RESEARCH ARTICLE

Molecular Nutrition & Food Research
www.mnf-journal.com

A lupin protein hydrolysate protects the central nervous system from oxidative stress in WD-fed ApoE$^{-/-}$ mice

Guillermo Santos-Sánchez, Eduardo Ponce-España, Ana Isabel Álvarez-López, Justo Pedroche, María del Carmen Millán-Linares, María-Soledad Fernández-Pachón, Patricia Judith Lardone, Ivan Cruz-Chamorro, and Antonio Carrillo-Vico**

Oxidative stress plays a crucial role in neurodegenerative diseases like Parkinson's and Alzheimer's. Studies indicate the relationship between oxidative stress and the brain damage caused by a high-fat diet. It is previously found that a lupin protein hydrolysate (LPH) has antioxidant effects on human leukocytes, as well as on the plasma and liver of Western diet (WD)-fed ApoE$^{-/-}$ mice. Additionally, LPH shows anxiolytic effects in these mice. Given the connection between oxidative stress and anxiety, this study aimed to investigate the antioxidant effects of LPH on the brain of WD-fed ApoE$^{-/-}$ mice. LPH (100 mg kg^{-1}) or a vehicle is administered daily for 12 weeks. Peptide analysis of LPH identified 101 amino acid sequences (36.33%) with antioxidant motifs. Treatment with LPH palliated the decrease in total antioxidant activity caused by WD ingestion and regulated the nitric oxide synthesis pathway in the brain of the animals. Furthermore, LPH increased cerebral glutathione levels and the activity of catalase and glutathione reductase antioxidant enzymes and reduced the 8-hydroxy-2'-deoxyguanosine levels, a DNA damage marker. These findings, for the first time, highlight the antioxidant activity of LPH in the brain. This hydrolysate could potentially be used in future nutraceutical therapies for neurodegenerative diseases.

1. Introduction

Bioactive peptides are defined as peptide sequences of between 2 and 20 amino acids in length, that are present within the food protein and exert physiological health benefits, beyond its known nutritional value.[1] They are released during enzymatic proteolysis and food processing.[2] Nowadays, there are numerous peptides derived from vegetable and animal food products that have demonstrated several health effects (antidiabetic, antihypertensive, cholesterol-lowering, anti-inflammatory, etc.).[2,3]

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses. A large body of evidence shows that oxidative stress is responsible for the onset and progression of several diseases,[4] and has been linked to several neurodegenerative conditions such as Alzheimer's disease, mild cognitive impairment in Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease.[5] For this reason, the potential beneficial role of antioxidants from a food source in reducing oxidative stress at the systemic level, and especially in the central nervous system, is a field of great interest. In this line, food-derived peptides such as those of walnut have been shown to increase superoxide dismutase (SOD) and catalase (CAT) activities and reduce the malonaldehyde levels (marker of lipid peroxidation) in the brain of mice treated with lipopolysaccharide (LPS).[6] Phytochemicals from medicinal plants have long been known to often offer a safer alternative to synthetic

G. Santos-Sánchez, E. Ponce-España, A. I. Álvarez-López, P. J. Lardone, I. Cruz-Chamorro, A. Carrillo-Vico
Instituto de Biomedicina de Sevilla
IBiS/Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla
Seville 41013, Spain
E-mail: icruz-ibis@us.es; vico@us.es

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/mnfr.202300503

DOI: 10.1002/mnfr.202300503

G. Santos-Sánchez, E. Ponce-España, A. I. Álvarez-López, M. del C. Millán-Linares, P. J. Lardone, I. Cruz-Chamorro, A. Carrillo-Vico
Departamento de Bioquímica Médica y Biología Molecular e Inmunología
Facultad de Medicina
Universidad de Sevilla
Seville 41009, Spain

J. Pedroche, M. del C. Millán-Linares
Department of Food & Health
Instituto de la grasa
CSIC, Ctra, Utrera Km 1, Seville 41013, Spain

M.-S. Fernández-Pachón
Área de Nutrición y Bromatología
Departamento de Biología Molecular e Ingeniería Bioquímica
Universidad Pablo de Olavide
Ctra. Utrera Km 1, Sevilla 41013, Spain

medications. They possess neuroprotective activity targeting various elements in pathogenic pathways due to their antioxidant, anti-inflammatory, and other properties.[7]

Lupin is a non-starchy grain legume characterized by a high protein content (31–52%) compared to other legumes such as chickpea (19%) or lentils (26%). Thus, in recent decades lupin seeds have gained attention as an important source of bioactive peptides. In this regard, we have recently demonstrated that a *Lupinus angustifolius* protein hydrolysate (LPH) improves antioxidant and anti-inflammatory status both in vitro[8] and in vivo,[9] as well as reduces abdominal adiposity and improves metabolic-associated fatty liver disease in mice.[10] Moreover, we have shown that the ingestion of a beverage based on LPH for 28 days improves the antioxidant status and reduces the cardiovascular risk ratio in humans.[11] Furthermore, LPH shows anxiolytic-like effects in Western Diet (WD)-fed ApoE$^{-/-}$ mice.[12] In this line, oxidative stress has been described as a key risk factor for the anxiety process and memory impairment.[13] LPHs biological effects have been recently revised in Cruz-Chamorro et al.[14]

Although we have evidence that LPH improves plasmatic and hepatic antioxidant capacity, there is no evidence of the effects of LPH on the oxidative status of the central nervous system (CNS) that could justify the anxiolytic effects of LPH. Therefore, the aim of this multidisciplinary study was to evaluate whether LPH can exert antioxidant effects on the CNS. First, a peptidomic characterisation of the LPH was performed, and the antioxidant motifs, present in the peptide sequences, were identified. Subsequently, in vitro tests were carried out to evaluate the antioxidant capacity of LPH. Finally, the effect of LPH on the CNS oxidative status was evaluated in ApoE$^{-/-}$ mice fed a WD. ApoE is a key protein related to the lipid metabolism whose deficiency has been shown to increase oxidative stress in the brain tissue,[15] and, furthermore, WD consumption increases oxidative stress in this mouse model.[16]

2. Experimental Section

2.1. LPH Preparation and Characterization

LPH was prepared at the "Instituto de la Grasa" (CSIC, Seville, Spain) by hydrolysis of a lupin (*Lupinus angustifolius*) seed-derived protein isolate. Hydrolysis was carried out with the food-grade Alcalase 2.4L (Novozymes, Bagsvaerd, Denmark) enzyme (2.4 AU g^{-1}) for 15 min, pH 8, 50°C. LPH was lyophilized, dissolved in a saline solution, filtered, autoclaved, and stored at −80°C until use, as previously described.[8]

The molecular weight profile of LPH was determined by molecular exclusion chromatography, as previously described.[8] Molecular weight estimation was performed by gel filtration chromatography in an Akta purifier system (GE Healthcare) equipped with a Superose 12 HR 10/30 column with an optimal separation range of 300–1000 Da. The elution was developed in 50 mM sodium phosphate 0.5 M NaCl, 0.02% (w/v) sodium azide buffer, and monitored at 280 nm. The analytical flow was 1.0 mL min^{-1}, and the protein concentrations of the samples were 1 mg protein mL^{-1}, with an injection volume of 0.5 mL in two elution column volume (50 mL).

