much higher due to the greater utilization of the oxidative pathway [34]. Accordingly, in the study performed by Li and Wang, [37] rats exhibited significant increases in brain CAT activity after the combination of running on a treadmill for 5 weeks (6 days per week with a gradual speed of 20-30 m/min for 30-60 min/day) with hypoxic conditions (3500 m altitude) considering that as an HIE protocol. Furthermore, others [57] have reported that plasma CAT activity was higher after a HIE protocol consisted of 4 series of 10–12 repetitions and 90 second intervals, 4 times per week, 65 % to 75 % of the one maximum repetition for 8 weeks. These assertions concur with our outcomes that point out to an increased brain CAT activity, which might be produced as auto-defence by the type of HIE carried out under our experimental conditions that led to a higher production of ROS.

The body of the literature indicates that Nrf2 is the primary transcriptional regulator of a majority of the antioxidants including hemoxygenase-1 (HO1), γ -glutamyl cysteine ligase-catalytic (γGCLC), NQO1, GPx and CAT [43]. It has been reported that moderate exercise enhances the promotion of an endogenous Nrf2/γGCLC antioxidant

system for the prevention of neurodegenerative diseases [60]. Moreover, a recent study has also demonstrated that acute exercise induces ROS production and activates Nrf2 and antioxidant responsive element functioning in myocardial tissue [43]. Under our experimental conditions, the production of ROS induced by HIE contributed to activate the transcriptional Nrf2 factor, and consequently the upregulation of CAT activity and GPx protein expression. Likewise, some authors observed that a moderate treadmill training (10–50 min/day of running at 40–60% of the maximal velocity 5 days per week for 5 weeks) increased GFAP content in the CA1 region of Wistar rats [51] and elevated the levels of GFAP in the hippocampus after a endurance training in diabetic rats [21]. Although, caution must be taken, the tendency in our study suggests that Nrf2 could up-regulate the GFAP expression after a HIE protocol

Our second concern was to characterize the potential adverse effects of prolonged use of AAS on brain redox status. The study performed by Tugyan et al. [61] analysed the neuroprotective effect of erythropoietin on brain damage induced by Nandrolone administration for 8 weeks. After this experimental period, the AAS group displayed some irreversible effects such as increased malondialdehyde (MDA) levels and apoptosis, as well as a significant decrease in GPx activity in

prefrontal cortex and hippocampus compared to the placebo group. In contrast, Celek et al. [13] revealed that Nandrolone and Testosterone were able to decrease the elevated MDA produced by ethanol ingestion in rat cerebellum. These results do not concur with our findings, in which the AAS administration did not appear to induce significant changes on the oxidative damage markers measured. However, emerging evidence suggests that Nrf2 may also play an important role in the regulation of brain inflammation, and some studies have suggested that Nrf2 has an antagonistic effect with the NF-κβ pathway, which is considered as a hallmark of inflammation [38]. Thus, the lack of changes in the expression of Nrf2 and the increased expression of inflammatory protein NF-κβ as well as the antioxidant enzymes GPx and GFAP after the Stanozolol treatment could be pointing out to the development of an inflammatory process derived from AAS administration that activate the defence mechanisms in brain.

Previous studies have analysed the effects of AAS or its combination with diverse exercise protocols on the oxidative status of other organs with controversial findings. Pey et al. [47] concluded that prolonged Stanozolol treatment, with or without moderate exercise training, induced oxidative stress that was reflected in higher TBARs

levels on rat liver, despite enhancements in the antioxidant activity of SOD, CAT and GPx enzymes. Likewise, Chaves et al. [15,16] showed that in spite of the beneficial effect of HIE on heart through increased activity of antioxidant enzymes, the Nandrolone administration induced lower SOD, GPx and glutathione reductase (GR) activity when compared to control and trained animals. Similarly, Sun et al. [58] demonstrated that the combination of Nandrolone and an aerobic physical training increased MDA and PCC, and decreased SOD and NQO-1 protein levels on aortic large vessels. On the other hand, a study performed by Saborido et al. [53] observed that Stanozolol administration might protect rat gastrocnemius mitochondria against oxidative damage of proteins and changes in membrane fatty acid composition induced by acute exercise. The above mentioned studies concur partially with the findings of the present work in which the combination of both effects (i.e. HIE and AAS) presented significant interactions in the brain oxidative stress markers except for CAT levels. In view of such findings we can conclude that the proliferation in antioxidant activity is linked as an auto-defence system against oxidative damage under both interventions. Therefore, the higher oxidative damage produced in the brain is associated with higher antioxidant activity produced as auto-defence mechanism.

Limitation and strengths

The present study has some limitations that need to be mentioned. The AAS are often used in combination with other drugs or substances, and it is difficult to separate their toxic effects. This study was conducted in animal models and not nearly as complex as carrying out long-term and well-controlled interventional studies in humans. However, it is important to highlight that this is the first study analysing the combined effects of HIE and AAS-administration on brain oxidative stress markers and antioxidant enzyme defence system in the same report, which allow a global picture about the effects of the combination of these two common behaviours.

CONCLUSIONS

Under our experimental conditions, the present results suggest that HIE appeared to reduce the AAS-mediated negative effect on brain redox status. However, despite of this beneficial consequence, HIE also induced potentially harmful effects on brain oxidative stress markers depending on whether the protocol of HIE was carried out autonomously or it was combined with AAS. Thus, this is of importance due to the fact that little is known in the literature regarding the effect of combining these two

interventions, which are generally carried out in combination by some individuals willing to gain muscle mass, on brain redox status. Therefore, further studies should be performed that involve the combination of HIE and AAS on oxidative stress in order to advise the general population against the deleterious consequences of unnecessary, uncontrolled AAS administration.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

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Table 1. Details of the high-intensity exercise program.

Week	Work time (min)	Sets	Time between sets (min)	Weight (% 1 RM)
1	2	10	1	55
2	2	10	1	60
3	2	10	1	65
4	2	10	1.5	70
5	2	10	1.5	70
6	2.5	10	1.5	75
7	2.5	12	1.5	75
8	2	12	2	80
9	2.5	12	2	80
10	1.5	12	2	85
11	2	12	2.5	85
12	1	12	2.5	85

RM, repetition maximum.

Table 2. Effects of the high-intensity exercise and anabolic androgenic steroids administration on final body weight, food intake and brain weight.

	Non AAS		AAS			P values		
	Untrained	HIE	Untrained	HIE	SEM	HIE	AAS	HIE*AAS
Food intake (g/day)	20.317b	15.622a	18.768b	16.500a	0.212	<0.001	0.434	0.007
Final body weight (g)	351.216b	313.042a	338.825a,b	321.673a,b	4.844	0.007	0.847	0.285
Brain (g)	1.925a	1.881a	1.903a	1.939a	0.015	0.899	0.554	0.195
Brain (g/100g body weight)	0.552a	0.605a	0.566a	0.606a	0.010	0.020	0.685	0.719

SEM, standard error of the mean; AAS, anabolic androgenic steroids; HIE, high-intensity exercise.

Values expressed as mean of 10 rats. The same letter in the same row indicates no significant difference between groups ($p>0.05$).

Table 3. Effects of the high-intensity exercise and anabolic androgenic steroids administration on brain oxidative stress markers.

	Non AAS		AAS			P values		
	Untrained	HIE	Untrained	HIE	SEM	HIE	AAS	HIE*AAS
TBARs (nmol MDA/mg protein)	19.684a	23.553a	22.238a	18.941a	0.727	0.845	0.484	0.019
PCC (nmol/mg protein)	3.496b	2.440a	2.953a,b	2.864a,b	0.087	0.002	0.735	0.009
tSOD (U/mg protein)	137.474a	154.578a,b	160.197b	138.480a,b	2.832	0.686	0.563	0.002
Mn-SOD (U/mg protein)	63.990a	71.828a,b	77.671b	65.123a,b	1.627	0.474	0.291	0.003
CuZn-SOD (U/mg protein)	73.484a	82.750a	82.526a	73.356a	1.726	0.989	0.960	0.011
CAT (μmolH2O2/min/mg protein)	2.852a	4.274b	3.407a,b	3.994b	0.118	<0.001	0.564	0.086

SEM, standard error of the mean; AAS, anabolic androgenic steroids; HIE, high-intensity exercise; TBARs, thiobarbituric acid-reactive substances; PCC, protein carbonyl content; tSOD, total superoxide dismutase, Mn-SOD, manganese superoxide dismutase; CuZn-SOD, cooper/zinc superoxide dismutase; CAT, catalase.
Values expressed as mean of 10 rats. The same letter in the same row indicates no significant difference between groups ($p>0.05$).