From the total sequences identified in LPH, sequences associated with the *L. angustifolius* species were selected by using the *L angustifolius* database (31386 sequences) from UnitProt. Identification of raw data spectra was performed using Proteome Discoverer v1.3 (Thermo) with the Mascot search engine v2.3.02. Furthermore, the BIOPEP-UWM database[17] was used to identify LPH peptide sequences with previously demonstrated bioactive motifs.[18] The hydrophobicity of these sequences was calculated using ToxinPred software.[19]

Protein concentrations were determined by elemental microanalysis as a percentage of nitrogen content × 6.25 using a Leco CHNS-932 analyzer (St. Joseph, MI, USA). The ash content was determined using the direct ignition method (550°C for 25 h). Total dietary fiber was determined according to Lee.[20] The oil content was measured using the AOAC method 945.16.[21] Soluble sugars and polyphenols were measured using standard glucose curves and chlorogenic acid, respectively.[22,23]

2.2. In vivo Experimental Approach

Twelve ApoE$^{-/-}$ mice (4-week-old male) were housed in the animal facility of the "Instituto de Biomedicina de Sevilla" (IBiS) under standard conditions (12/12 light/dark cycles, temperature 22 ± 2°C, and humidity < 55%) with free access to water and diet. Of these, four mice were fed a standard diet (SD) and eight mice with WD (Test Diet 58v8, 45% energy from fat), provided by the Special Diet Production Section of the University of Granada (Granada, Spain). The composition of diets is reported in Table S1 (Supporting Information).

After 2 weeks (6-week-old mice) the WD-fed mice were divided into two groups: 1) animals intragastrically treated with LPH (100 mg kg^{-1}) (WD + LPH group, $n = 4$) and **2**) mice intragastrically treated with saline (WD group, $n = 4$). SD-fed mice were also intragastrically treated with saline (SD group, $n = 4$). Treatment for each group was 12 weeks (up to mice were 18-weeks-old) and 5 days per week. Individual body weight was recorded weekly.

At the end of the experiment, 12 h fasted mice were sacrificed, each brain was collected, and the hypothalamus was separated, which was rapidly frozen, and stored at −80°C until use. The whole frozen brain (without hypothalamus) was homogenized in 4 mL of PBS containing a protease inhibitor cocktail (Sigma Aldrich, Darmstadt, Germany) using the Tissue Ruptor II (Qiagen, Hilden, Germany). The samples were then centrifuged at 12 000 × *g* for 15 min at 4°C and the supernatant was collected and stored at −20°C until use. The experimental protocol was approved by the Ethics Committee (reference 21/06/2016/105).

2.3. Antioxidant Activity

The antioxidant activity was measured in raw LPH or in the brain using several assays. Whole brain homogenate was used for the FRAP, GSH, and NO assays, and the results were referred to mg of protein. The protein content of each brain was quantified using the Bradford method. Briefly, 5 µL of homogenate brain sample was mixed with Bradford reagent in a final volume of 250 µL. The absorbance at 595 nm was measured using a CLARIOstar Plus microplate reader (BMG Labtech, Ortenberg, Germany). Protein content was extrapolated using a bovine serum albumin (BSA, 0–2 mg mL^{-1}) curve. The hypothalamus was employed for the gene expression analysis.

2.3.1. Ferric Reducing Antioxidant Power Assay (FRAP)

To quantify the total antioxidant capacity, the ferric reducing antioxidant power (FRAP) assay was performed. 20 µL of LPH at the final concentration of 0.25, 0.5, and 1 mg mL^{-1} or 20 µL brain homogenate were mixed with 280 µL FRAP reagent. The FRAP reagent was prepared by mixing 1.3 mL of a 10 mM TPTZ solution (Sigma-Aldrich) in 40 mM HCl, 1.3 mL of 20 mM $FeCl_3 \times 6H_2O$ and 13 mL of 0.3 M acetate buffer (pH 3.6). After 30 min of incubation at 37°C, the absorbance was recorded at 595 nm with a Synergy HT-multimode microplate reader (BioTek instruments, Winooski, VT, USA). The results were obtained by data extrapolation with a standard curve using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, Sigma).

2.3.2. Total Glutathione

Total glutathione (GSH) levels were quantified using the Glutathione Assay Kit (Cayman Chemical, MI, USA), according to the manufacturer's instructions. Briefly, 50 µL of samples were mixed with 150 µL of the Assay Cocktail (MES buffer, cofactor mixture, enzyme mixture, water, and DTNB) and incubated in the dark on an orbital shaker for 30 min. The absorbance at 405 nm was recorded using the Synergy HT-multimode microplate reader (Biotek Instruments).

2.3.3. Nitric Oxide Quantification

Nitric oxide (NO) was quantified in the brain homogenate using the Griess test (Sigma-Aldrich, St. Louis, MO, USA). 50 µL of the Griess reagent was incubated with 50 µL of the sample for 15 min at room temperature in the dark. The absorbance at 540 nm was measured using the Synergy HT-multimode microplate reader (Biotek Instruments).

2.3.4. Relative Expression of Nitric Oxide Synthase mRNA

The gene expression of neuronal and inducible NO synthase (nNOS and iNOS, respectively) was evaluated by RT-quantitative PCR (RT-qPCR). Total mRNA was obtained from the frozen hypothalamus using TRIsure (Meridian Bioscience, Cincinnati, OH, USA) standard protocol according to the manufacturer's instructions. cDNA from 3 µg of RNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostic). qPCR was performed using the LightCycler 480 SYBR Green I Master kit and the Lightcycler 480 thermocycler (Roche), at 55°C (iNOS) or 58°C (nNOS) for 40 cycles (80 ng cDNA per well). The hypoxanthine phosphoribosyl transferase (*hprt*) gene was used as a housekeeping gene and the relative expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were: *hprt* (NM_013556): Forward (Fwd) 5′-TGTTGGATATGCCCTTGACTA-3′, Reverse (Rev) 5′-TGCGCTCATCTTAGGCT-3′; *nnos* (NM_008712): Fwd 5′-TATTCCATCAGCTCCTCTCCA-3′, Rev 5′-GGATGTCAAA TTGTCGCTGTT-3′; *inos* (NM_001313921): Fwd 5′-ACGGA CGAGACGGATAG-3′, Rev 5′-GGGCTTCAAGATAGGGA-3′.

2.3.5. Antioxidant Activities Assays

Enzymatic activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GR, Randox Laboratories Ltd, Crumlin, UK) and catalase (CAT) (Cayman Chemical, MI, USA) were performed in the brain homogenate according to the manufacturer's instructions.

2.3.6. 8-Hydroxy-2′-Deoxyguanosine (8-OHdG) Assay

The 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels were quantified by the 8-OHdG Check (high sensitive) ELISA (IBL International GmbH, Hamburg, Germany) following the kit instructions.

2.4. Statistical Analysis

Data are represented as mean ± standard error of the mean (SEM). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunn's post hoc test. A p-value ≤ 0.05 was considered statistically significant. In addition, the size effect was analyzed using Cohen's test, and a d-value ≥ 0.80 was considered as "large effect size". Moreover, a nonparametric Spearman's correlation was applied. Data were analyzed with GraphPad Prism v.8 (GraphPad Software, San Diego, CA, USA) and Jeffreys's Amazing Statistics Program (JASP v. 0.16.3, Amsterdam, Netherlands).

3. Results

3.1. LPH Characterization

LPH was contained by 80.37 ± 0.09% of proteins with molecular weights less than 1 kDa, as can be observed in the molecular weight profile (**Figure 1A**).

Furthermore, LPH contained a low content of fiber (2.88 ± 0.08%), lipids (0.58 ± 0.00%), soluble sugar (0.02 ± 0.00%), and polyphenols (0.06 ± 0.00%). Furthermore, 8.62 ± 0.09% of the LPH was ash and 7.97 ± 0.19% was moisture, as reported in **Table 1**.