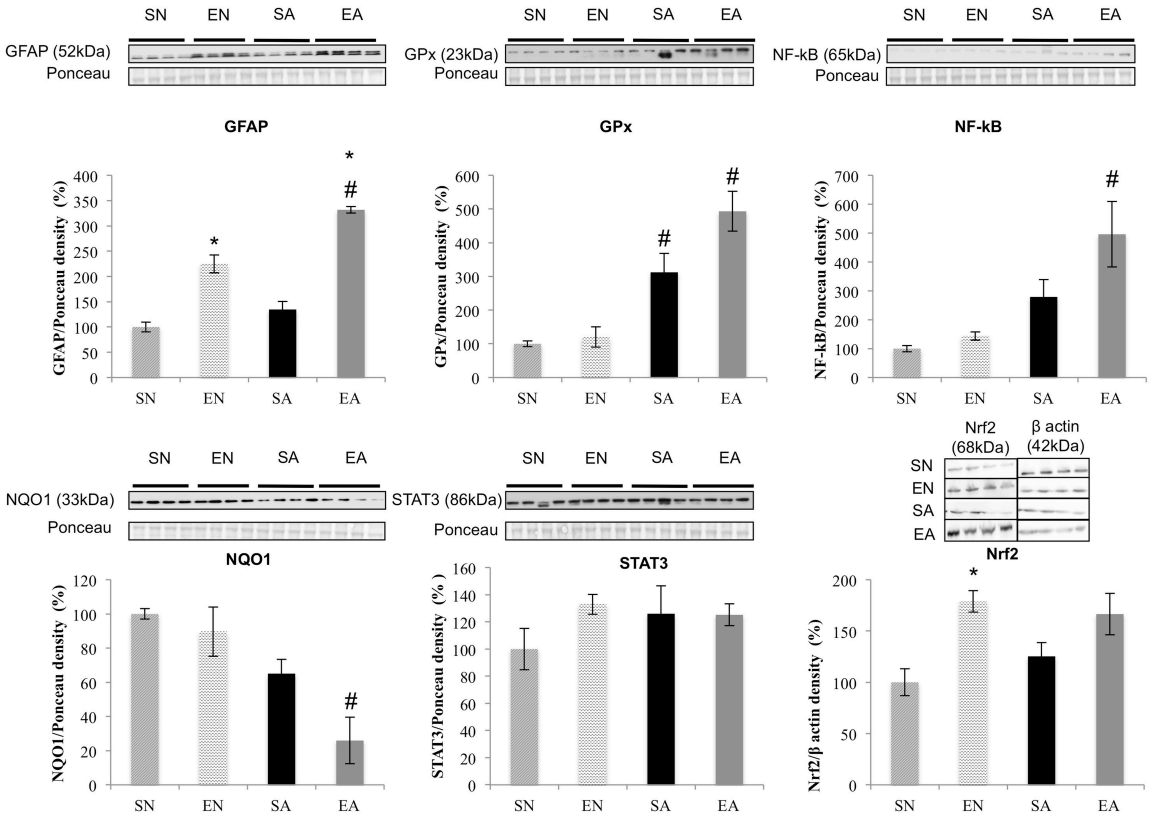
SN EN SA EA
GFAP (52kDa)
Ponceau
GPx (23kDa)
NF-kB (65kDa)
GFAP
GPx
NF-kB
GFAP/Ponceau density (%)
GPx/Ponceau density (%)
NF-kB/Ponceau density (%)
NQO1 (33kDa)
STAT3 (86kDa)
Nrf2 (68kDa)
β actin (42kDa)
NQO1
STAT3
Nrf2
NQO1/Ponceau density (%)
STAT3/Ponceau density (%)
Nrf2/β actin density (%)

FIGURE LEGEND

Figure 1. Effects of the anabolic androgenic steroids administration and high-intensity exercise on brain GFAP, GPx, NF-κβ, NQO1, STAT3 and Nrf2 protein levels, n=4.

Results are means ± SD. Significant differences between *untrained and exercised groups and #Stanozolol-treated and non-treated groups: $p<0.05$.

The representative western blots show the GFAP, GPx, NF-κβ, NQO1, STAT3 and Nrf2 bands (upper lines) and the ponceau bands used as a loading control (lower lines).

Abbreviations: SN, Untrained and Non anabolic androgenic steroids administration; EN, Exercise and Non anabolic androgenic steroids administration; SA, Untrained and anabolic androgenic steroids administration; EA, Exercise and anabolic androgenic steroids administration; GPx, glutathione peroxidase; NQO1, NAD(P)H dehydrogenase, Quinone 1; Nrf2, NF-E2-Related Factor 2; GFAP, glial fibrillary acidic protein; NF-κβ, nuclear factor kappa B; STAT3, signal transducer and activator of transcription 3.

2.2. KIDNEY

High-intensity exercise attenuates the oxidation of renal lipids and proteins caused by Stanozolol administration

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

The effects of AAS-administration and HIE on final body weight, carcass weight, kidney weight and food intake are shown in **Table 6.** The AAS groups exhibited higher carcass weight, kidney wet mass expressed in absolute value, kidney wet mass referred to the final body weight and kidney wet mass referred to the carcass weight when compared to the non-AAS groups (all, $p<0.05$).

Oxidative stress markers

The effects of the HIE and AAS-administration on kidney oxidative stress markers are shown in **Table 7.** The AAS groups showed significantly higher values of TBARs content and lower levels of PCC when compared to the non-AAS groups ($p<0.001$ and $p=0.033$, respectively). In addition, kidney Mn-SOD, CAT and GPx activities were significantly lower in the AAS groups when compared to the non-AAS groups ($p=0.001$, $p=0.003$ and $p<0.001$, respectively).

A significant HIE*AAS interaction was found for kidney TBARs content derived from a higher HIE-induced decrease in that marker for AAS groups that was not observed in the non-AAS groups (both, $p<0.001$). A significant HIE*AAS interaction was also found for PCC derived from a

higher HIE-induced decrease in that marker for the non-AAS groups that was not observed in the AAS groups (both, $p<0.001$). In addition, significant HIE*AAS interactions were observed for t-SOD and CuZn-SOD activities derived from a higher HIE-induced increase in the AAS groups that was not observed in the non-AAS groups (both, $p<0.001$).

Plasma and renal morphology parameters

The effects of AAS-administration and HIE on plasma and kidney morphology parameters are shown in **Table 8.** Plasma total protein and creatinine content was significantly lower in the AAS when compared to the non AAS-administered groups ($p=0.034$ and $p<0.01$, respectively).

With regard to renal morphology, the AAS groups exhibited significantly higher glomerular area when compared to the non-AAS groups ($p=0.025$). No significant alterations caused by AAS administration were to be observed in any of the rest of renal morphology parameters studied.

Table 6. Effects of the high-intensity exercise and anabolic androgenic steroids administration on final body weight, carcass weight, food intake and kidney weight.

	Non AAS		AAS		*p* values			
	Untrained	HIE	Untrained	HIE	SEM	AAS	HIE	AAS*HIE
Food intake (g/day)	20.32c	15.62a	18.55b	16.50a	0.210	0.296	<0.001	0.003
Final body weight (g)	351.22b	313.04a	332.86a,b	321.67a,b	4.725	0.610	0.013	0.162
Carcass weight (g)	172.12a	163.21a	181.27a	178.09a	2.591	0.027	0.251	0.584
Kidney (g)	0.92a	0.87a	1.09b	1.02a,b	0.020	<0.001	0.125	0.806
Kidney (g/100g body weight)	0.26a	0.28a,b	0.33c	0.32b,c	0.005	<0.001	0.951	0.225
Kidney (g/100g carcass weight)	0.54a	0.53a	0.60b	0.57a,b	0.008	0.004	0.305	0.404

SEM, standard error of the mean; HIE, high-intensity exercise; AAS, anabolic androgenic steroids

Values expressed as mean of 10 rats. The same letter in the same row indicates no significant difference between groups ($p>0.05$).

Table 7. Effects of the high-intensity exercise and anabolic androgenic steroids administration on kidney oxidative stress markers.

	Non AAS		AAS			*p* values		
	Untrained	HIE	Untrained	HIE	SEM	AAS	HIE	AAS*HIE
TBARs (nmol MDA/mg protein)	12.8a	18.6a	59.8b	23.4a	1.891	<0.001	<0.001	<0.001
PCC (nmol/mg protein)	5.1c	2.1a	3.1b	2.9a,b	0.126	0.033	<0.001	<0.001
t-SOD (U/mg protein)	247.3b	215.5a,b	193.0a	243.2b	4.438	0.144	0.311	<0.001
Mn-SOD (U/mg protein)	136.9b	109.2a	109.8a	98.4a	2.717	0.001	0.001	0.143
CuZn SOD (U/mg protein)	110.4a	106.3a	83.2a	144.8b	4.131	0.498	0.001	<0.001
CAT (μmolH2O2/min/mg protein)	125.3b	123.6b	110.9a,b	88.3a	3.888	0.003	0.128	0.187
GPx (nmol NADPH/min/mg protein)	28.6a,b	44.9c	21.2a	30.1b	1.094	<0.001	<0.001	0.100

SEM, standard error of the mean; HIE, high-intensity exercise; TBARs, thiobarbituric acid-reactive substances; PCC, protein carbonyl content; t-SOD, total superoxide dismutase; Mn-SOD, manganese superoxide dismutase; CuZn-SOD, cooper and zinc superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

Values expressed as mean of ten rats. The same letter in the same row indicates no significant difference between groups ($p>0.05$).

Table 8. Effects of the high-intensity exercise and anabolic androgenic steroids administration on plasma and kidney morphology.

	Non AAS		AAS			*p* values		
	Untrained	HIE	Untrained	HIE	SEM	AAS	HIE	AAS*HIE
Plasma								
Urea (mg/dl)	24.52a	30.82a	27.60a	27.42a	0.844	0.926	0.078	0.063
Total Protein (g/dl)	5.62a	5.49a	5.40a	5.36a	0.039	0.034	0.286	0.544
Creatinine (mg/dl)	0.66b	0.48a	0.49a	0.42a	0.017	0.002	0.001	0.099
Albumin (mg/dl)	2.75a	3.18a	3.28a	2.47a	0.114	0.701	0.413	0.010
Morphology								
Interstitial connective tissue (%)	2.71a	3.97b	2.65a	3.42a,b	0.16	0.347	0.003	0.457
Interstitial connective tissue (μm²)	3657a,b	5278b	3404a	4493a,b	214.588	0.235	0.003	0.540
Glomerular tuft I (%)	16.74a,b	22.13b	15.16a	22.35b	0.902	0.709	0.001	0.620

Glomerular tuft I area (µm²)	6616a	9181a	6815a	9416a	385.891	0.780	0.002	0.981
Glomerular tuft II (%)	37.08a	52.46b	34.34a	53.45b	1.977	0.827	<0.001	0.641
Glomerular tuft II area (µm²)	14573a	21448b	15278a	22021b	797.01	0.691	<0.001	0.967
Mesangium (%)	64.74a	62.32a	64.63a	61.86a	0.659	0.830	0.057	0.896
Mesangium area (µm²)	4172a	5673a	4282a	5812a	226.569	0.786	0.002	0.975
Glomerular area (µm²)	40405a	41328a,b	45324b	42407a,b	637.789	0.025	0.440	0.141
Fibrosis-T (%)	2.09a,b	3.02b	2.03a	2.48a,b	0.122	0.221	0.008	0.334
Fibrosis-T area (µm²)	3660a,b	5279b	3547a	4329a,b	213.372	0.221	0.008	0.334

SEM, standard error of the mean; HIE, high-intensity exercise.

Values expressed as mean of 10 rats. The same letter in the same row indicates no significant difference between groups ($p>0.05$).

GENERAL DISCUSSION [DISCUSIÓN GENERAL]

GENERAL DISCUSSION [DISCUSIÓN GENERAL]

The raised interest of soy as source of protein (86) and high-protein diets consumption in the last decades to improve physical status (142) is an obviousness. Additionally, it is well established that AAS are widely used among professional athletes, competitive and recreational body builders or even non-athletic adolescents (103). Therefore, owing to the beneficial effects of soy high-protein diets intake and exercise on brain (96,97) and kidney (86), and given that it is the main macronutrient type experienced by AAS abusers and sportsmen (86,87,104,126), it is of importance to clarify the physiological effects of soy high-protein diets, HIE and AAS administration on the oxidative stress status of two tissues, specially sensitive to the oxidative damage. Thus, the purpose of the present Thesis was to complete the scarce information about the effects of high-protein diets, AAS administration and hypertrophy resistance training protocol (i.e. HIE) on brain and renal redox status.

1. Oxidative effects of high-protein diets and high-intensity exercise.

First we analysed the influence of high-protein diets and HIE on brain and kidney oxidative stress markers. The main findings of this study at brain level were: 1) high-protein diet increased TBARs and PCC concentrations, t-SOD, Mn-SOD, CuZn-SOD and CAT activity and the expression of Nrf2 protein, and 2) HIE decreased PCC concentrations and the expression of NQO1, increasing CAT activity and the expression of GFAP, GPx, and Nrf2 protein.

The main findings at kidney level were: 1) high-protein diet increased TBARs content, whereas it decreased t-SOD, Mn-SOD, CuZn-SOD, GPx activity, and 2) HIE reduced TBARs, PCC, whereas it augmented CuZn-SOD and GPx activity.

High-protein and brain and kidney oxidative stress

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. However, regarding to renal redox status, high-protein diets produced slightly opposite effects.

Thus, it could be observed that high-protein diets induced an oxidative status, derived from an imbalance between oxidative damage markers and the antioxidant defence system.

Studies in animals as well as in humans have illustrated that high-protein diets (143,144) provide higher satiety levels than other macronutrients, thus leading to a decrease of food intake. These assertions are in agreement with our findings in the high-protein groups that displayed a reduced food intake when compared to the normal-protein groups. Despite the decrease in food intake reported for the high-protein groups, there was a higher protein content on the above mentioned diets that were adjusted to 45% of dietary amount, leading to an elevated N intake, which may affect the antioxidant status in the tissues studied.

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 (145), 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 (91) have suggested that high-protein diet intake may cause an imbalance between ROS generation and the capacity of the antioxidant defence system in

digestive organs of mice such as duodenum, liver and pancreas, which lead to an induction of oxidative stress. This imbalance is reflected in a diminished antioxidant defence system and increased concentration of 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. (146), 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, at brain level, high-protein diets appeared to increase antioxidant activity as well as the overexpression of Nrf2, although this increment may be in response to the production of higher levels of brain lipid 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. Nonetheless, the opposite tendency was observed at kidney level where the deleterious effects produced by high-protein diets were reflected in a reduced antioxidant defence status. Thus, the higher the kidney lipid peroxidation detected in the high-protein groups, the lower the antioxidant enzyme activity (**Figure 2 and 3**). Therefore, the oxidative damage produced at kidney

level does not produce a compensatory antioxidant response, which lead us to consider that kidney is more sensitive than brain to the oxidative stress produced by the high-protein diets. This higher sensitivity can be attributed to the essential role of kidney in the excretion of N overload induced by high-protein diets.