The LPH peptide analysis allowed us to identify 278 sequences belonging to the taxonomy *L. angustifolius* of which 101 sequences (36.33%) showed at least a motif related to antioxidant effects (**Table 2**). The hydrophobicity of the antioxidant peptides ranged from −0.73 to 0.34. In particular, 15.84% of the sequences had a hydrophobicity of < −0.40, 26.73% were between −0.20 and −0.40, 37.62% had −0.01 to −0.19 and 19.80% had a hydrophobicity ranging −0.34 to 0.00. Furthermore, these 101 LPH sequences contained >6 (1.98%), 5–6 (8.91%), 3–4 (33.6%), and 1–2 (45.54%) aromatic rings. Only 9.90% of the sequences had no aromatic rings (Figure 1B). In this way, 234 out of 1425 (16.42%) amino acids that were part of the antioxidant sequences had at least an aromatic ring. Finally, 39.16% of the amino acids containing in the antioxidant sequences were nonpolar amino acids (39.16%), although the most representative amino acid was glutamic acid (E, 14.11%) (Figure 1C). Regarding the 41 antioxidant motifs identified in 101 sequences mentioned above, these contained 20.0% Leu, 19.0% His, 8.6% Pro, 7.6% Tyr, 7.6% Trp, and

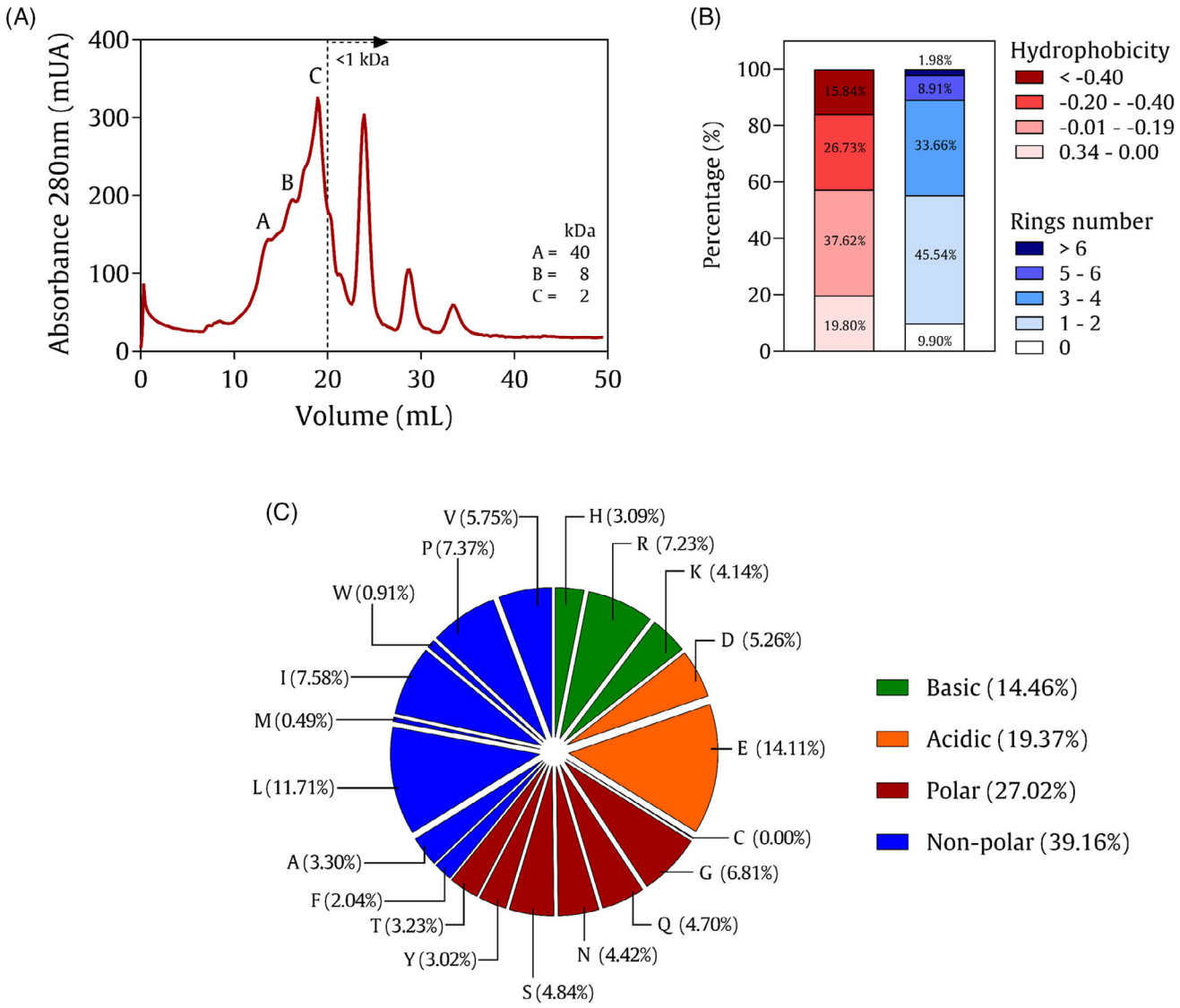

Figure 1. Molecular weight distribution of the entire *Lupinus angustifolius* protein hydrolysate (LPH) (A), percentage of hydrophobicity and number of rings (B), and amino acid composition (C) of the sequences with demonstrated antioxidant motifs.

4.8% Val. In addition, IR (ID 8215), LK (ID 8217), EL (ID 7888), and LLPH (ID 3314) were the motifs more represented taking part of 28, 14, 12, and 9 sequences, respectively (Table 2).

Table 1. LPH composition.

Components	LPH
Proteins	80.37%
Fiber	2.88%
Lipids	0.58%
Soluble sugar	0.02%
Polyphenols	0.06%
Ash	8.62%
Moisture	7.97%

Data are expressed as percentages on a dry basis. LPH, lupin protein hydrolysate.

3.2. In vitro Antioxidant Activity of LPH

To evaluate the antioxidant capacity exerted by LPH, an in vitro FRAP assay test was performed. LPH significantly increased FRAP levels in a dose-dependent manner. Specifically, the 0.25 mg mL^{-1} LPH concentration reached a Trolox equivalent antioxidant capacity (TEAC) equal to 9.54 ± 0.76 µM ($p < 0.0001$), while 0.5, and 1.0 mg mL^{-1} reached 14.41 ± 6.03 µM ($p < 0.0001$) and 30.40 ± 1.51 µM ($p < 0.0001$) TEAC, respectively (**Figure 2**).

3.3. LPH Does Not Modify the Body Weight of the Mice

As shown in **Figure 3**A, no differences ($p > 0.05$) were observed in the body weight of the animals in the three experimental groups weekly and throughout the experiment. However, a significant increase in the final body weight and in the body weight gain

Table 2. Bioactive motifs with antioxidative demonstrated activity.

ID[1]	Bioactive motifs[a)]	No. sequences	Secuences[b)]
3300	PHH	1	RHRPHHHEEEEEEEEWSHQ
3301	HLH	1	HLHTDEEIRFA
3305	LH	5	GLHLPSYTNYPQL
			GLHLPSYTNYPQIIM
			GLHLPSYTNYPQLIF
			HLHTDEEIRFA
			GLHLPSYTNYPQ
3314	LLPH	9	INEGGLLLPH
			EINEGALLLPHYNSK
			FTEINEGALLLPHYNSK
			LIFTEISEGALLLPHYNSK
			TEINEGALLLPHYNSK
			TEISEGALLLPHYNSK
			FIEINEGGLLLPHYNSK
			FIEINEGALLLPHYNSK
			LTFTEINEGALLLPHYNSK
3317	HL	7	GLHLPSYTNYPQL
			HLPSYTNYPQIIM
			GLHLPSYTNYPQIIM
			GLHLPSYTNYPQLIF
			HLHTDEEIRFA
			GLHLPSYTNYPQ
			HLPSYTNYPQIIM
3319	HH	6	KGILDKIKDKLPGHHN
			RHRPHHHEEEEEEEEWSHQ
			HHEEEEEEEEWSH
			HHEEEEEEEEWSHQ
			HHEEEEEEEEWS
			HHEEEEEEEEW
7862	IKK	1	TVGNVGEKIKKPFESITK
7866	AY	4	KLRHNIGQSTSPDAYNPQAGRL
			KLRHNIGQSTSPDAYNPQAGRLK
			TSPDAYNPQAGRL
			AYNDEDLIRIL
7872	LY	2	RADLYNPNAGRIS
			IADPTRADLYNPTAGRIS
7886	AH	4	AHIPGVVEL
			SIRALPLDVVAH
			ALPLDVVAH
			WLPPWLDAH
7888	EL	12	AHIPGVVEL
			RIGPVELPYTL
			EETVRSIELPGLL
			GRQQEQQLEGELE
			KAKNELRDSFKLL
			IERVLLGDDELQ
			RVLLGDDELQEN
			SELSGKRPLFGPDLPQTE
			VVDEGEGNYELVGIR
			IERVLLGDDELQE
			IERVLLGDDELQEN
			SVLSPEELLAVR
7898	WY	1	NHPEVVAEEPWYGIE
7918	GHH	1	KGILDKIKDKLPGHHN
7980	HHH	1	RHRPHHHEEEEEEEEWSHQ
7995	LHL	4	GLHLPSYTNYPQL
			GLHLPSYTNYPQIIM
			GLHLPSYTNYPQLIF
			GLHLPSYTNYPQ
8001	LHT	1	HLHTDEEIRFA
8038	PHY	8	EINEGALLLPHYNSK
			FTEINEGALLLPHYNSK
			LIFTEISEGALLLPHYNSK
			TEINEGALLLPHYNSK
			TEISEGALLLPHYNSK
			FIEINEGGLLLPHYNSK
			FIEINEGALLLPHYNSK
			LTFTEINEGALLLPHYNSK
8045	PWL	1	WLPPWLDAH
8053	PWY	1	NHPEVVAEEPWYGIE
8063	RHN	2	KLRHNIGQSTSPDAYNPQAGRL
			KLRHNIGQSTSPDAYNPQAGRLK
8065	RHR	3	RTTRHRRAQGEEGEEEEEETSTR
			RHRRAQGEEGEEEEEETSTR
			RHRPHHHEEEEEEEEWSHQ
8076	RWL	1	TSLDFPILRWL
8103	VKL	1	ARDIWNIEPVKLP
8107	IKL	3	YEGGIKLPLDVI
			YEGGIKLPTNVL
			YEGGIKLPLDVIST
8114	GGE	1	RLINEPVEGGEGNLL
8133	KVI	5	KVISPPTLRPR
			VKEGLKVISPPTLRPR
			GLKVISPPTLRPR
			EVKEGLKVISPPTLRPR
			EGLKVISPPTLRPR
8134	KD	4	KGILDKIKDKLPGHHN
			KDPNYVDEEDEEEEDVKGF
			NMVDPDEKDSTGNLPSRAL
			RDSFGNDIIKDL
8190	PW	2	NHPEVVAEEPWYGIE
			WLPPWLDAH
8214	RW	1	TSLDFPILRWL
8215	IR	28	SSNIIRFF
			NDEDLIRIL
			VGGKIIREPGPLPGL
			LIFPGSAEDVERLIR
			QNPNERIKQIIRVE

(*Continued*)

Table 2. (Continued)

ID[1]	Bioactive motifs[a)]	No. sequences	Secuences[b)]
			NQRNFLAGSEDNVIRQL
			HLHTDEEIRFA
			VGGKIIREPGPLPGLK
			SEDNVIRQL
			LAGSEDNVIRQL
			SIRALPLDVVAH
			IIRVEEGLGVISPK
			SIRAGLQFPVGRIG
			QIIRVEEGLGVISPK
			VVDEGEGNYELVGIR
			QIIRLLL
			QVEDPQINDDEVLIR
			LENVIRDAVTY
			IIREPGPLPGL
			SDALDKIRFL
			ADENQRNFLAGSEDNVIRQL
			IFPGSAEDVERLIR
			PGSAEDVERLIR
			LDIVIPTIR
			QGDIIRVPSGTPVYL
			IFPGSAEDVERLIRNQ
			AYNDEDLIRIL
			LIFPGSAEDVERLIRNQ
8216	LKP	1	YSLKPLVPRLS
8217	LK	14	LEVPTIDLK
			ERLKQIVRVK
			KLRHNIGQSTSPDAYNPQAGRLK
			QNPDERLKQIVRVK
			VKEGLKVISPPTLRPR
			GLKVISPPTLRPR
			EVKEGLKVISPPTLRPR
			VGGKIIREPGPLPGLK
			EGLKVISPPTLRPR
			LKLPEGSNDVLLK
			KFVVDMPGLK
			KTNDQATTSPLKQVFRGIPAE
			YSLKPLVPRLS
			FVVDMPGLK
8218	KP	2	TVGNVGEKIKKPFESITK
			YSLKPLVPRLS
8219	TY	1	LENVIRDAVTY
8224	VY	1	QGDIIRVPSGTPVYL
8459	TW	1	SIIEEFPRLVTW
8461	VW	1	FVMDSPDDVWRIS
9879	SVL	1	SVLSPEELLAVR
10000	LPL	5	YEGGIKLPLDVI
			HFWQVDKGHTLPL
			SIRALPLDVVAH
			ALPLDVVAH
			YEGGIKLPLDVST
10003	LQL	1	IMLQLVILP
10051	RY	5	EQEEEYEQGEEEVRRY
			DQQRQQDEQEEEEEEVRRY
			QRQQDEQEEEYEQGEEEVRRY
			DQQRQQDEQEEEYEQGEEEVRRY
			QDEQEEEYEQGEEEVRRY

[a)] According to BIOPEP-UWM Database.[17] [b)] 1-letter amino acid code.

was observed in the WD group (27.83 ± 2.30 g, $p = 0.015$; +8.60 ± 1.34 g, $p = 0.014$) and in the WD + LPH group (27.35 ± 2.48 g, $p = 0.047$; +8.10 ± 1.95 g, $p = 0.028$) compared to the SD group (24.53 ± 1.24 g and 5.30 ± 1.10 g, respectively) (Figure 3B and C). However, no significant differences were observed in the final body weight ($p = 0.312$) and in the body weight gain ($p = 0.384$) when mice were fed WD and treated with the LPH, compared to the WD group (Figure 3B and C).

As shown in Figure 3D, no statistical changes ($p = 0.966$) were observed in the brain weight between groups at the endpoint (12 weeks of treatment; SD: 0.42 ± 0.02 g; WD: 0.42 ± 0.02 g; WD + LPH: 0.42 ± 0.01 g).

3.4. LPH Treatment Increases Total Antioxidant Activity

To evaluate the antioxidant activity exerted by LPH on the brain, the FRAP assay was performed in the brain of the animals. As shown in **Figure** 4A, WD ingestion significantly decreased ($p = 0.003$) FRAP by 18.31% (0.83 ± 0.03 mmol equivalent Trolox per liter per milligram protein) compared to SD-fed mice (1.01 ± 0.04 mmol equivalent Trolox per liter per milligram protein). LPH treatment for 12 weeks overcame these effects, showing

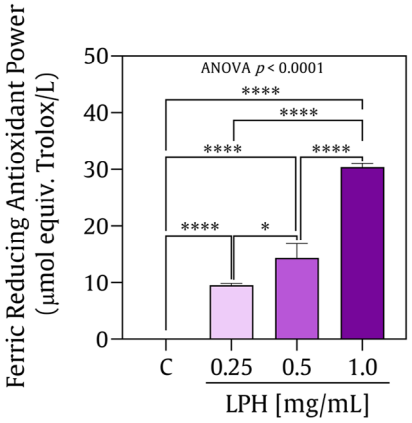

Figure 2. Evaluation of LPH in vitro antioxidant power by ferric reducing antioxidant power (FRAP) assay. Data were represented as mean ± SEM ($n = 6$). Data were analyzed by One-way ANOVA followed by Dunn's post-hoc test. *, $p \leq 0.05$; ****, $p \leq 0.0001$. C, control; LPH, lupin protein hydrolysate; TEAC, Trolox equivalent antioxidant capacity.