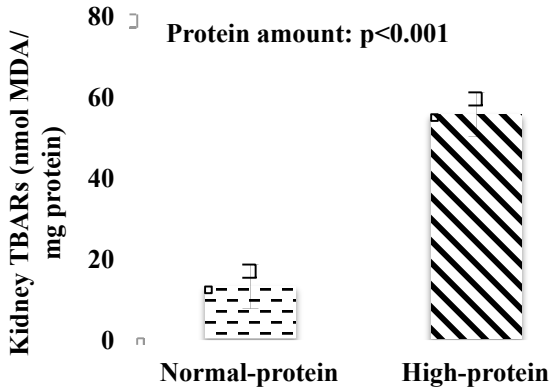


Figure 2. Effects of high-protein diet on renal lipid peroxidation.

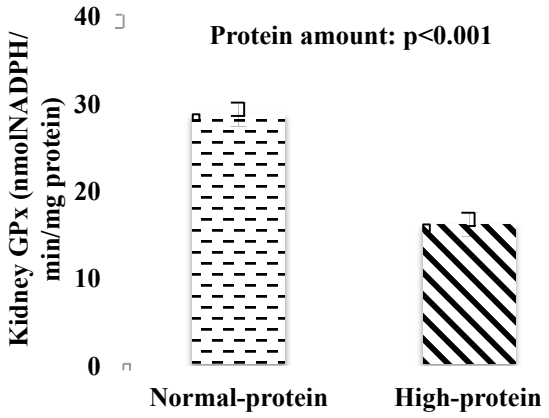


Figure 3. Effects of high-protein diet on GPx activity.

High intensity exercise and brain and kidney oxidative stress

The HIE protocol carried out caused a high stress situation, as reflected by higher levels of plasma levels of corticosterone (81), and was accompanied by a decreased food intake found in exercised compared to untrained animals.

Overall, the same trend related to exercise was observed for brain and kidney oxidative stress. HIE reduced protein oxidation and increased some antioxidant markers such as CAT activity and the expression of GFAP, GPx, and Nrf2 protein on brain. Likewise, regarding the renal redox status, HIE reduced lipid and protein oxidation and augmented CuZn-SOD and GPx activity.

Controversial findings have been reported regarding the effects of HIE on brain and kidney oxidative stress (5,80). On the one hand, some authors suggest that intermittent anaerobic exercise and acute exhaustion exercise increase brain antioxidant capacity and do not induce lipid peroxidation (147,148). On the other hand, under HIE training, ROS production may be strongly and persistently increased, and the antioxidant response may not be effective to reset the system to the original level of brain and kidney redox homeostasis (77,132,149,150). Concerning oxidative damage, despite the high levels of lipid

peroxidation in brain tissue due to the high content of polyunsaturated fatty acids and the free radical metabolism (46), some studies have demonstrated that HIE does not alter lipid peroxidation in whole brain (5), hippocampus (147,151), cerebellum (151), prefrontal cortex and striatum (147,152). In contrast, HIE has been shown to induce oxidative stress and increase lipid peroxidation in mouse brain (153,154). Taking into account these contradictory results in the literature our findings agree with some of the aforementioned studies in that no significant evidence was observed in brain TBARs levels after a HIE protocol. However, the type of exercise carried out under our experimental conditions reduced kidney TBARs levels (**Figure 4)**. Thus, this protocol could be beneficial in terms of reducing renal lipid peroxidation. In view of the results obtained in brain and kidney after the implementation of HIE, it appears that the intensity and length of training protocol assayed in the present study might lead to different effects depending the type of tissue studied.

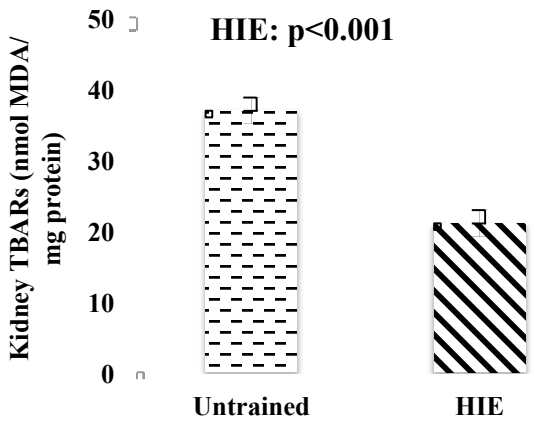


Figure 4. Effects of high-intensity exercise on renal lipid peroxidation.

Reactive oxygen species may also lead to the production of oxidative damage to proteins that is accompanied by an increase in the number of carbonyl residues. In this regard, Tsakiris et al. (155) reported that either short or prolonged forced swimming exercise increase ROS production, which could cause protein damage. The same trend was observed in the study by Aydin et al. (156), where brain PCC was significantly increased after swimming until exhaustion. In contrast, lower levels of PCC in the hippocampus of 12-month old rats were found after daily moderate intensity exercise for 15 weeks (18 m/min, on a 0% incline, for 30 min) (157). In accordance to previous findings, our results suggest that HIE might be responsible for lower levels of brain and

kidney PCC when compared to untrained groups, suggesting that the oxidants produced by HIE were not able of affecting brain and kidney proteins (**Figure 5**).

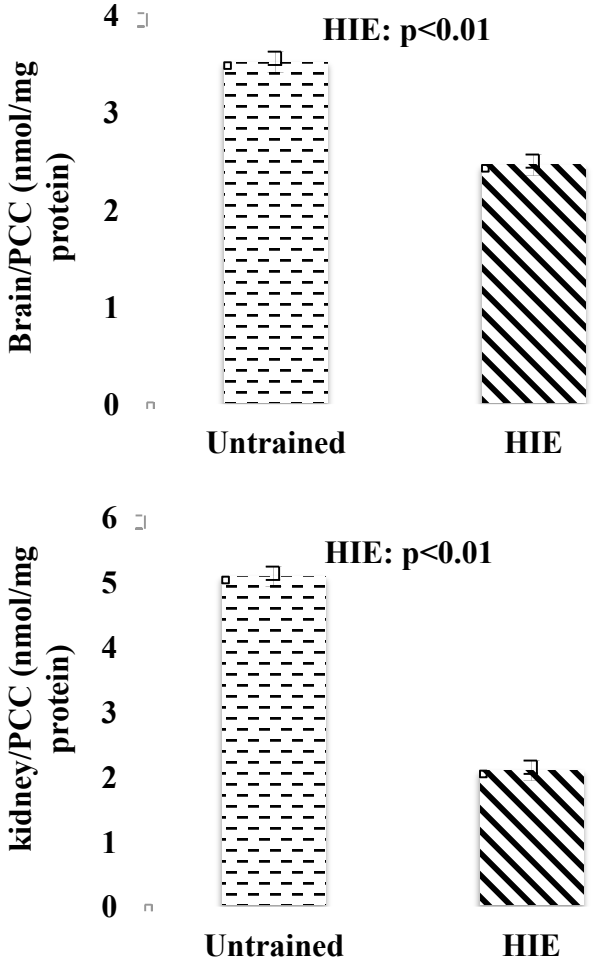


Figure 5. Effects of high-intensity exercise on brain and renal protein oxidation.

In the present Thesis, brain CAT activity levels increased after 12 weeks of HIE (**Figure 6**) whereas Mn-SOD and CuZn-SOD brain activity were not altered. However, slightly different results were observed at kidney level where the HIE protocol increased GPx and decreased Mn-SOD activity while CAT levels were not altered (**Figure 7**).

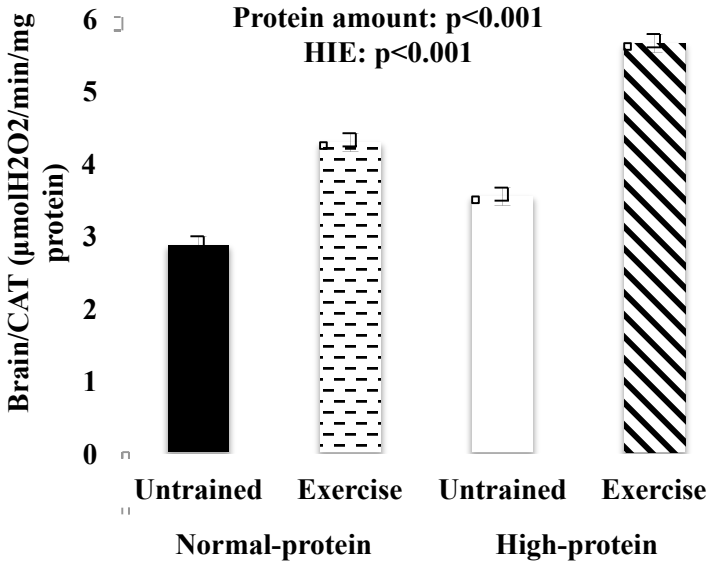


Figure 6. Effects of high-protein diet and high-intensity exercise on brain catalase activity.