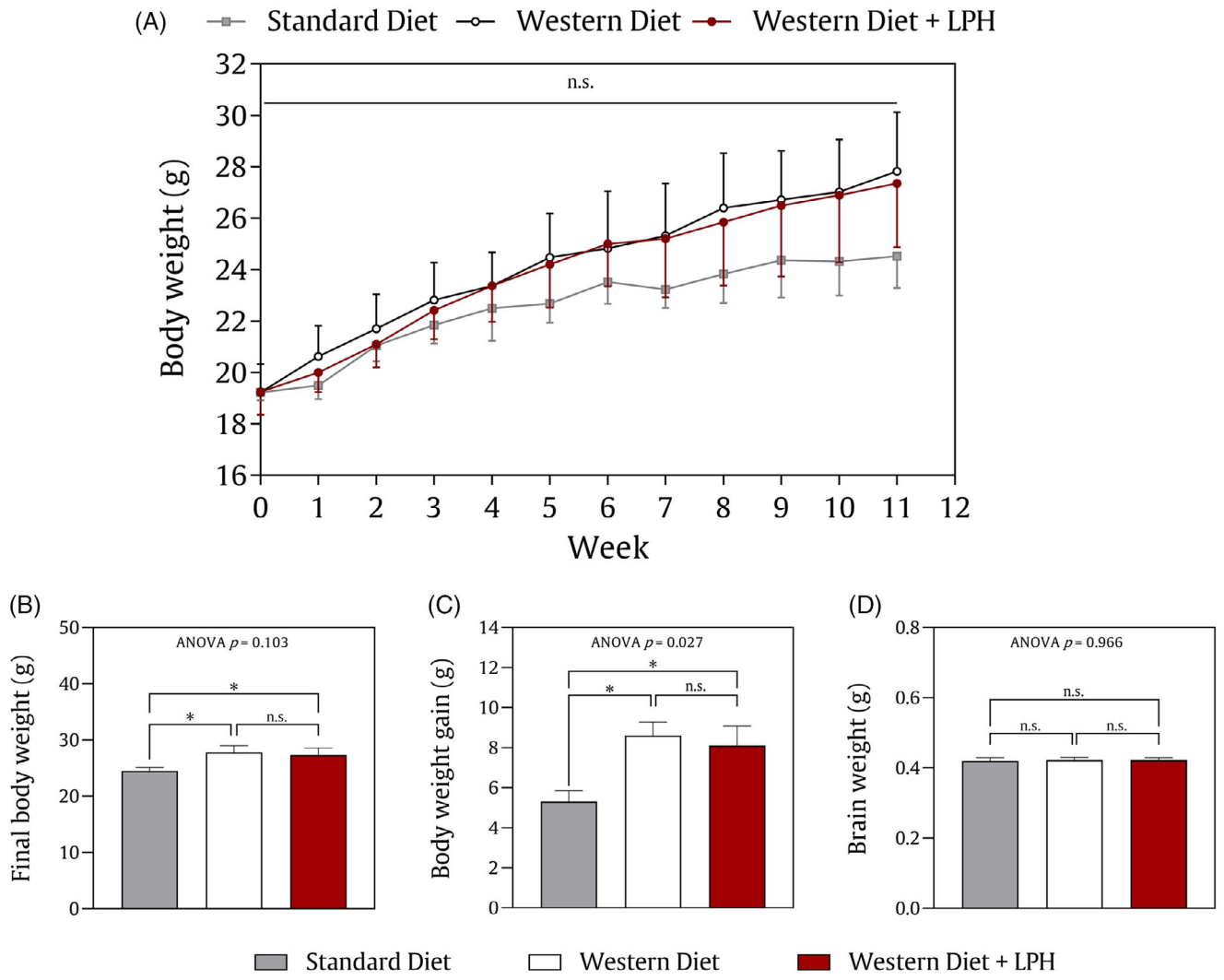

Figure 3. Weekly body weight (A), final body weight (B), body weight gain (C), and brain weight (D) of the in vivo experiments carried out in male ApoE$^{-/-}$ mice. Data were represented as mean ± SEM ($n = 4$). Data were analyzed by Two-way ANOVA followed by Sidak's post-hoc test (A) and One-way ANOVA followed by Dunn's post-hoc test (B-D).*, $p \leq 0.05$; LPH, lupin protein hydrolysates; n.s., not significant.

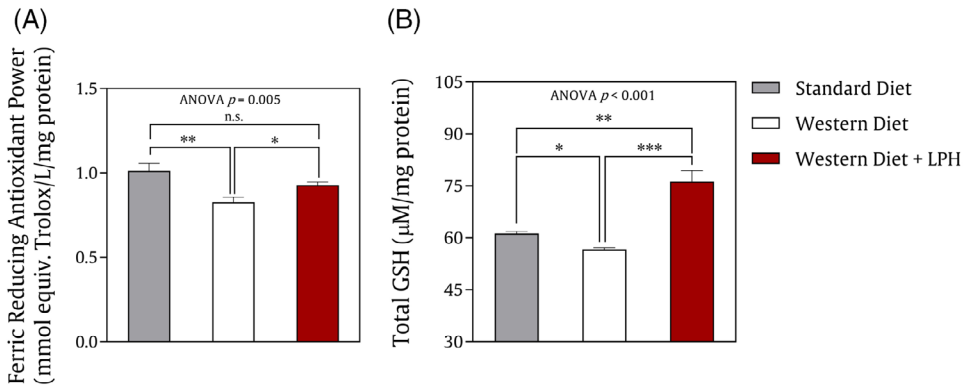

Figure 4. Ferric reducing antioxidant power (FRAP) (A) or glutathione (GSH) content (B) in the brain of male ApoE$^{-/-}$ mice from the three experimental groups. Data were represented as mean ± SEM ($n = 4$). Data were analysed by One-way ANOVA followed by Dunn's post-hoc test. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; GSH, glutathione; LPH, lupin protein hydrolysates; n.s., not significant.

Table 3. Spearman's correlations between total antioxidant power and GSH.

Group	FRAP vs GSH	
	r	*p*-value
SD	0.886	**0.033**
WD	−0.300	0.683
WD + LPH	0.893	**0.012**

GSH, glutathione; FRAP, ferric reducing antioxidant power; SD, standard diet; WD, western diet; LPH, lupin protein hydrolysate. The bold value indicates a statistical difference.

a significant increase of 12.29% ($p = 0.037$) in brain FRAP (0.93 ± 0.02 mmol equivalent Trolox per liter per milligram protein) compared to the WD group. No significant differences ($p = 0.165$) were observed between the WD + LPH and SD-fed mice groups.

3.5. LPH Treatment Increases GSH Levels in the CNS

The cerebral GSH content was evaluated in all experimental groups. Mice fed WD showed significantly lower levels (−7.48%, $p = 0.041$) of cerebral GSH (56.68 ± 0.44 µM per mg protein) compared to the SD group (61.26 ± 0.59 µM per mg protein). LPH was able not only to counteract this effect (76.21 ± 3.22 µM per mg protein, $p < 0.001$), increasing it by 34.46%, but also significantly increased ($p = 0.01$) the GSH levels even compared to the SD group (Figure 4B).

The nonparametric Spearman's correlation was applied to study the possible relationship between total antioxidant power and GSH content. As shown in **Table 3**, a strong positive correlation was observed between FRAP and GSH in the SD group. However, this was lost in the WD group. Significantly, a strong positive correlation between FRAP and GSH was restored in LPH-treated mice (WD + LPH). Graphs with individual data points for each group are shown in Figure S1 (Supporting Information).

3.6. Nitric Oxide Synthesis Is Downregulated by the LPH in the CNS

To verify the possible effect of LPH on brain NO synthesis, the relative expression of the nNOS and iNOS mRNA, and the NO concentration were evaluated. As shown in **Figure 5**, WD-fed mice showed a significant increase of 65.55% and 63.67% in the gene expression of nNOS (1.80 ± 0.56-fold change, $p = 0.004$) (Figure 5A) and iNOS (1.41 ± 0.17-fold change, $p = 0.008$) (Figure 5B), respectively, as well as by 19.50% in the NO production (4.36 ± 0.07 O.D. per mg protein, $p = 0.045$) (Figure 5C) compared to SD-fed mice. Treatment with LPH for 12 weeks reduced by 49.66% and 18.84% both iNOS gene expression (0.70 ± 0.18-fold change, $p = 0.002$) and NO production (3.49 ± 0.18 O.D. per mg protein, $p = 0.01$), respectively, compared to the WD group, without affecting the nNOS gene expression ($p = 0.192$). No differences between WD + LPH and SD groups were observed.