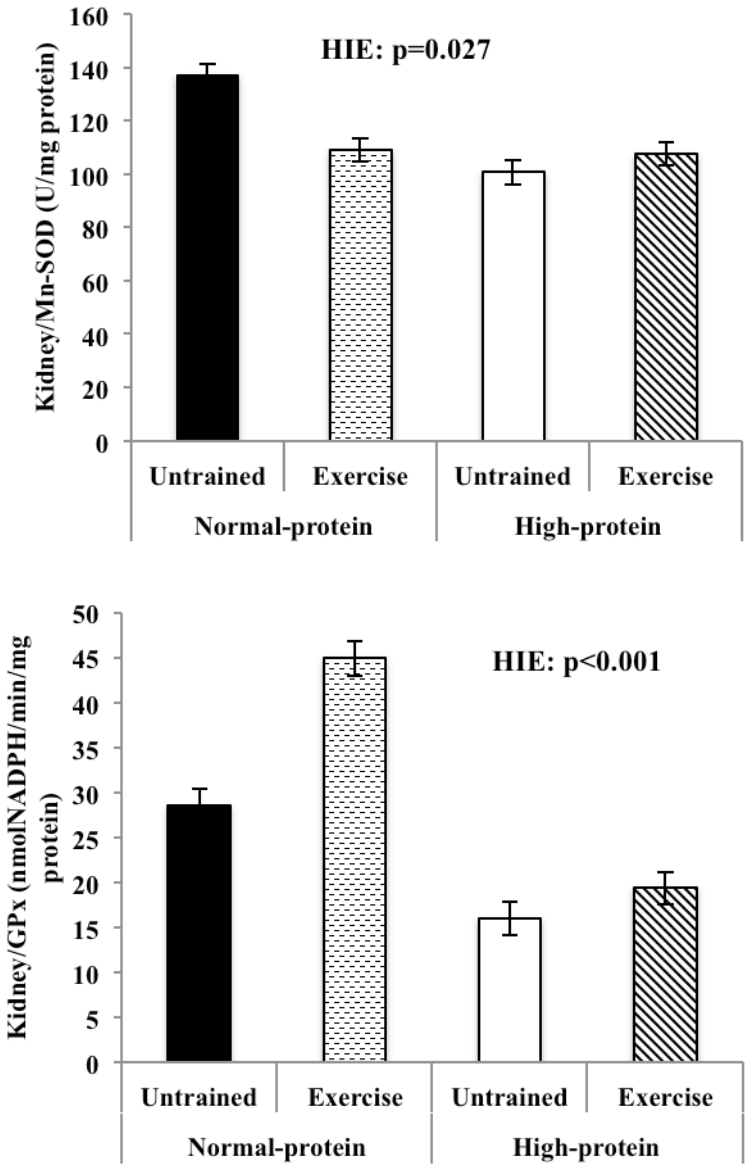


Figure 7. Effects of high-protein diet and high-intensity exercise on kidney Mn-SOD and GPx activities.

In a previous study carried out in humans, plasma CAT activity did not change in response to resistance training until the participants showed symptoms of overtraining (158). In addition, Margonis et al. (158) 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 matched with the maximum training load and performance decrement. The training protocol carried out in this study was found to induce overtraining (159) and may explain our findings related to the increased CAT activity in brain. Nevertheless, it should be taken into consideration that such activity may not represent a significant proportion of the tissue total antioxidant activity due to its low values (159).

At brain level, we have also determined the expression of Nrf2 protein, which is a transcription factor that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation (21). Thus, the body of the literature indicates that Nrf2 is the primary transcriptional regulator of a majority of the antioxidants including hemoxygenase-1 (HO1), γ -glutamyl cysteine ligase-catalytic (γGCLC), NQO1, GPx and CAT (160). It has been reported that moderate exercise enhances the promotion of an

endogenous Nrf2/γGCLC antioxidant system for the prevention of neurodegenerative diseases (161). Moreover, a recent study has also demonstrated that acute exercise induces ROS production and activates Nrf2 and antioxidant responsive element functioning in myocardial tissue (160). Under our experimental conditions, the production of ROS induced by HIE contributed to activate the transcriptional Nrf2 factor, and consequently the upregulation of CAT activity and GPx protein expression. Likewise, some authors observed that a moderate treadmill training (10–50 min/day of running at 40–60% of the maximal velocity 5 days per week for 5 weeks) increased GFAP content in the CA1 region of Wistar rats (118) and elevated the levels of GFAP in the hippocampus after a endurance training in diabetic rats (162). Although, caution must be taken, the tendency in our study suggests that Nrf2 could up-regulate the GFAP expression after a HIE protocol. In this regard, HIE might increase GFAP expression leading to a beneficial increase of astrocytes after the HIE protocol conducted in this Thesis.

An important aspect of the effects of the previously discussed interventions on brain and kidney redox status is the presence of significant interactions between them. Thus, some effects of the HIE protocol assayed may be affected by the intake of a high protein diet. One

example of such interaction is exemplified by differential effects of exercise on kidney PCC depending on normal-protein or high-protein diet intake (**Figure 8**). HIE reduces renal PCC when implemented in normal protein groups whilst it increases them in the high-protein groups.

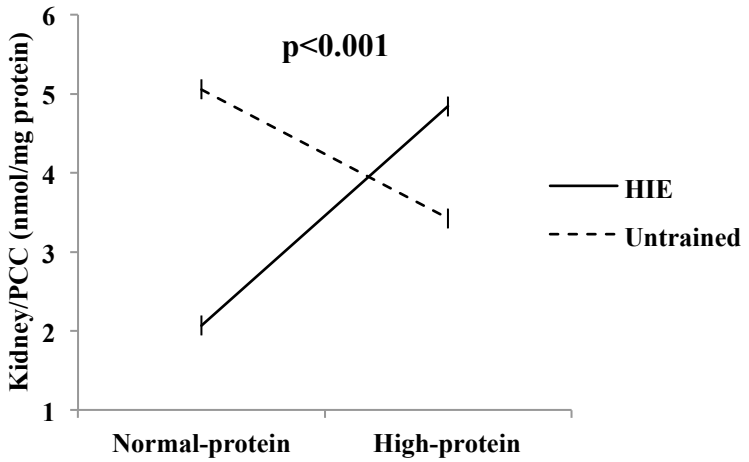


Figure 8. Interactions taking place between high-protein diet and high-intensity exercise on kidney protein carbonyl content.

Another example is represented by the differential effects of HIE on brain TBARS content that is higher associated to normal-protein diet intake and it is reduced in the high-protein groups (**Figure 9**). This result highlights the importance of HIE that did not worsen the deleterious

effect caused by the high-protein diets and may be an efficient way to protect the brain against high dietary protein aggression.

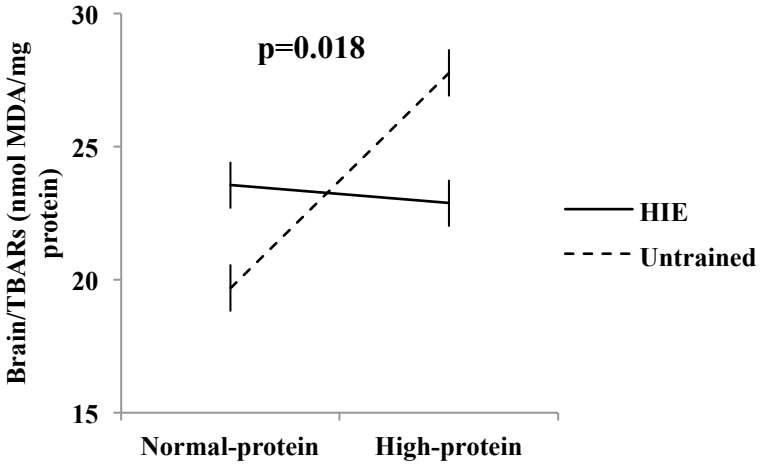


Figure 9. Interactions taking place between high-protein diet and high-intensity exercise on brain lipid peroxidation.