3.7. LPH Treatment Increases the Activity of CAT and GR Enzymes and Decreases the DNA Damage in the CNS

Once the increase in the brain of total antioxidant activity was demonstrated in mice treated with LPH, the activity of the main antioxidant enzymes as well as oxidative damage to DNA were evaluated. As shown in **Figure 6**, treatment with LPH significantly increased the activity of the antioxidant enzymes CAT (+ 31.29%, $p = 0.011$) and GR (+ 26.11, $p = 0.043$), compared to the WD group. On the other hand, LPH treatment did not modify the activity of the antioxidant enzymes SOD ($p = 0.122$) or GPx ($p = 0.193$). Furthermore, a significant decrease in 8-OHdG levels (-27.99%, $p = 0.031$) was shown in the brain of LPH-treated mice, compared to the WD group (Figure 6).

4. Discussion

This study highlights the antioxidant effects of LPH treatment on the brain of WD-fed ApoE$^{-/-}$ mice. Specifically, LPH palliated the oxidizing effects on the brain caused by WD feeding. The effects

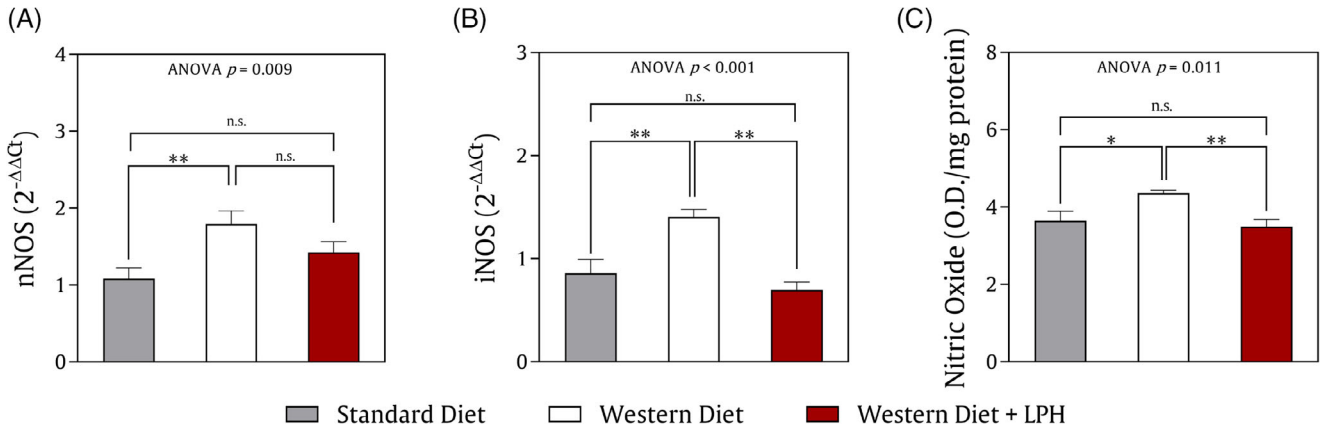

Figure 5. Neuronal (A) and inducible (B) nitric oxide synthase (nNOS and iNOS) gene expression in hypothalamus, and nitric oxide production (C) in brain of the male ApoE$^{-/-}$ mice from the different experimental groups. Data were represented as mean ± SEM ($n = 4$). Data were analyzed by One-way ANOVA followed by Dunn's post-hoc test. *, $p \leq 0.05$; ** $p \leq 0.01$; LPH, lupin protein hydrolysates; n.s., not significant.

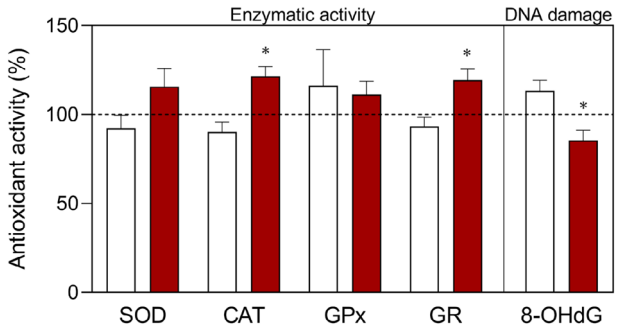

Figure 6. Percentage of the antioxidant activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and DNA damage (8-OHdG) in the brain of male ApoE$^{-/-}$ mice. After referring the data to mg of protein, results are expressed as a percentage of the standard diet group. Data were represented as mean ± SEM (n = 4). Data were analyzed by One-way ANOVA followed by Dunn's post-hoc test. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$ versus the control group (standard diet, dashed line). 8-OHdG, 8-hydroxy-2′-deoxyguanosine; LPH, lupin protein hydrolysates.

of LPH were not related to body weight gain, final body weight, or daily food intake (Figure S2, Supporting Information), which remain unchanged after LPH treatment.

LPH is a mixture of peptides obtained after hydrolysis of a lupin (*L. angustifolius*) protein isolate using the food-grade endopeptidase Alcalase. The protein content of the hydrolysate was higher than that of the previous lupin flour. Additionally, the use of Alcalase generated a hydrolysate with peptides mainly smaller than 8 kDa in size, with a peak in <1 kDa. This fact is due to Alcalase is an endopeptidase that can cleavage between a wide range of amino acids (Glu, Met, Leu, Tyr, Lys, and Gln), providing a hydrolysate with many peptides of small size and hydrophobic characteristics. In this sense, it is well known that the bioactive effect of peptides is influenced by structural properties, such as molecular weight, peptide size, and amino acid composition.[24] In particular, low-molecular-weight peptides are associated with a powerful antioxidant capacity compared to larger peptides.[25] Furthermore, peptides containing i) hydrophobic amino acids such as Leu or Val in their N-terminal regions, ii) imidazole ring-containing His, iii) nucleophilic sulfur-containing amino acid residues (Cys and Met), iv) pyrrolidine ring (Trp and Pro), and/or v) aromatic amino acid residues (Phe, Trp, and Tyr) have been shown to possess strong antioxidant properties.[25] In this line, a previous study by our group demonstrated that LPH is rich in the amino acids Leu, Val, Pro, His, Thr, Tyr, and Phe.[9]

Other food-derived hydrolysates generated with Alcalase have shown potent antioxidant effects, such as pea, corn, soy, zein, amaranth, and rice bran protein hydrolysates.[26–30] Furthermore, numerous scientific articles have shown that hydrolysates obtained from Alcalase have greater antioxidant effects than hydrolysates generated with other proteases such as protamex, neutrase, flavourzyme, papain, and pepsin.[31,32]

The characterization of LPH revealed the presence of 101 *L. angustifolius* peptides (36.33%) that contained at least a known antioxidant motif. The physicochemical analysis of these sequences showed a high content of imidazole rings (90.1% of sequences with at least 1 ring) and hydrophobic amino acids such as Leu (11.71%) and Val (5.75%). In this way, a high proportion of Leu, Val, and amino acids with imidazole rings have been associated with strong antioxidant capacity through direct electron transfer.[24] Moreover, these considerations are supported by the LPH in vitro FRAP test, in which a dose-dependent increase in antioxidant capacity was observed from a LPH concentration of 0.25 mg mL^{-1}.

In addition, Wang et al. have previously demonstrated that His-Tyr, Pro-Tyr, and Leu-Pro-Phe motifs, also present in the LPH (6.84%), exert anti-inflammatory and antioxidant effects on the BV-2 microglial cell line. Specifically, these motifs have been shown to reduce LPS-induced nitrite and ROS production after 24 h of cell treatment.[6,18]

In the present study, oxidative stress was induced in ApoE$^{-/-}$ mice by feeding a WD. As expected, WD-fed mice showed reduced cerebral levels of FRAP and GSH, as well as increased levels of nNOS, iNOS, and NO, compared to SD-fed mice. These results agree with previous studies that observed an increase in brain oxidative stress caused by high-fat diet feeding.[33,34] Oxidative stress has been implicated in the progression of several neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease.[5]

Here, we show that LPH treatment palliates the exacerbated oxidative stress triggered by WD consumption in the brain of animals. In particular, LPH treatment counteracts the decrease in FRAP levels and GSH concentration, as well as restores iNOS gene expression and NO content in the brain of WD-fed animals to those of SD-fed mice.