High-intensity exercise and renal morphology

The findings of the present Thesis show that rats involved in a HIE protocol displayed a worse renal morphological profile when compared to the untrained group, which might present a higher risk for incidence of kidney disease in the long-term. The stress induced by the type of exercise performed under our experimental design associated to changes in blood circulation pattern during performance of the high-intensity protocol may be related to this poorer morphological renal status.

In the general healthy population, exercise appears to improve global renal status (76,163–165). Exercise could improve microalbuminuria (76,165,166), and we have observed higher albumin concentrations in our trained group, which could mean a reduction of the microalbuminuria.

Hypertension is also an important risk factor for CKD, and regular exercise can efficiency help to decrease blood pressure (167,168). In the study by Agarwal et al. (169), spontaneously hypertensive rats performed 16 weeks of moderate-intensity exercise on a treadmill (5 days per week; 60 min per day at 20 m/min, which corresponds approximately to 60 % of maximal aerobic velocity), and this exercise protocol preserved renal hemodynamic and structure. Furthermore, exercise-induced effects, at least in part, were found to be pressure-independent (169).

Patients with chronic renal failure usually present the syndrome of "protein-energy malnutrition", which is a relevant factor for morbidity and mortality in this population and requires early detection and vigorous treatment (170). These patients could benefit from resistance training interventions (171). Indeed, Ding et al. (172) explored the effects of long-term weightlessness on the renal tissue and investigated the simulated

microgravity on the renal morphological damage and related molecular mechanisms in rats. Resistance training (4 sets, 12 repetitions for each set at 65–75 % of 1RM, 5 times per week for 8 weeks) reduced kidney cell apoptosis and expression of HSP70 protein and attenuated the kidney impairment imposed by weightlessness (172). Quadriceps N (protein) content was higher in trained animals, which might confirm the effectiveness of the strength training protocol performed in the present study on increasing muscle mass.

The maintenance of urinary acid/base homeostasis is also important in order to preserve renal health (170). A decrease in urinary pH, hypocitraturia and hypercalciuria are risk factors for kidney stone formation (173,174). In our study, no noticeable differences in these urinary parameters were observed, and consequently both groups presented similar risk of nephrolithiasis. However, the ~37 % higher urinary volume together with the ~20 % higher levels of plasma urea found in the HIE group could mean a higher renal filtration (i. e., hyperfiltration) in the trained group (175). Moreover, most of the morphological renal variables studied exhibited a worse profile, with higher kidney interstitial connective tissue, glomerular tufts and mesangium areas in the HIE group. Different hypotheses could explain

these findings:

1) During heavy physical exercise (such as that performed in our strength training protocol), 2 phenomena occur: the decrease of the glomerular filtration rate and the release into the blood of some molecules from muscles such as creatine kinase (CK), lactate dehydrogenase (LDH) and metmyoglobin (176–179). Renal filtration of metmyoglobin released from damaged muscle and filtered at the glomerulus is known to cause acute renal injury in exercise rhabdomyolysis (177,180,181). A 10-fold increase of CK is common in athletes after exercise (176,182). In humans, serum CK 5 times higher than normal usually confirms rhabdomyolysis (177). We have observed higher levels of CK in our HIE group, but in a lower magnitude. Therefore, the higher levels of CK may indirectly suggest that metmyoglobin has been liberated. Also noteworthy, yet without statistical significance, is the 3 times higher level of LDH observed in the HIE group. In fact, in the study by Colombini et al. (176) CK activity from 9 professional cyclists during the Giro d'Italia 3-week stage race increased during the second part of the race, and LDH activity progressively increased during the entire course of the race. There was a negative correlation between CK activity and the delta prerace-day 12 of glomerular filtration rate. The authors concluded that

the effect of prolonged strenuous muscular effort on biochemical laboratory parameters in professional road cyclists was confirmed. In agreement with our results, the authors also observed that creatinine is unaffected by response to physical stress-induced muscular damage (176).

2) Cortisol is a glucocorticoid released from the adrenal cortex in response to stress, which is believed to play an important role in the remodelling of tissue (183) in response to intense exercise such as ours (184–186). Indeed, resistance HIE protocols such as a 10-station heavy-resistance exercise protocol with 3 sets of 10 RM and very short rest periods between sets, or a sprint intervals protocol (185,186) that stimulate the greatest lactate response are correlated with high plasma cortisol levels. Moreover, protocols that result in the greatest concentrations of circulating CK 24-h post-exercise, also result in the greatest rises in circulating cortisol (187). Moreover, high plasma corticosterone levels have been reported in rats after a moderate aerobic treadmill exercise protocol (60 min/d, 5 d/wk at 42 cm/s and 0 % grade) (188). We have confirmed these findings and observed higher levels of plasma corticosterone in our HIE groups. Sustained delivery of supraphysiological levels of corticosterone play a role in modifying

kidney structure and function (189).

Our trained group also presented ~60 % less testosterone than the untrained group. Gonadal dysfunction is a frequent finding in men with CKD and with end-stage renal disease. Testosterone deficiency is present in 26–66 % of men with different degrees of renal failure (190). Experimental and clinical evidence suggests that testosterone may have important clinical implications with regard to kidney disease progression (190).

3) Finally, disturbances promoted by intense resistance training, such as hypoxia, glucose depletion or oxidative stress, may lead to endoplasmic reticulum dysfunction, which can induce endoplasmic reticulum stress. Accumulating evidence indicates that endoplasmic reticulum stress contributes to glomerular and tubular damage (191,192).

The differences found between the HIE and the untrained group in corticosterone-testosterone, as well as in testosterone-corticosterone ratios, may indicate a possible overtraining status in the HIE group (193). This overload may also influence the 3 hypotheses stated above.

2. Oxidative effects of anabolic androgenic steroids and high-intensity exercise

Results related to the HIE intervention have been discussed in the previous subsection. Therefore, the present subsection was focused in the AAS effect and its interaction with HIE. The main findings related to the administration of AAS and the combination of AAS and HIE on brain oxidative stress markers were: 1) AAS administration increased the expression of brain GFAP, GPx, NF-κβ protein whereas it decreased NQO1 expression, 2) brain PCC was more markedly reduced in the non-AAS groups than in the AAS groups when HIE and AAS were combined, 3) the opposite effect was raised on brain TBARs content where HIE decreased lipid peroxidation in the AAS-administered groups that was not observed in the non-AAS animals. On the other hand, the main findings of the combination of AAS and HIE at kidney level were: 1) AAS administration increased TBARs concentration, whereas it decreased Mn-SOD, CAT and GPx activity, 2) the same tendency observed at brain level was also shown for kidney TBARs and PCC, with a higher reduction of PCC caused by the HIE in the non-AAS when compared to AAS-administered groups. The opposite phenomena was

found on kidney TBARs content where HIE noticeably decreased lipid peroxidation in the AAS-administered groups; a finding that was not observed in the non-AAS animals.

Overall, the AAS administration appeared to promote brain inflammation and kidney lipid peroxidation, negatively affecting the antioxidant defence system. The HIE protocol was significantly affected by AAS administration.

Our first concern was to characterize the potential adverse effects of prolonged use of AAS on brain and kidney redox status. The study performed by Tugyan et al. (106) analysed the neuroprotective effect of erythropoietin on brain damage induced by Nandrolone administration for 8 weeks. After this experimental period, the AAS group displayed some irreversible effects such as increased MDA levels and apoptosis as well as a significant decrease in GPx activity in prefrontal cortex and hippocampus compared to the placebo group. In contrast, Celek et al. (194) revealed that Nandrolone and Testosterone were able to decrease the elevated MDA produced by ethanol ingestion in rat cerebellum. These results do not concur with our findings at brain level, in which the AAS administration did not appear to induce significant changes on lipid and protein oxidation, although it significantly decreased the expression

of NQO1. On the other hand, the HIE protocol reduced brain PCC and NQO1 expression (**Figure 10**). When AAS administration and HIE interventions were merged, the exercise clearly reduced lipid oxidation only in AAS group and protein oxidation only in non-AAS group, leading to a better brain oxidative status. Such improvement was reinforced by the stronger HIE effect on NQO1 expression in AAS vs. non-AAS administered groups (**Figure 11**).

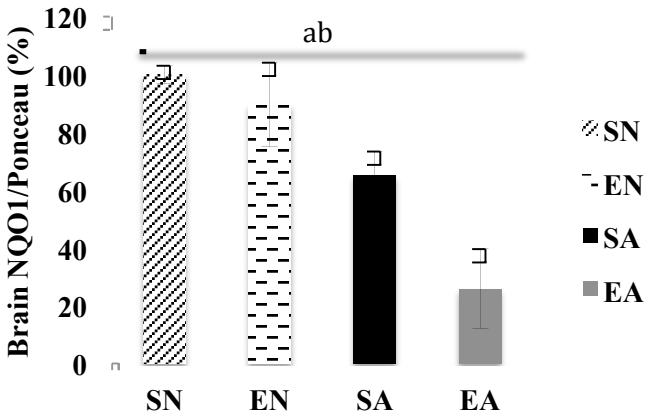


Figure 10. Effects of high-intensity exercise and the anabolic androgenic steroids administration on brain NQO1 protein level, n=4.
Results are means ± SD. Annotation indicates significant effect of a = exercise and b = anabolic androgenic steroids. $p<0.05$. Abbreviations: SN, Untrained and Non-AAS administration; EN, HIE and Non-AAS administration; SA, Untrained and AAS administration; EA, HIE and AAS administration; NQO1, NAD(P)H dehydrogenase, Quinone 1.

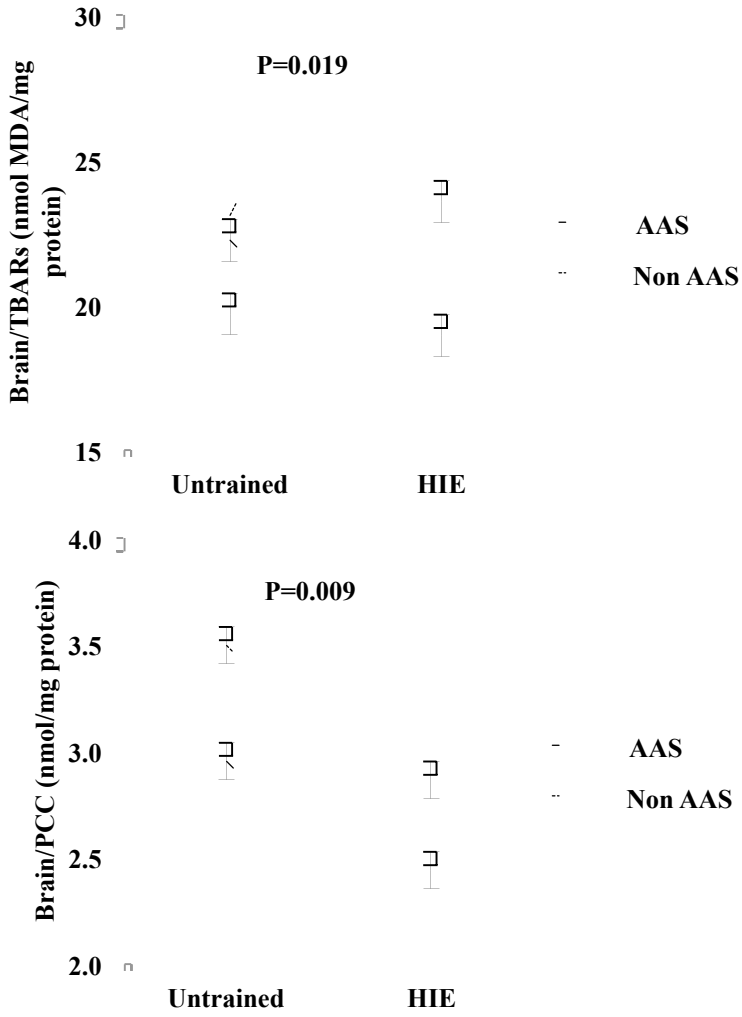
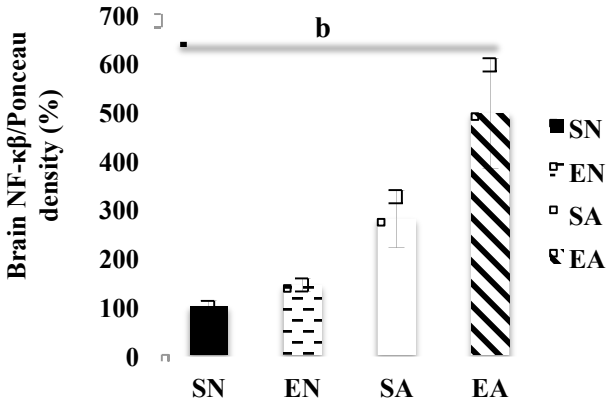
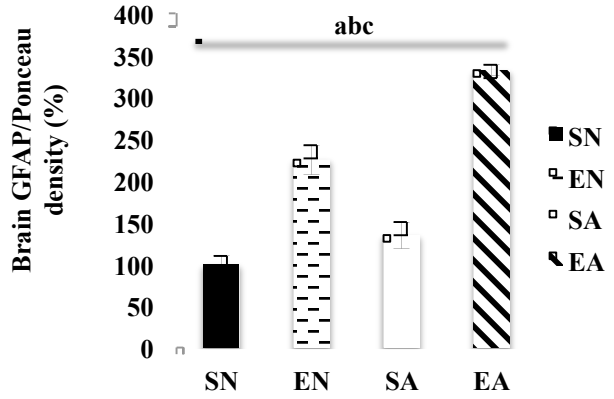


Figure 11. Interactions taking place between high-intensity exercise and anabolic androgenic steroids administration on brain oxidative damage markers.

Emerging evidence suggests that Nrf2 may also play an important role in the regulation of brain inflammation, and some studies have suggested that Nrf2 has an antagonistic effect with the NF-κβ pathway, which is considered as a hallmark of inflammation (195). Thus, the lack of changes in the expression of Nrf2 and the increased expression of inflammatory protein NF-κβ as well as the antioxidant enzymes GPx and GFAP after the Stanozolol treatment could be pointing out to the development of an inflammatory process derived from AAS administration that activate the defence mechanisms in brain **(Figure 12)**.


b
Brain NF-κβ/Ponceau density (%)
700
600
500
400
300
200
100
0
SN
EN
SA
EA
SN
EN
SA
EA


abc
Brain GFAP/Ponceau density (%)
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350
300
250
200
150
100
50
0
SN
EN
SA
EA
SN
EN
SA
EA

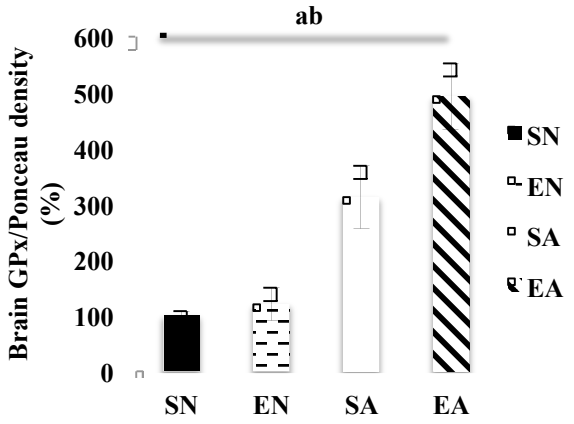
ab
Brain GPx/Ponceau density (%)
600
500
400
300
200
100
0
SN
EN
SA
EA
SN
EN
SA
EA

Figure 12. Effects of high-intensity exercise and the anabolic androgenic steroids administration on brain NF-κβ, GFAP and GPx protein level, n=4.
Results are means ± SD. Annotation indicates significant effect of a = exercise and b = anabolic androgenic steroids, or c = significant exercise*steroids interaction: $p<0.05$. Abbreviations: SN, Untrained and Non-AAS administration; EN, HIE and Non-AAS administration; SA, Untrained and AAS administration; EA, HIE and AAS administration; NF-κβ, nuclear factor kappa β; GFAP, glial fibrillary acidic protein; GPx, glutathione peroxidase.