While iNOS is produced by pro-inflammatory stimuli such as cytokines, nNOS is constitutively expressed and involved in several physiological procedures in the CNS. In fact, normal NO levels synthesised by nNOS are involved in neuronal plasticity, regulation of blood flow in the CNS, and neurotransmitter release, among others.[35] LPH does not significantly alter the gene expression of nNOS in WD-fed mice, supporting the idea that decreased NO production in the LPH-treated mice is primarily mediated by decreased iNOS mRNA expression, which in turn is involved in a decrease in pro-inflammatory/pro-oxidant status generated in the CNS of mice fed WD.

On the other hand, GSH, the most important antioxidant tripeptide (Glu–Cys–Gly) in biological systems, participates in excessive ROS scavenging. It is also important to note that the increase in GSH content in the brain of LPH-treated mice is highly correlated with the increase in total antioxidant power (FRAP). Thus, LPH treatment restores and increases the GSH content, favoring a better response against oxidative stress. Furthermore, an increase in the activity of the antioxidant enzymes CAT and GR and a decrease in 8-OHdG levels were shown in the brain of LPH-treated mice. Thus, the increase of antioxidant system allows the preservation of the integrity of nucleic acids, reducing the severity of danger of the oxidation of the DNA, and so improving the cell's survival. In this sense, an increase in the activity of antioxidant enzymes was observed previously in plasma and liver of mice treated with LPH.[10,36]

Interestingly, a previous study using the same mouse model has shown an anxiolytic role of LPH treatment.[12] Therefore, we suggest that the antioxidant effects observed in the CNS of LPH-treated mice could be a possible explanation for the previous anxiolytic effects demonstrated. All these results are in agreement with our previous studies that showed antioxidant effects of LPH

on i) in vitro cultured human leukocytes[8]; ii) plasma and liver of 12 weeks LPH-treated ApoE$^{-/-}$ mice fed a WD,[9,10] and iii) leukocytes of participants in the clinical study Lupine-1 after 4 weeks ingestion of a LPH-based beverage.[11]

The antioxidant effects observed by the LPH treatment on both liver and brain tissues could be explained by the close interaction between the liver and the CNS through the liver-brain axis.[37] In this sense, previous studies have demonstrated that liver overload with toxic metabolites and immune mediators such as tumor necrosis factor (TNF), interleukin (IL)-1β, and IL-6 leads to an increase in systemic load that reaches the brain via altered blood-brain barrier (BBB) or brain regions without BBB, such as the circumventricular organs and the choroid plexus, causing brain cell toxicity and increased oxidative stress.[38] In addition, although the peptide passage through the BBB is not well defined, previous studies have defined that this may occur through receptor-mediated transcytosis or by specific transporters, such as peptide transporter (PepT), the large neutral amino acid transporter (LAT), and the peptide histidine transporter (PHT).[39] In this line, future studies must be dedicated to elucidate this aspect.

The present study has certain solvable limitations. Although the number of mice was limited, i) it was enough to achieve significant differences, ii) different antioxidant assays were performed to confirm the effects, and iii) the Cohen's test analysis (one of the most common ways to measure statistical power and effect size) shows a large size effect on the variables studied (Table S2, Supporting Information). If the size effect is large ($\geq$0.80), the difference between groups is meaningful and has practical significance. In addition, it is important to highlight that the SD group was necessary only to confirm that the WD ingestion causes oxidative stress at the CNS level.

On the other hand, the main strength of this study is the multidisciplinary strategy used ranging from an in silico study to identify antioxidant peptides in the LPH, to evaluate not only in vitro antioxidants effects but also in vivo antioxidant capacity in the brain of WD-fed ApoE$^{-/-}$ mice.

Although other food-derived peptides, such as those of walnut and wheat, have been shown to alleviate brain oxidative stress, improving the learning and memory deficits in rats and mice,[6,40,41] this is the first study to elucidate the antioxidant effect on the brain exerted by lupin-derived peptides.

5. Conclusions

To our knowledge, there is no previous scientific evidence for the in vivo antioxidant effects on the CNS of any legume-derived peptides. In this study, we demonstrate that LPH is capable of improving the antioxidant status in the CNS of mice with exacerbated oxidative stress, induced by a high-fat-rich diet (Western diet). We show that the LPH treatment improves total antioxidant capacity in the CNS, reducing the expression of the iNOS gene and NO and 8-OHdG levels, and increasing CAT and GR activities and GSH levels, the most important non-enzymatic antioxidant molecule.

The described antioxidant effects could be due to the different and specific chemical composition of this LPH. Specifically, the analysis of amino acid sequences reveals that LPH contains numerous encrypted antioxidant motifs. In addition, LPH is mainly composed of small peptides (<1Kda) and is rich in Leu, Val, Pro, His, Thr, Tyr, and Phe, characteristics that have been shown to enhance the antioxidant effect.

This investigation opens the door to investigating the possible mechanisms of vegetable-derived peptides in the CNS with the aim to design antioxidant functional food in the short-medium term.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

The authors thank the staff from the IBiS Animal Facility for their valuable assistance. This research was funded by the Andalusian Government Ministry of Health PC-0111-2016-0111, PEMP-0085-2020 (co-financed with FEDER funds, call Resolution of 7 July 2021 of the General Secretary for Research, Development and Innovation in Health, which calls for grants to finance research, development and innovation in biomedicine and health sciences in Andalusia by 2021) and the PAIDI Program from the Andalusian Government [CTS160]. G.S.-S. was supported by FPU grants from the Spanish Ministerio de Educación, Cultura y Deporte, [FPU16/02339]. I.C.-C. was supported by a postdoctoral fellowship from the Andalusian Government Ministry of Economy, Knowledge, Business, and University [DOC_00587/2020]. A.I.A.-L. was funded by the Andalusian Government Ministry of Health [PI-0136-2019]. E.P.-E. was supported by the VI Program of Inner Initiative for Research and Transfer of the University of Seville [VI PPIT-US]. The experiments were performed under the Spanish legislation and the EU Directive 2010/63/EU for animal experiments and were approved by the Virgen Macarena and Virgen del Rocío University Hospitals ethical committee (reference 21/06/2016/105).

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

The following are the authors' contributions: Conceptualization: A.C.-V., I.C.-C.; Methodology: G.S.-S., I.C.-C., A.I.A.-L., E.P.-E.; Resources: A.C.-V., J.P., M.C.M.-L, P.J.L., M.-S.F.-P.; Formal analysis: G.S.-S, I.C.-C., A.I.A.-L., E.P.-E.; Drafting of the manuscript: G.S.-S., I.C.-C., A.C.-V.; Supervision: A.C.-V., I.C.-C.; Funding acquisition: A.C.-V., J.P., M.C.M.-L., P.J.L., M.-S.F.-P. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data available on request from the authors.

Keywords

brain, glutathione, iNOS, nitric oxide, peptides

Received: July 17, 2023
Revised: December 22, 2023
Published online: February 3, 2024

[1] D. Bhandari, S. Rafiq, Y. Gat, P. Gat, R. Waghmare, V. Kumar, *Int. J. Pept. Res. Ther.* **2020**, *26*, 139.