Otherwise, in terms of renal oxidative status the general effects of AAS administration described in brain agree with our results and reflects an oxidative damage to the tissue, where AAS administration increased lipid oxidation (**Figure 13**) and decreased Mn-SOD, CAT and GPx enzymatic activity.

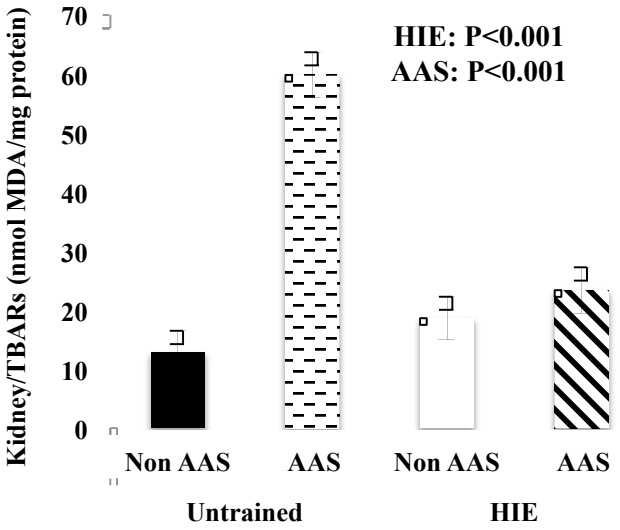

Figure 13. Effects of high-intensity exercise and anabolic androgenic steroids on renal lipid peroxidation.

Previous studies have analysed the effects of AAS or its combination with diverse exercise protocols on the oxidative status of other organs with controversial findings. Pey et al. (196) concluded that prolonged Stanozolol treatment, with or without moderate exercise training, might induce oxidative stress by the increase of TBARs levels on rat liver, despite enhancement in the antioxidant activity of SOD, CAT and GPx enzymes. Likewise, Chaves et al. (111,132) showed that in spite of the beneficial effect of HIE on heart through increased activity of antioxidant enzymes, the Nandrolone administration induced lower SOD,

GPx and GR activity when compared to control and trained animals. Similarly, Sun et al. (197) demonstrated that the combination of Nandrolone and an aerobic physical training increased MDA and PCC, and decreased SOD and NQO-1 protein levels on aortic large vessels. On the other hand, a study performed by Saborido et al. (113) reported that Stanozolol administration might protect rat gastrocnemius mitochondria against oxidative damage of proteins and changes in membrane fatty acid composition induced by acute exercise. The above mentioned studies concur partially with the findings of the present work in which the combination of both effects (i.e. HIE and AAS) presented significant interactions in the oxidative stress markers. Thus, owing to the fact that the antioxidant activity, oxidative damage markers and the glial activation of GFAP protein increased by HIE as well as AAS administration, we can conclude that the induction of antioxidant activity is linked as an auto-defence system against oxidative damage under both interventions. Therefore, the higher oxidative damage produced is associated with higher antioxidant activity produced as auto-defence mechanism.

Finally, we could elucidate that the pathways responsible for HIE and AAS effects on brain and kidney status might have upregulated or

downregulated by the aforementioned proteins in a different manner. Further studies are needed in order to deepen on this topic.

LIMITATIONS AND STRENGTHS [LIMITACIONES Y FORTALEZAS]

LIMITATIONS AND STRENGTHS [LIMITACIONES Y FORTALEZAS]

Limitations

The present PhD Thesis study has several limitations and strengths that need to be mentioned:

- It may be beneficial to compare our results with different sources of protein for the interpretations of the present findings.
- The protein carbonyl assay could suffer confounding factors.
- The physiological responses observed in rodents must be confirmed in humans. In other words, the responses found after 3 months of training using our experimental exercise protocol in rodents cannot be directly extrapolated to the potential effects over decades in human subjects.
- Although we have tried to mimic the training methodology performed by humans, this HIE protocol does not exactly reflect human strength training.

- Measuring additional markers of renal function such as the glomerular filtration rate would have been of interest in the interpretation of the PhD Thesis results.

Strengths

- However, it is important to highlight that this PhD Thesis possesses novel data analysing the combined effects of:
    - A high-protein diet and a HIE, based on a hypertrophy resistance training protocol, on brain and kidney oxidative stress.
    - HIE and AAS-administration on brain and kidney oxidative stress markers and antioxidant enzyme defence system in the same report, which allow a global picture about the effects of the combination of these two common behaviours.
    - High-intensity strength training on renal morphology in healthy animals under normal experimental conditions.

CONCLUSIONS [CONCLUSIONES]

CONCLUSIONS

1. High-protein diets may cause oxidative damage to the brain by means of lipid and protein oxidation, which could explain the induction of the endogenous antioxidant defence system.
2. High-intensity exercise protocol improved the deleterious effects caused by high-protein diet and may be an efficient way to protect the brain against high dietary protein aggression.
3. High-protein diets led to a prooxidant status at kidney level. However, the beneficial effect of high-intensity exercise observed on brain, did not appear at kidney level.
4. The high-intensity exercise protocol displayed a worse renal morphological profile, which might be associated with a higher risk for incidence of kidney disease in the long-term. The stress induced by the type of exercise performed in the present Thesis could be related to this worse morphological renal status.
5. Under our experimental conditions, the present results suggest that high-intensity exercise reduce the negative effects of anabolic androgenic steroids on brain redox status. High-intensity exercise

also improved the harmful effects caused by the anabolic androgenic steroids administration on kidney lipid and protein oxidation.

Overall conclusion:

The results of the current Thesis underline that high-protein diets intake and the anabolic androgenic steroids administration instigated brain and kidney damage by means of the induction of lipid and protein oxidation. Despite the apparently beneficial effect of high-intensity exercise among the others two interventions studied, cautiousness should be taken with this protocol regarding to brain and kidney overproduction of their antioxidant defence systems.

CONCLUSIONES

1. Las dietas altas en proteínas pueden causar daño oxidativo en el cerebro por medio de la oxidación de lípidos y proteínas, lo que podría explicar la estimulación del sistema de defensa antioxidante endógeno.
2. El protocolo de ejercicio de alta intensidad mejoró los efectos nocivos provocados por la dieta alta en proteínas, y puede ser un medio eficaz para proteger el cerebro contra la agresividad producida por dicha dieta.
3. Las dietas altas en proteínas conducen a un estado prooxidante a nivel renal. Por otra parte, el efecto beneficioso del ejercicio de alta intensidad observado en el cerebro, no se mostró a nivel del riñón.
4. El protocolo de ejercicio de alta intensidad muestra un peor perfil morfológico renal, lo que podría estar asociado con un mayor riesgo de incidencia en enfermedades renales a largo plazo. El estrés inducido por el tipo de ejercicio realizado en la presente Tesis podría estar relacionado con este peor estado morfológico renal.
5. Bajo nuestras condiciones experimentales, los resultados sugieren

que el ejercicio de alta intensidad reduce el efecto negativo de los anabolizantes androgénicos esteroideos sobre el estado redox del cerebro. El ejercicio de alta intensidad también mejoró el daño producido por la administración de anabolizantes androgénicos esteroideos en la oxidación de lípidos y proteínas del riñón.

Conclusión general:

Los resultados de la presente Tesis doctoral subrayan que el consumo de dietas ricas en proteínas y la administración de anabolizantes androgénicos esteroideos desencadenan daño cerebral y renal a través de la inducción de la oxidación de lípidos y proteínas. A pesar del aparente efecto beneficioso del ejercicio de alta intensidad frente a las otras dos intervenciones ensayadas, se debe tener cautela con este protocolo respecto a la estimulación del sistema de defensa antioxidante tanto del cerebro como del riñón.

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