[2] E. B.-M. Daliri, D. H. Oh, B. H. Lee, *Foods* **2017**, *6*, 32.
[3] I. Cruz-Chamorro, G. Santos-Sánchez, C. Bollati, M. Bartolomei, J. Li, A. Arnoldi, C. Lammi, *J. Agric. Food Chem.* **2022**, *70*, 577.
[4] M. Sharifi-Rad, N. V. Anil Kumar, P. Zucca, E. M. Varoni, L. Dini, E. Panzarini, J. Rajkovic, P. V. Tsouh Fokou, E. Azzini, I. Peluso, *Front Physiol* **2020**, *11*, 694.
[5] K. J. Barnham, C. L. Masters, A. I. Bush, *Nat. Rev. Drug Discovery* **2004**, *3*, 205.
[6] S. Wang, L. Zheng, T. Zhao, Q. Zhang, Y. Liu, B. Sun, G. Su, M. Zhao, *J. Agric. Food Chem.* **2020**, *68*, 2381.
[7] A. Surguchov, L. Bernal, A. A. Surguchev, *Biomolecules* **2021**, *11*, 624.
[8] I. Cruz-Chamorro, N. Álvarez-Sánchez, M. del Carmen Millán-Linares, M. del Mar Yust, J. Pedroche, F. Millán, P. J. Lardone, C. Carrera-Sánchez, J. M. Guerrero, A. Carrillo-Vico, *Food Res Int* **2019**, *126*, 108585.
[9] G. Santos-Sánchez, I. Cruz-Chamorro, A. I. Álvarez-Ríos, N. Álvarez-Sánchez, B. Rodríguez-Ortiz, A. I. Álvarez-López, M. S. Fernández-Pachón, J. Pedroche, F. Millán, M. C. Millan-Linares, P. J. Lardone, I. Bejarano, A. Carrillo-Vico, *J. Agric. Food Chem* **2022**, *70*, 8243.
[10] G. Santos-Sánchez, I. Cruz-Chamorro, A. I. Álvarez-Ríos, J. M. Fernández-Santos, M. V. Vázquez-Román, B. Rodríguez-Ortiz, N. Álvarez-Sánchez, A. I. Álvarez-López, M. d. C. Millán-Linares, F. Millán, *Antioxidants* **2021**, *10*, 1222.
[11] I. Cruz-Chamorro, N. Álvarez-Sánchez, A. I. Álvarez-Ríos, G. Santos-Sánchez, F. Millan, J. Pedroche, C. Carrera-Sánchez, M. S. Fernández-Pachón, M. C. Millan-Linares, A. Martínez-López, P. J. Lardone, I. Bejarano, J. M. Guerrero, A. Carrillo-Vico, *Mol. Nutr. Food Res.* **2021**, 2100139.
[12] G. Santos-Sánchez, E. Ponce-España, J. C. López, N. Álvarez-Sánchez, A. I. Álvarez-López, J. Pedroche, F. Millán, M. C. Millán-Linares, P. J. Lardone, I. Bejarano, I. Cruz-Chamorro, A. Carrillo-Vico, *Int. J. Mol. Sci.* **2022**, *23*, 9828.
[13] A. D. G. Fedoce, F. Ferreira, R. G. Bota, V. Bonet-Costa, P. Y. Sun, K. J. Davies, *Free Radic. Res.* **2018**, *52*, 737.
[14] I. Cruz-Chamorro, G. Santos-Sánchez, A. I. Álvarez-López, J. Pedroche, P. J. Lardone, A. Arnoldi, C. Lammi, A. Carrillo-Vico, *Trends Food Sci. Technol.* **2023**.
[15] T. B. Shea, E. Rogers, D. Ashline, D. Ortiz, M.-S. Sheu, F. Radic, *Biol. Med.* **2002**, *33*, 1115.
[16] M.-C. Tung, Y.-W. Lan, H.-H. Li, H.-L. Chen, S.-Y. Chen, Y.-H. Chen, C.-C. Lin, M.-Y. Tu, C.-M. Chen, *Sci. Rep.* **2020**, *10*, 8802.
[17] P. Minkiewicz, A. Iwaniak, M. Darewicz, *Int. J. Mol. Sci.* **2019**, *20*, 5978.
[18] G. Santos-Sánchez, I. Cruz-Chamorro, C. Bollati, M. Bartolomei, J. Pedroche, F. Millan, M. C. Millan-Linares, A. L. Capriotti, A. Cerrato, A. Laganà, A. Arnoldi, A. Carrillo-Vico, C. Lammi, *Food Funct.* **2022**, *13*, 4158.
[19] S. Gupta, P. Kapoor, K. Chaudhary, A. Gautam, R. Kumar, G. P. Raghava, *Methods Mol Biol.* **2015**, *1268*, 143.
[20] S. Lee, L. Prosky, J. W. de Vries, *J. AOAC Int.* **1974**, *75*, 22.
[21] A. Arlington, *AOAC Official Methods of Analysis*, **1995**.
[22] R. M. C. Moores, D. Demott, T. Wood, *Anal. Chem.* **1948**, *28*, 5.
[23] M. Dubois, K. Gilles, J. Hamilton, P. Rebers, F. Smith, *Anal. Chem.* **1956**, *28*, 7.
[24] S. Ketnawa, M. Wickramathilaka, A. M. Liceaga, *Food Chem.* **2018**, *254*, 36.
[25] I. D. Nwachukwu, R. E. Aluko, *J Food Biochem* **2019**, *43*, e12761.
[26] S. Y. Park, J. S. Lee, H. H. Baek, H. G. Lee, *J Food Biochem* **2010**, *34*, 120.
[27] H. M. LI, X. Hu, P. Guo, P. Fu, L. Xu, X. Z. ZHANG, *J Food Biochem* **2010**, *34*, 44.
[28] X. Tang, Z. He, Y. Dai, Y. L. Xiong, M. Xie, J. Chen, *J. Agric. Food Chem.* **2010**, *58*, 587.
[29] V. A. Tironi, M. C. Añón, *Food Res Int* **2010**, *43*, 315.
[30] L. Wattanasiritham, S. Kubglomsong, C. Theerakulkait, *Pak. J. Nutr.* **2015**, *14*, 647.
[31] M. F. Sbroggio, M. S. Montilha, V. R. G. D. Figueiredo, S. R. Georgetti, L. E. Kurozawa, *Food Sci. Technol.* **2016**, *36*, 375.
[32] M. D. Sharmila, T.-T. Chai, F.-C. Wrong, *Xiandai Shipin Keji* **2019**, 35.
[33] A. M. Ajayi, K. A. John, I. B. Emmanuel, E. O. Chidebe, A. D. Adedapo, *Metabol Open* **2021**, *9*, 100077.
[34] L. Zhang, J. Bo, W. Chen, S. Li, Y. Wang, L. Yan, L. Wu, Y. Zhang, *Biol. Trace Elem. Res.* **2021**, *199*, 2247.
[35] S. S. S. Saravi, *Nitric Oxide Synthase - Simple Enzyme-Complex Roles*, InTech, **2017**.
[36] G. Santos-Sánchez, I. Cruz-Chamorro, A. I. Álvarez-Ríos, N. Álvarez-Sánchez, B. Rodríguez-Ortiz, A. I. Álvarez-López, M. S. Fernández-Pachón, J. Pedroche, F. Millán, M. C. Millan-Linares, P. J. Lardone, I. Bejarano, A. Carrillo-Vico, *J. Agric. Food Chem.* **2022**.
[37] Y. Matsubara, H. Kiyohara, T. Teratani, Y. Mikami, T. Kanai, *Neuropharmacology* **2021**, 108915.
[38] H. Gupta, K. T. Suk, D. J. Kim, *J Clin Med* **2021**, *10*, 541.
[39] M. Tanaka, S. Dohgu, G. Komabayashi, H. Kiyohara, F. Takata, Y. Kataoka, T. Nirasawa, M. Maebuchi, T. Matsui, *Sci. Rep.* **2019**, *9*, 5769.
[40] N. Ding, H. Meng, C. Wu, H. Hong, Y. Luo, Y. Tan, *Food Biosci.* **2023**, 103460.
[41] N. Ding, H. Meng, C. Wu, W. Yokoyama, H. Hong, Y. Luo, Y. Tan, *Nutrients* **2023**, *15*, 1228.